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Cancer testis antigen OY-TES-1: analysis of protein expression in ovarian cancer with tissue microarrays

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Summary

Objectives: The purpose of this study was to determine the potential of cancer testis antigen OY-TES-1 as a vaccine for ovarian cancer (OC). *Materials and Methods:* A tissue microarray (TMA) containing 107 samples from OC tissues and 48 samples from OC adjacent tissues was analyzed by immunohistochemistry with the OY-TES-1 polyclonal antibody. The correlation between OY-TES-1 and clinic pathological traits of OC was statistically analyzed. *Results:* The expression of OY-TES-1 protein was found in 81% (87/107) of OC tissues and 56% (27/48) of OC adjacent tissues. The immunostaining intensity of OY-TES-1 in OC tissues was significantly higher than that in OC adjacent tissues tested ($p = 0.040$). OC adjacent tissues only demonstrated lower immunostaining intensity, whereas some of OC tissues presented higher immunostaining intensity and majority showed the heterogeneity of protein distribution. There was no statistically significant correlation found between OY-TES-1 expression and any other clinicopathological traits such as age, FIGO stage, pathological grade, and histological type. *Conclusions:* OY-TES-1 was expressed in OC tissues with a high proportion, and some of OC tissues presented OY-TES-1 expression in high level vs OC adjacent tissues. OY-TES-1 could be an attractive target for immunotherapy for OC in the future.

Key words: OY-TES-1 protein; Ovarian cancer; Immunohistochemistry; Antigen; Immunotherapy.

Introduction

Ovarian cancer (OC) is the ninth most common cancer and the fifth leading cause of cancer death in women [1]. Survival rates approaching 90% have been reached among OC patients who were diagnosed at an early stage. Nonetheless, a challenge for early detection of OC still remains because non-specific symptoms of early ovarian lesions go unnoticed until the patient presents with an abdominal distension due to late-stage tumor growth and accumulation of ascites. Despite great improvements in surgical resection, chemotherapy, and radiotherapy, the long-term survival rate is only at 20% to 30% for advanced OC [2]. These "high-risk" patients have a short remission duration of 10 to 12 months and a recurrence rate of >70% [3]. Therefore, additional treatment such as immunotherapy for prolonging the life-span of patients with OC is needed [4].

An ideal candidate antigen for immunotherapy of any cancer type should show both inherent immunogenicity and differential expression in the cancer tissues. Cancer testis (CT) antigens represent a unique class of tumor antigens, which are expressed in a variety of cancerous tissues and are silent in normal tissues except for the testis. So far, there are many reports demonstrating that patients with tumors are able to elicit both specific cellular and humoral immune responses to these antigens. Therefore, CT antigens are considered to be ideal candidates for novel cancer immunotherapies with encouraging preliminary results [5-7].

OY-TES-1 has been firstly characterized as a CT antigen by Ono *et al*. [8]. It is the human homologue of acrosin binding protein (ACRBP), which was as the precursor of sp32 (sperm protein 32) originally identified in porcine, guinea pig, and mouse [9]. As of yet, it has been reported that OY-TES-1 can express in different cancerous tissues, but restrictively or doe not express in normal adult tissues except for testis. Moreover, it can raise humoral response in patients with a variety of tumor types including in bladder, prostate, liver, colon, lung, and ovary [8, 10]. However, there is limited information regarding the OY-TES-1 expression in OC tissues, and expression status of OY-TES-1 protein in OC adjacent tissues is not yet available. Therefore, in this study, the authors determined the frequency and intensity of expression of OY-TES-1 protein in OC tissues as well as adjacent tissues of OC, and evaluated the relationship between OY-TES-1 expression and clinicopathological parameters.

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Table 1. — *Correlation between OY-TES-1 expression and clinicopathological parameters.*

		Positive/total test (%)	*p* value	IOD**	95% CI	*p* value
OC tissues		87/107 (81)		542.30	341.26 - 2,965.14	
OC adjacent tissues		27/48 (56)	0.001	243.42	234.66 - 461.26	0.040
Age (years)	< 30	10/13 (77)		454.74	< 1767.30	
	30-60	64/79 (81)		542.30	711.82 - 1,474.19	
	> 60	13/15 (87)	0.798	630.08	< 15,200.56	0.423
FIGO Stage	I	40/49 (82)		529.09	578.74 - 1,094.82	
	II	17/23 (74)		608.49	348.39 - 1,454.07	
	III	15/17 (88)		521.03	334.88 - 3,597.98	
	IV	7/9 (77)		715.57	< 26,072.78	
	Not available*	8/9 (89)	0.776	495.36	228.42 - 1,322.69	0.900
Pathological Grade	1	23/30 (77)		556.40	442.92 - 113.66	
	2	27/32 (84)		479.73	469.40 - 1,347.75	
	3	37/45 (82)	0.723	630.08	< 5,904.13	0.612
Histology type	Papillary serous	65/78 (83)		596.10	148.61 - 3,746.24	
	Clear cell	4/7 (57)		529.09	238.68 - 692.28	
	Dysgerminoma	2/3 (67)		439.20	< 1,507.70	
	Mucinous	2/3 (67)		407.21	< 6,337.00	
	Metastatic	8/9 (89)		495.36	228.42 - 1,322.69	
	Others	6/7 (86)	0.524	630.08	< 3,126.52	0.885

*samples with metastasis; **the median of integrated optical density (IOD).

