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Original Article

Cancer testis antigen OY-TES-1 expression and serum immunogenicity in colorectal cancer: its relationship to clinicopathological parameters

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Abstract: Cancer testis (CT) antigens are attractive targets for cancer immunotherapy because their expression is restricted in normal germ line tissues but frequently detected in variety of tumors. OY-TES-1 is identified as a member of CT antigens. Current knowledge about OY-TES-1 expression in colorectal cancer (CRC) is solely based on mRNA analysis. None of previous researches has studied OY-TES-1 at protein level. In this study, OY-TES-1 polyclonal antibody was generated. The expression of OY-TES-1 mRNA and protein was detected by RT-PCR and immunohistochemistry in 60 CRC and paired adjacent non-tumor tissues, 24 colorectal adenoma and 3 normal colon tissues, respectively. Sera from 73 CRC patients were also tested for OY-TES-1 antibody by ELISA. Our results showed that the frequency of OY-TES-1 mRNA expression was statistically higher in CRC (73.3%, 44/60) than that in adjacent non-tumor tissue (55.0%, 33/60) and colorectal adenoma (45.8%, 11/24). For the first time, OY-TES-1 protein expression was found in (43.3%, 26/60) of CRC tissues, but absent in any of adjacent non-tumor and colorectal adenoma tissues. No OY-TES-1 expression was found in normal colon by either RT-PCR or immunohistochemistry. Furthermore, OY-TES-1 protein expression was correlated with tumor invasion stage (P=0.004) and histological grade (P=0.040). Anti-OY-TES-1 antibody was detected in (9.6%, 7/73) of CRC patients' sera but not in 76 healthy donors. This finding demonstrates that OY-TES-1 is frequently expressed in CRC and is able to induce humoral immune response spontaneously in CRC patients, suggesting that it might be a promising immunotherapy target for CRC.

Keywords: Cancer testis antigen, OY-TES-1, colorectal cancer

Introduction

Colorectal cancer (CRC) is one of the top three causes of cancer deaths. There are over one million of new cases being diagnosed annually and about half million deaths worldwide [1]. Current CRC diagnosis, prognosis and treatments have not been advanced in the past several decades [2]. Therefore, there is an urgency to develop novel therapies for CRC. Recently, new targeted therapeutic strategies, including immunologic and biologic approaches, have been explored as complementary treatments for patients with CRC, but without satisfactory therapeutic effects [3]. This might be partially attributed to the difficulty in selecting appropriate target genes.

Cancer testis (CT) antigens are a group of tumor antigens, which could induce immune response during tumorigenesis. One of the most common characteristics of CT antigens is their tissue-specific expression in germ line tissues, but aberrant expression in a wide range of different tumors types. These properties render them as attractive candidates for cancer immunotherapy [4, 5]. So far, more than 200 CT antigens have been reported in previous studies, which belong to more than 70 distinct subfamilies [6]. Promising therapeutic benefits have been demonstrated for several members of CT antigens

in clinical trials, such as NY-ESO-1 and MAGE-3 [7, 8]. Unfortunately, CRC is generally considered as a poor CT antigen expresser. In fact, less than 10% of studied CT antigen genes have been shown to express in CRC at a frequency of 30% or more [4, 9, 10]. Hence, it is clinically beneficial to identify a CT antigen that has high expression frequency in CRC.

OY-TES-1 is a member of CT antigens, which was originally identified as the human homologue of proacrosin binding protein sperm protein 32 precursor. OY-TES-1 mRNA was expressed not only in testis but also in different malignant tissues, including bladder, breast, lung, liver, colon and epithelial ovarian cancers. About 3.5% to 10.5% of cancer patients have developed humoral immune response to OY-TES-1 [11, 12]. A HLA-A24-binding peptide was identified and recognized by $CD8^+$ T-cell, and thus caused cytotoxicity to tumor cells expressing OY-TES-1 [13]. More recently, researches indicated that OY-TES-1 normalized mitotic spindle function to promote cancer cell proliferation [14], and expressed in mesenchymal stem cells [15]. In the mouse, two functional forms of OY-TES-1 were produced by pre-mRNA alternative splicing, and may play different role in spermiogenesis and fertilization [16].

Current knowledge about OY-TES-1 in CRC is only from one previous study, in which OY-TES-1 mRNA expression was observed in 2 of 13 colon cancer and sera antibody against OY-TES-1 was tested in 6 of 58 colon cancer patients [11]. However, little is known about the expression of OY-TES-1 at the protein level. In this study, we detected OY-TES-1 mRNA and protein expression in CRC and their paired adjacent non-tumor tissues, colorectal adenomas and normal colon tissues. The presence of anti-OY-TES-1 antibody in the sera of CRC patients was also evaluated by ELISA. Furthermore, the correlation among OY-TES-1 expression, antibody production and clinicopathological parameters in CRC patients was analyzed.

Materials and methods

Tissues and sera

Tissues and sera were obtained from the First Affiliated Hospital of Guangxi Medical University, approved by Hospital Ethic Review Committee with informed consent from patients. The tissues consisted of 60 CRC with their adjacent non-tumor tissues, 24 colorectal adenomas and 3 normal colons. CRC tissues were from 43 men and 17 women with a mean age at diagnosis of 55.53 ± 14.42 years (ranging 30-86 years). Sera were collected from 73 CRC patients (55.37 ± 13.69 years, ranging 27-90 years) at diagnosis prior of therapy. 76 sera from healthy donors were obtained from routine physical examination, containing men and women half of each. Tumor stage and disease grade were classified according to the TNM classification of the Union for International Cancer Control [17]. Testis tissues were taken from prostate cancer patients undergoing surgery by castration.

Generation of recombinant OY-TES-1 protein and polyclonal antibody

OY-TES-1 coding sequence from nucleotides 28 to 1723 was amplified from human testis cDNA by PCR with Primers (sense: 5'-GCGGGCGGATCTTCTCCGGCCATG-3'; antisense: 5'-ACGGGATCCTTATCAGTTGGGCTGGGGTGT-3'). PCR products were inserted into plasmid pMAL-C_2 (New England Biolabs, USA), which was confirmed by sequencing. Recombinant OY-TES-1 protein was then produced by isopropyl-1-thio-D-galactopyranoside (IPTG, Sigma, USA) induction and purified as described previously [18]. The purified protein was subjected to electrophoresis on 12% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE). Target bands excised from the gel were analyzed by tandem mass spectrometry/mass spectrometry (MS/MS) to confirm the protein sequence.

New Zealand rabbits were injected with recombinant OY-TES-1 protein in Freund's adjuvant (Sigma, USA). For the first immunization, 1 mg of recombinant OY-TES-1 was admixed 1:1 with complete Freund's adjuvant; for the next two booster injections (on days 21 and 35), incomplete Freund's adjuvant (Sigma, USA) was used. Antiserum was purified with Affi-Prep protein A (Bio-Rad, USA) and antigen affinity columns using Affigel-15 gel (Bio-Rad, Hercules, CA) in accordance with the manufacturer's instructions. The specificity of the antiserum was validated by ELISA and Western blot, respectively.

RT-PCR

Total RNA was extracted by using TRIzol reagent (Invitrogen, CA) according to the manufacturer's instructions. Reverse-transcription was performed with 2 μg total RNA by using

RevertAid™ First Strand cDNA Synthesis Kit (MBI Fermentas, USA). cDNAs were then tested for integrity by amplification of p53 gene [19]. RT-PCR was performed with OY-TES-1 specific primers as described earlier [11, 12]. The thermal cycling conditions were in accordance with previous report by Cen et al [15]. Human testis cDNA was used as a positive control in PCR. The expression of OY-TES-1 was counted as positive, only if the RT-PCR reaction repeated at least twice with same result.

Immunohistochemistry

Tissue sections were deparaffinized, and heated in antigen retrieval buffer ethylene diamine tetraacetic acid (EDTA, pH8.0) in a pressure cooker for 20 min. After the inactivation of endogenous peroxidase with 3% H_2O_2 for 10 min, the sections were treated with normal goat serum for blocking. OY-TES-1 polyclonal antibody was added at a 1:1000 dilution and incubated for overnight at 4°C in humid chamber. Subsequently, secondary antibody of Supervision™ horse radish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Long Island Biotech, China) was incubated for 30 minutes at room temperature. Immunodetection was visualized with 3, 3'-diaminobenzidine (DAB) (Maixin Biotec, China) and couterstained with hematoxylin. Negative controls using rabbit serum collected before immunization were also incubated in parallel. Positive immune-reactivity was recorded according to the number of positive tumor cells as follows: focal or ≤ 5%, negative; > 5-20%, weak; ≥ 20-50%, moderate; > 50%, strong [20]. Two independent pathologists who did not know patients' clinical information evaluated all the sections.

ELISA

Recombinant OY-TES-1 protein (1 μg/ml) was coated on the 96-well plates (Corning, USA) at 4°C overnight. Maltose binding protein (MBP) protein was used as a blank control. The plates were washed with phosphate buffer saline (PBS)/Tween and blocked with 5% nonfat milk at room temperature for 1 h. Serum dilutions were added and incubated at 37°C for 1 h, followed by incubation of HRP-conjugated sheep antihuman IgG (Jackson ImmunoResearch, West Grove, DA, 1:5000 dilution). Detection was accomplished using 100 μl of tetramethylbenzidine substrate. The reaction was stopped by using 3 mol/l sulfuric acid in the absence of light. The absorbance at 450 nm was measured with a microplate reader (Bio-Rad, USA). A positive reaction was defined as an optical density (OD) value for 1:64 diluted sera that exceeded the mean OD value of sera from healthy donors by three standard deviations (SDs). All OD values of serum samples were calculated from triplicates and indicated as mean. Specificity of each positive serum sample was examined by testing reactivity after pre-incubating with recombinant OY-TES-1 protein.