Materials and Methods

Patients and specimens

A panel of formalin-fixed paraffin-embedded human tissue microarray (TMA) included 107 cases of OC and 48 cases of OC adjacent tissues (Table 1). All tumors were classified according to World Health Organization (WHO) criteria [11]. The OC adjacent tissues were taken from the periphery of the lesion. The absent of pathological cells was confirmed by microscope. The testis tissue was collected an elderly patient with prostate cancer. The use of tissue was approved by Ethics Committee of the Hospital and informed consent was obtained from all patients.

siRNA transfection

The OC cell line SKOV3, known to express OY-TES-1, [10] was obtained from Shanghai Cell Collection of the Chinese Academy of Science and used for RNA interference (RNAi). The siRNA (sense: 5'- GACUAUAUCCAGUACCCAATT-3', antisense: 5'- UUGGGUACUGGGAUAUAGUCTG -3) targeting OY-TES-1 from 1486 to 1504 nucleotides (UniGene no. Hs.123239) and a scrambled siRNA were designed and synthesized. Briefly, transfection procedure was as followings: 1×10^5 cells/well was incubated in 24-well board, when cell confluence reached around 50-60%, the culture medium was changed with serum-free L-DMEM 24 hours before the transfection, then the cells were transfected with OY-TES-1 siRNA by using X-treme GENE siRNA transfection reagent. OY-TES-1 protein was detected in 24, 36, 48, and 72 hours after siRNA transfection with immunocytochemistry using OY-TES-1 antibody (Cat no. ab64809).

Immunocytochemistry and immunohistochemistry

TMA sections were deparaffinized in xylene, rehydrated by transfer through graded concentrations of ethanol to distilled water, and submitted to antigen retrieval in an 800W microwave containing 0.01 mol/L citrate buffer (pH = 6) for 15 minutes. Then, endogenous peroxidase activity was blocked by incubation with 0.03% H_20_2 for five minutes at room temperature. The slides were then incubated with polyclonal OY-TES-1 antibody (5 μg/ml) at 4°C overnight followed by horseradish peroxidase (HRP)-conjugated second antibody at room temperature for one hour. For immunocytochemistry the cells treated with siRNA were cultured on the cover slide and fixed with methanol, following immunostaining as above mentioned, but without antigen retrieval. All slides were developed by diaminobenzidine (DAB) for ten minutes and lightly counterstained with hematoxylin. Stained slides were dehydrated consecutively in graded ethanol, and finally transferred into xylene and mounted [12].

The expression status of OY-TES-1 was scored based on the number of immunostaining cells in three different fields of each slide. The extent of expression was graded as follows: negative, staining of single cells or small clusters of cells (<5% of cells stained); +, 5%-25%; ++, >25%-50%; +++, >50%-75%; ++++, >75% of cell stained. For quantitative analysis of OY-TES-1 protein the integrated optical density (IOD) was used by special image analysis software.

Statistical analysis

The associations between OY-TES-1 expression and clinicopathological parameters were evaluated using the χ^2 test or Kruskal Wallis test as appropriate. Statistical program for social sciences (SPSS) software (version 15) was used in all statistical analysis. A *p* value less than 0.05 was statistically considered significant.

Results

Study population

The characteristics of the study population are presented in Table 1. The median age of patients was 48 years (range 12-75). The 107 cases tested were in Federation International of Gynecology and Obstetrics (FIGO) Stage I to IV and included nine cases with metastatic cancer from other sites, all specimens presented pathological grades 1 to 3