Statistical analysis

Statistical analysis was performed using the SPSS for Windows software package (Version 16.0, SPSS). To test differences between groups, the Kruskal-Wallis test was used. A possible association between OY-TES-1 based on its positivity and clinicopathological parameters was compared by χ^2 test or fisher's exact test. Differences were considered significant when the *P*-value was less than 0.05.

Results

Verification of recombinant OY-TES-1 protein and polyclonal antibody

Recombinant OY-TES-1 protein was detected on SDS-PAGE with a main band just below 116 kDa, which was consistent with the theoretical molecular mass. After removing MBP by protease factor Xa, a single band of approximately 61 kDa was observed, which was consistent with the molecular mass of OY-TES-1 protein (**Figure 1A**). To further verify the recombinant OY-TES-1 protein, the bands were exc-ised from SDS-PAGE gel and analyzed by MS/MS. A search from the GenBank database confirmed that the fragmented peptides from excised gel were matched with the sequence of OY-TES-1 (**Figure 1B**).

After purification, affinity of OY-TES-1 antiserum was checked by ELISA. The result demonstrated that the titer of antiserum can reach more than 1:12800 after final immunization, while pre-immune serum was negative (data not shown). Next, Western blot analysis was carried out to confirm the specificity of rabbit anti-OY-TES-1 polyclonal antibody. We found that the antiserum can specifically detect both the recombinant OY-TES-1 protein and endogenous OY-TES-1 protein extracted from testis (**Figure 1C**), whereas pre-immune serum was fail to detect the recombinant OY-TES-1 protein as well as testis (data not shown).

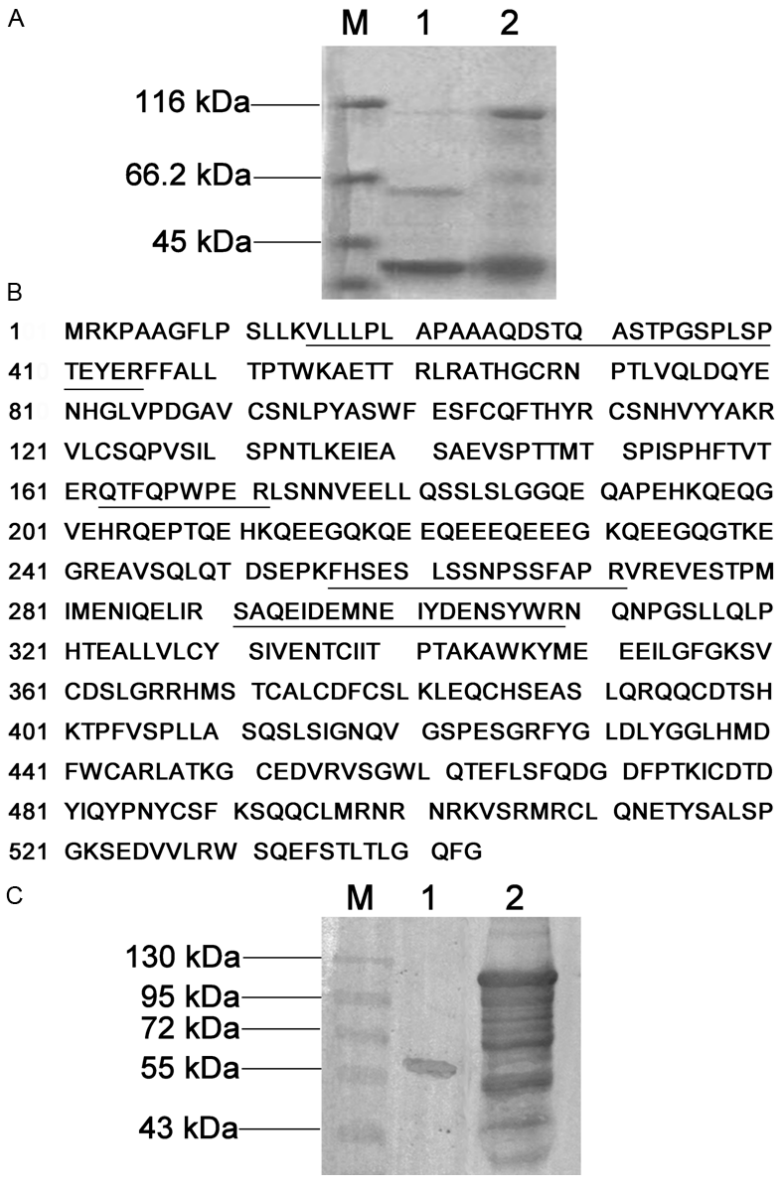

Figure 1. Verification of recombinant OY-TES-1 protein and antiserum. A: SDS-PAGE analysis of OY-TES-1 proteins. Lane 1, recombinant OY-TES-1 protein was digested by protease Factor Xa to removal MBP tag. Lane 2, recombinant OY-TES-1 protein displayed expected molecular weight of about 104 kDa. M, marker. B: MS/MS analysis for confirmation of recombinant OY-TES-1 protein. Peptides with underline represent the matched sequence of OY-TES-1. C: Western blot analysis for the specificity of OY-TES-1 polyclonal antibody. Recombinant OY-TES-1 (lane 1) and total protein from testis (lane 2) were used to test specificity of OY-TES-1 antibody, Expected bands at the level of 104 kDa and 61 kDa were detected respectively. M, marker.

Expression of OY-TES-1 mRNA

OY-TES-1 mRNA was detected in 73.3% (44/60) of CRCs, 55.0% (33/60) of adjacent non-tumor tissues and 45.8% (11/24) of colorectal adenomas, respectively (**Figure 2**). Statistical analysis revealed that there were significant differences between CRC and adjacent non-tumor tissue (P=0.036), CRC and colorectal adenoma (P=0.017), but not adjacent non-tumor tissue and colorectal adenoma (P=0.447). No OY-TES-1 mRNA expression was detected in 3 normal colons.

Expression of OY-TES-1 protein

In testis, OY-TES-1 protein was mainly localized in the germ cells of seminiferous tubules. The immunostaining increased towards the tubular lumen. The more mature germ cells, i.e. spermitids and spermatozoa, showed predominantly stronger cytoplasmic staining, although some positive signals could also be seen in the nuclei. Weaker immunostaining was seen in some early stage of germ cells and interstitial Leydig cells. Sertoli cells showed no detectable OY-TES-1 immunostaining signals (**Figure 3B**).

In CRC, OY-TES-1 staining intensity showed intratumoral variability and was primarily located in the cytoplasm. OY-TES-1 protein was detected in 26 out of 60 (43.3%) CRCs. Among these immunopositive tissue sections, strong (**Figure 3H**), moderate (**Figure 3L**) and weak (**Figure 3P**) stainings were showed in seven, thirteen and six samples, respectively. Notably, heterogeneous expression of OY-TES-1 protein was observed in a significant number of CRC samples, which varied from single positive cells, small nests or clusters of cells to uniform staining of tumor cells. The expression of

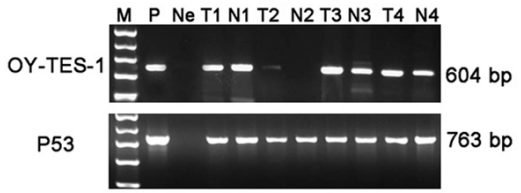

Figure 2. RT-PCR analysis of OY-TES-1 mRNA expression in CRC tissues (T), paired adjacent non-tumor tissues (N). M, DNA marker; P, positive control (testis); Ne, negative control (no cDNA template). P53 was used as internal control for the parallel PCR analysis of the same sample.

OY-TES-1 protein was mainly restricted to malignant cell but absent in the stromal components of neoplastic lesions or the surrounding normal tissues. None of paired adjacent non-tumor tissues (**Figure 3F**, **3J** and **3N**), colorectal adenomas (**Figure 3D**) and normal colons (data not shown) was positive for OY-TES-1 protein immunostaining. In the present study, 60 CRC samples had been evaluated by all assays for OY-TES-1 mRNA and protein expression. **Table 1** demonstrated the comparison of OY-TES-1 expression pattern within the same patient. Of 26 OY-TES-1 protein-positive samples evaluated, 24 of them demonstrated OY-TES-1 mRNA-positive, while the remaining 2 samples showed OY-TES-1 mRNA-negative.

Antibody response to OY-TES-1

Given the coordinate expression of OY-TES-1, we then investigated its immunogenicity using recombinant OY-TES-1 protein by ELISA. OY-TES-1 antibody production was observed in 7 of 73 (9.6%) CRC patients' sera, but in none of 76 healthy donors (**Figure 4A**). **Figure 4B** shows titration curves of selected OY-TES-1 antibody-positive and -negative sera. No antibody against MBP protein was detected in sera from OY-TES-1 seropositive patients. Of 73 sera screened, 25 sera were collected from CRC patients whose tissues were also assessed for OY-TES-1 expression. As shown in **Table 1**, 2 of 22 (9.1%) CRC patients with OY-TES-1 mRNA-positive tumors had antibody against OY-TES-1. No anti-OY-TES-1 antibodies were detected in the remaining 3 patients with OY-TES-1 mRNA-negative tumors. Interestingly, one CRC patient with serum OY-TES-1 antibody-positive demonstrated strong positive expression of OY-TES-1 protein, while the other did not show protein reactivity.