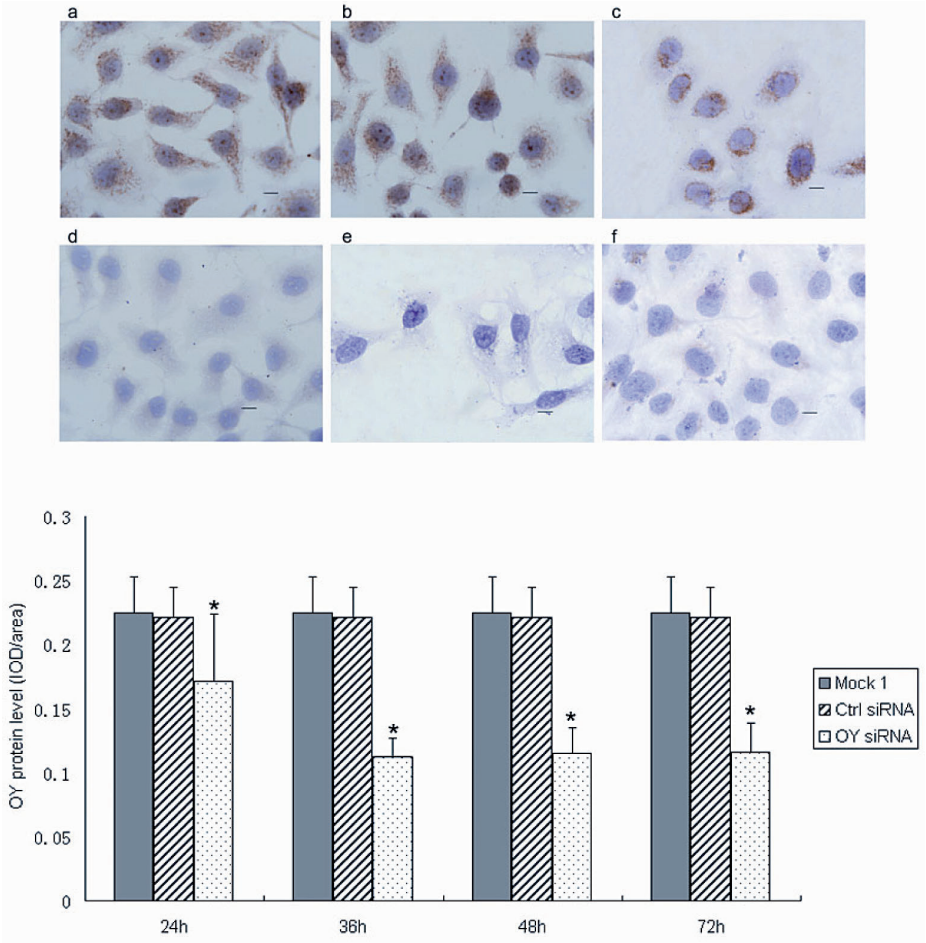

Figure 1. — Validation of OY-TES-1 antibody by RNAi reduced OY-TES-1 expression in SKOV3. (a) cells without transfection (Mock 1); (b): cells transfected with scrambled siRNA (Ctrl siRNA); (c-f) cells transfected with OY siRNA for 24, 36, 48, and 72 hours (OY siRNA), respectively. All sections were counter-staining with Hematoxylin (Bar: 20 μm). Bar graph represents quantitative analysis of OY-TES-1 protein with integrated optical density (IOD). Error bars represent average error from the mean (asterisk: $p < 0.05$).

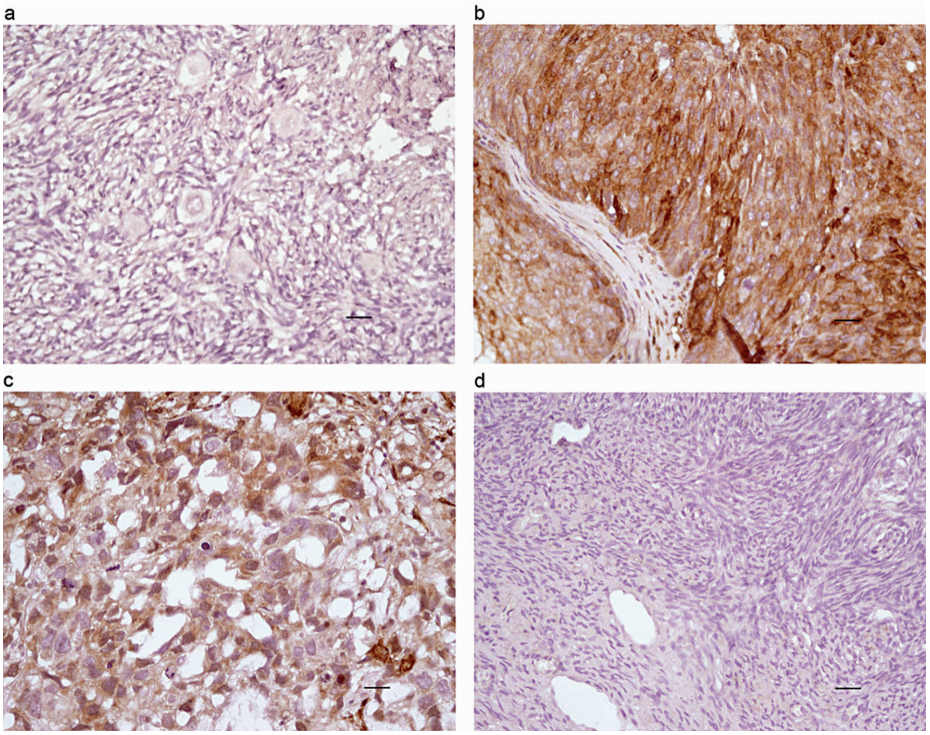

Figure 2. — Immunohistochemical staining of OY-TES-1 protein with OY-TES-1 antibody. (a, d): OC adjacent tissues; b-c: OC tissues; All sections were counter-staining with Hematoxylin (Bar: 50 μm).