Correlation among OY-TES-1 expression, antibody production and clinicopathological parameters in CRC

Next, we analyzed the possible correlation among OY-TES-1 expression, antibody production and clinicopathological parameters in CRC. The results were summarized in **Table 2**. It is worth noting that OY-TES-1 protein expression was associated with tumor invasion stage (P=0.004). OY-TES-1 protein was positive in only 7.7% (1/13) cases with early stage (T1 and T2), whereas in more than 50% (25/47) cases with late stage (T3 and T4). In addition, high frequency of OY-TES-1 protein expression can be detected in 66.7% (12/18) of poorly differentiated CRCs (G3), followed by 38.5% (10/26) of moderately differentiated CRCs (G2) and 25.0% (4/16) of well differentiated CRCs (G1), which showed statistical difference (P=0.040). No significant correlation was observed among OY-TES-1 mRNA expression, antibody production and clinicopathological parameters.

Discussion

One of the major barriers to antigen-specific immunotherapy in CRC is the lack of well-defined immunogenic tumor antigens. There is an ever-increasing need to search for new targets for immunotherapy. In this study, we characterized OY-TES-1 gene, a new member of CT antigen family that was thought to be a promising therapeutic candidate in many types of tumors. Prior to utilize OY-TES-1 for CRC, it is important to assess its expression at both mRNA and protein level in normal, benign and malignant tissues.

Our result demonstrated that 73.3% of CRCs expressed OY-TES-1 mRNA. This is in contrast to a previous study, which revealed the low expression frequency (15.4%, 2/13) of OY-TES-1 mRNA in colon cancers by using the same PCR primers we used in this study [11]. The discrepancy might be caused by the heterogeneity of gene expression in tumors and sample variations due to genetic or ethnic differences. Scanlan et al [4] reported that colon cancer had low CT expression with only 4/25 (16%) of CT antigens examined had an expression frequency > 20%. Another study had demonstrated the expression of several CT antigens at mRNA level in CRC, such as MAGE-3 (27.3%), MAGE-4 (22.3%), LAGE-1 (15.7%), MAGE-1 (11.6%), NY-ESO-1 (9.9%), CT-10 (6.6%), SSX-1

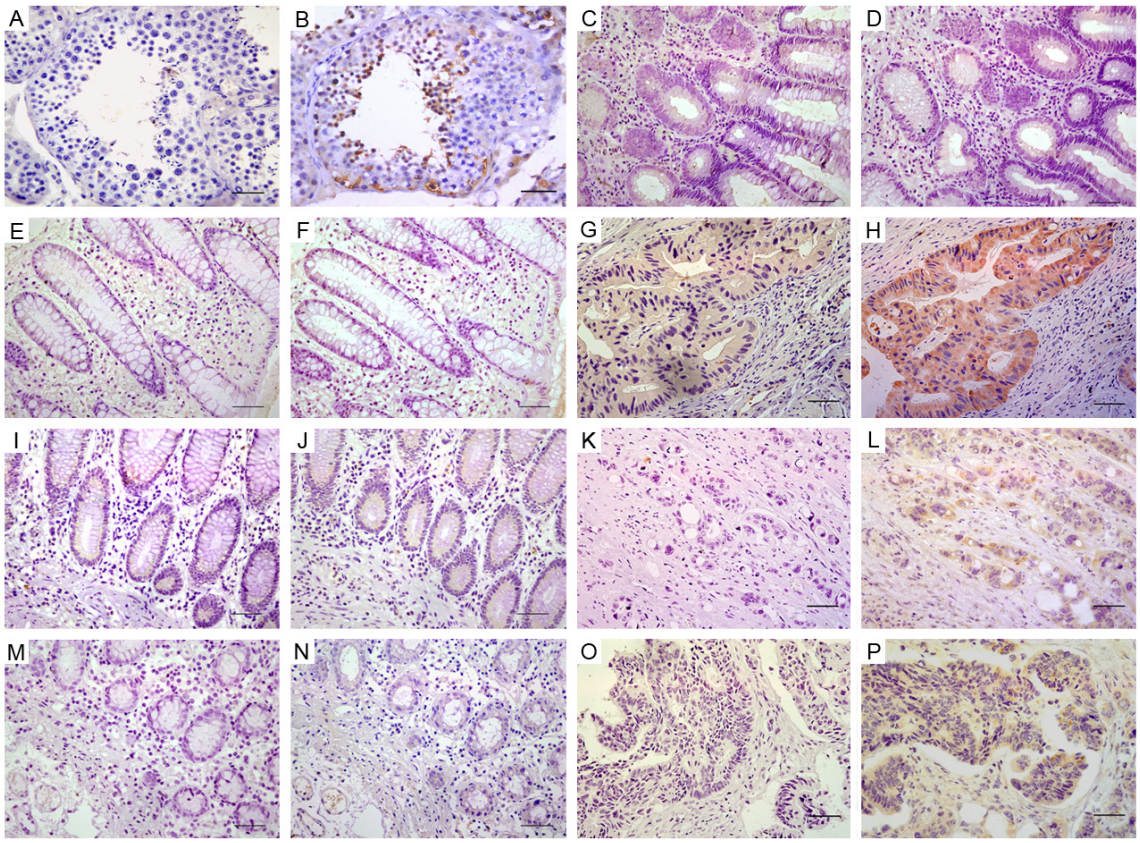

Figure 3. Immunohistochemical staining of OY-TES-1 protein in normal testis (A, B), CRC tissues (G, H, K, L, O, P), paired adjacent non-tumor tissues (E, F, I, J, M, N) and colorectal adenomas (C, D). Seminiferous tubules with strong intratubular staining of mostly late stages of spermatogenic cells in normal testis (A). Strong, moderate, weak immunoreactivity of OY-TES-1 protein immunostaining were shown in H, L and P respectively, using polyclonal OY-TES-1 antibody. No positive reactivity was observed in a serial section of colorectal adenoma (D), paired adjacent non-tumor tissues (E, J, N) probed with pre-immune serum (A, C, E, G, I, K, M, O). Bar, 50 μm.

Table 1. Summary of OY-TES-1 expression and humoral immunogenicity in CRC

OY-TES-1 expression		Number	Antibody against OY-TES-1
mRNA	Protein		Positive /Total
+	+	24	1/12
+	-	20	1/10
-	+	2	0/1
-	-	14	0/2
ND	ND	48	5/48

Abbreviation: ND, not done.

(5%), SSX-2 (2.5%), SSX-4 (2.5%) and SCP-1 (1.7%) [9]. Therefore, our data suggests that OY-TES-1 mRNA has a higher expression frequency comparing to other CT antigens reported in CRC.

In spite of detecting OY-TES-1 mRNA in CRC adjacent non-tumor tissues as well as colorectal adenomas, OY-TES-1 protein can not be detected in both of them. Only CRC samples showed positive staining for OY-TES-1 protein. This suggests that OY-TES-1 is likely to be associated with the immortalised and malignant cells. The expression frequency of OY-TES-1 protein (43.3%, 26/60) in our study is obviously higher than other CT antigens reported in CRC, such as the well-defined CT antigens, i.e. NY-ESO-1 (0-10%) and MAGE-A3 (0-8%), which have been used in clinical trial for tumor immunotherapy [10, 21, 22]. Our results also demonstrated that the expression of OY-TES-1 mRNA and protein was not well paralleled. In 44 OY-TES-1 mRNA positive samples, only 20 samples (45%) were positive in protein immunostaining, which means OY-TES-1 protein was undetectable in more than half of CRC samples. The discrepancy between mRNA and protein expression might be considered as the following factors. First, the PCR-based technique is

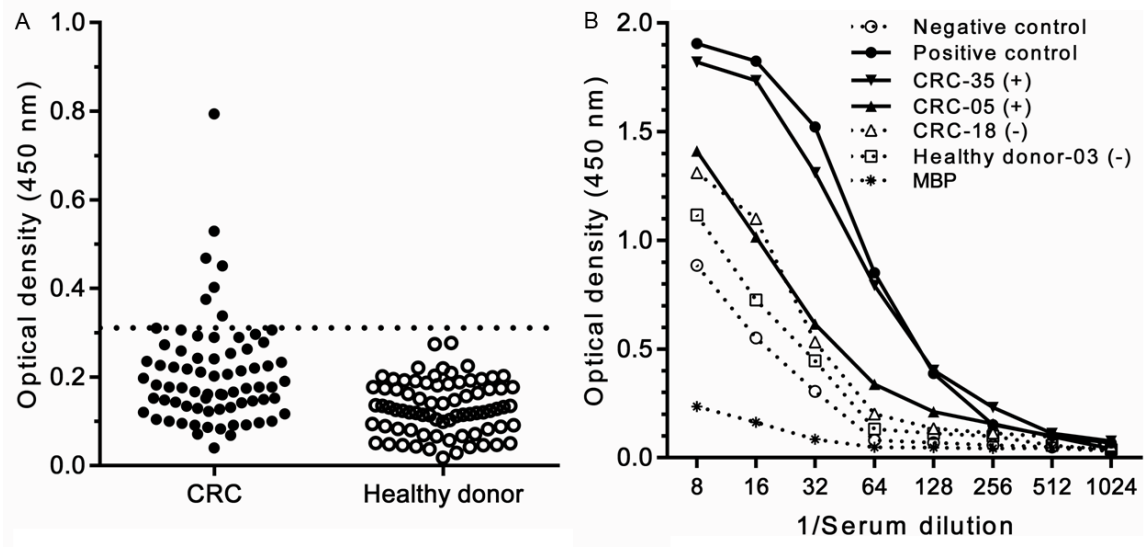

Figure 4. Antibody against OY-TES-1 in sera by ELISA. A: Detection of anti-OY-TES-1 antibody in the sera from 73 CRC patients and 76 normal donors. Three standard deviations above the mean absorbance in the sera from normal donors were used as cutoff (0.311) for a positive result (dotted line). B: Sera titration curves of dilution series of recombinant OY-TES-1 protein against eight different concentrations. Sera from three CRC patients (▼, ▲, △) and one healthy donor (□) were shown, together with a positive control and a negative control. MBP protein (*) was used as a blank control. Patient 35 and 5 demonstrated the highest (▼) and the lowest (▲) OY-TES-1 antibody titer among 7 seropositive CRC patients, respectively.

more sensitive than immunohistochemistry method. Second, different areas of the same tumor were sampled for PCR and immunohistochemistry test, respectively. Third, additional process such as post-transcriptional regulation, translation modification, and proteasomal degradation, might also play a role [23, 24]. Accordingly, it emphasizes the need to perform protein analysis rather than mRNA analysis in order to confirm the actual translation of OY-TES-1.

Here, the localization of OY-TES-1 protein demonstrated was mainly in the cytoplasm of CRC tumor cells, which was different from a recent report that showed OY-TES-1 protein in both cytoplasm and nucleus of mesenchymal stem cells derived from the human bone marrow [15]. OY-TES-1 protein distribution in different regions in various cells may reflect its functional disparity. Compared with mesenchymal stem cells in which the expression of OY-TES-1 protein was highly homogeneous, heterogeneous expression was present in CRC. This is consistent with epithelial ovarian cancer [12]. This heterogeneity appears to be a consistent morphologic feature of other CT antigens in the many tumor types tested thus far [21, 22, 24-26]. The underlying reason is still largely unknown. It is possible that the expression of CT antigen in tumor cells is not regulated genetically but epigenetically. For example, abnormal DNA methylation has been frequently observed in a subset of CT antigens [27, 28]. However, whether the heterogeneous expression pattern of OY-TES-1 in CRC is also related to DNA methylation needs further investigation.

As high percentage of OY-TES-1 expression in CRC, we further explore the possible relationship between OY-TES-1 expression and clinicopathological parameters. Our data suggested that OY-TES-1 protein expression was significantly correlated with tumor invasion stage and histological grade in CRC. Using TNM grading system, the depth of tumor invasion defines the T stage and increases from T1 to T4. As the depth of tumor invasion increases, the risk for nodal and distant spread also grows [29]. We observed OY-TES-1 protein expression at high frequency (51.1%) in T3 and T4 stage, but at low frequency (7.7%) in T1 and T2 stage. In addition, OY-TES-1 protein was more frequently identified in poorly differentiated CRC than moderately and well differentiated CRC. Since it is well known that the lower differentiation CRC are, the higher malignancy, and the worse prognosis [30-32]. Thus, OY-TES-1 may be used as a potential target for prognosis of CRC. A follow-up should be done to further illuminate

Table 2. Correlation among OY-TES-1 expression, antibody production and clinicopathological parameters in CRC

Clinicopathological parameters	mRNA		Protein		Antibody	
	Positive/Total (%)	*P* value	Positive/Total (%)	*P* value	Positive/Total (%)	*P* value
Gender						
Male	32/43 (74.4)	0.762	16/43 (37.2)	0.128	7/49 (14.3)	0.088
Female	12/17 (70.6)		10/17 (58.8)		0/24 (0)	
Age (year)						
≤ 55	27/33 (81.8)	0.100	16/33 (48.5)	0.373	6/36 (16.7)	0.103
> 55	17/27 (63.0)		10/27 (37.0)		1/37 (2.7)	
Tumor location						
Colon	16/24 (66.7)	0.578	10/24 (41.7)	0.832	3/36 (8.3)	1.000
Rectum	28/36 (77.8)		16/36 (44.4)		4/37 (10.8)	
Tumor size (cm)						
≤ 5	26/38 (68.4)	0.258	15/38 (39.5)	0.428	6/50 (12.0)	0.546
> 5	18/22 (81.8)		11/22 (50.0)		1/23 (4.3)	
Depth of tumor invasion						
T1-T2	8/13 (61.5)	0.277	1/13 (7.7)	0.004**	2/21 (9.5)	1.000
T3-T4	36/47 (76.6)		25/47 (53.2)		5/52 (9.6)	
Lymph node metastasis						
N0	33/45 (73.3)	1.000	20/45 (44.4)	0.764	4/48 (8.3)	0.931
N1-2	11/15 (73.3)		6/15 (40.0)		3/25 (12.0)	
Distant metastasis						
M0	40/54 (74.1)	1.000	23/54 (42.6)	1.000	7/66 (10.6)	1.000
M1	4/6 (66.7)		3/6 (50.0)		0/7 (0)	
TNM stage						
I + II	30/40 (75.0)	0.680	17/40 (42.5)	0.854	4/43 (9.3)	1.000
III + IV	14/20 (70.0)		9/20 (45.0)		3/30 (10.0)	
Histological type[a]						
Non-mucin-producing	39/53 (73.6)	1.000	21/53 (39.6)	0.234	2/8 (25.0)	0.351
Mucin-producing	5/7(71.4)		5/7 (71.4)		5/65 (7.7)	
Histological grade[b]						
G1	11/16 (68.8)		4/16 (25.0)		3/17 (17.6)	
G2	20/26 (76.9)	0.838	10/26 (38.5)	0.040*	1/36 (2.8)	0.144
G3	13/18 (72.2)		12/18 (66.7)		3/20 (15.0)	

[a]Non-mucin-producing cancer includes tubular and (or) papillary adenocarcinoma, Mucin-producing cancer includes mucinous cancer and signet-ring cell cancer. [b]Histologic grade (G): G1, well differentiated; G2, moderately differentiated; G3, poorly differentiated. **P=0.004. *P=0.040. G1 vs G2: P=0.574; G1 vs G3: P=0.020; G2 vs G3: P=0.066.

the correlation between OY-TES-1 expression and disease outcome.

In our non-tumor tissue setting, OY-TES-1 mRNA was detected in colorectal adenoma and CRC adjacent non-tumor tissues, but not in normal colons tested. It might be caused by transformation from benign to malignancy or by infiltration of surrounding tumors. Since the adenoma-to-carcinoma sequence in colorectal carcinogenesis was firstly brought forward in 1996, more and more studies demonstrated colorectal adenoma as a precursor lesion for CRC [33-35]. The significance for OY-TES-1 mRNA positive in colorectal adenoma and CRC adjacent tissues is hard to determine at this time. Interestingly, OY-TES-1 protein can not be totally detected in all of non-tumor tissues we tested except testis. Similar results have been shown in some CT antigens. Sugita et al [36] found a high frequency of NY-ESO-1 mRNA in 68% (21/31) of benign breast lesions by RT-PCR, while none of these cases showed positively stained by immunohistochemistry. Ano-

ther study revealed that although NY-ESO-1 mRNA was detected in both pancreas and liver, no corresponding protein has ever been reported in these tissues analyzed by immunohistochemistry [4]. Therefore, we speculates that OY-TES-1 protein in those non-tumor tissues was either extremely low if it presented, or unstable due to rapid protein degradation.

In an effort to evaluate the inherent immunogenicity of OY-TES-1 in CRC patients, 73 sera were studied for antibody status. We observed 9.6% (7/73) of CRC patients had antibody against OY-TES-1. In terms of antibody frequency, our data was similar with the previous study, which has reported anti-OY-TES-1 antibody was detected in 10.3% (6/58) of colon cancer patients [11]. In our survey of 25 CRC patients, where tumor and serum from the same patient were available, 2 of 22 CRC patients with OY-TES-1 mRNA-positive produced OY-TES-1 antibodies. One sero-positive patient showed strong positive expression of OY-TES-1 protein, while the other did not show protein reactivity. It can be speculated that the specific tumor is originally OY-TES-1-positive to elicit humoral antibody response. The lymphocytes can invade the tumor and disrupt malignant cells, thus the leaving cells have no or low OY-TES-1 expression for immunohistochemistry detection, while the anti-OY-TES-1 antibodies could remain in the patient's blood for some time. Another possible explanation is tumor heterogeneity not represented in the small tissue fragment analyzed by immunohistochemistry. We did not detect significant correlation between OY-TES-1 antibody production and clinicopathological parameters of CRC patients.

In summary, our findings demonstrate that OY-TES-1 exhibits the CT antigen characteristics. OY-TES-1 is frequently expressed in CRC and shows inherent immunogenicity. Hence, OY-TES-1, just like other important members of CT antigens, may be a potential target for active specific immunotherapy in CRC. Defining the prognostic significance and cell-mediated immune responses to this antigen are currently ongoing and will help elucidate the role of OY-TES-1 in CRC tumorigenesis.

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Disclosure of conflict of interest

None.

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