Table 2. — *Immunostaining in OC tissue and OC adjacent tissue.*

Tissues	Staining intensity N (%)				χ^2 value	*p* value
	+	++	+++	++++		
OC adjacent tissues (n=27)	21 (78)	6 (22)	0 (0)	0 (0)		
OC tissues (n=87)	35 (40)	24 (28)	17 (20)	11 (12)	14.829	0.002
Grade 1 (n=23)	10 (44)	4 (17)	6 (26)	3 (13)		
Grade 2 (n=27)	7 (26)	11 (41)	5 (18)	4 (15)		
Grade 3 (n=37)	18 (48)	9 (24)	6 (16)	4 (11)	5.718	0.455

with different histological types. The majority of specimens was in FIGO Stage I (46%, 49/107), pathological grade 3 (42%, 45/107), and papillary serous (73%, 78/107). The OC adjacent tissues were taken from the periphery of the lesion. The absent of pathological cells was confirmed by microscope. The median age of patients was 40 years (range 14-69).

Validation of OY-TES-1 antibody

To validate the specificity of OY-TES-1 antibody, OY-TES-1 positive cell line of SKOV3 was used to RNAi. Down-regulation of OY-TES-1 was observed after transfection of OY-TES-1 siRNA in SKOV3 for 24 hours (Figure 1), which suggested that the OY-TES-1 antibody can specifically target OY-TES-1 protein.

Expression of OY-TES-1 in OC and OC adjacent tissues

The location of OY-TES-1 protein showed a predominantly, although not exclusively, cytoplasmic staining in OC tissues (Figure 2). The OY-TES-1 protein was detected in 81% (87/107) of OC tissues and 56% (27/48) of OC adjacent tissues, respectively. The expression frequency of OY-TES-1 protein in OC tissues was significantly higher than OC adjacent tissues ($p = 0.001$). Furthermore, IOD was used for quantitative analysis of OY-TES-1 protein. The results showed the difference of IOD median between the OC tissues and OC adjacent tissues ($p = 0.040$), and confirmed the higher expression of OY-TES-1 protein in OC tissues (IOD median = 542.30) as compared to OC adjacent tissues tested (IOD median = 243.42).

All specimens of OY-TES-1 protein positive from OC adjacent tissues only demonstrated lower immunostaining intensity (+ and ++). Whereas 87 specimens of OY-TES-1 protein positive from OC presented in different immunostaining intensity from + to ++++, among which 68% (59/87) of OC specimens with staining intensity of + and ++ demonstrated apparent heterogeneity of OY-TES-1 protein distribution (Table 2).

Correlation between OY-TES-1 expression and clinicopathological parameters

To evaluate whether the OY-TES-1 expression might be related to the clinicopathological parameters, 107 specimens from OC were analyzed. There was no statistically significant correlation between OY-TES-1 expression and any other clinicopathological traits (age, FIGO stage, pathological grade, and histology type) as shown in Table 1.

Discussion

OY-TES-1 has been annotated into a CT database by Ludwing Institute for Cancer Research as CT23 according to its list in the database (http://www.cta.lncc.br). In previous studies, the mRNA expression spectrum of OY-TES-1 in tumors included bladder cancer, breast cancer, colon cancer and liver cancer [8, 12, 13]. However, only a single study has been conducted with OC in which mRNA and protein of OY-TES-1 were detected in 23% (23/100) and 60% (60/100), respectively [10]. Here, the authors present the result with 81% of OC expressing OY-TES-1 protein. The discrepancy of OY-TES-1 protein expression rate in OC may relate to the different race and histological types. It may also result in use of different antibody. Tammela *et al.* [10] used monoclonal antibody against OY-TES-1, while the present authors applied the polyclonal antibody which should be lower specificity than monoclonal antibody, which was commonly considered to lower specificity than monoclonal antibody. However, this antibody the present authors used has been validated through combination of recombinant OY-TES-1 protein and RNAi, confirming to be specific for the target antigen. Although the present result demonstrated more than half of OC adjacent tissues with OY-TES-1 protein positive, its expressive level was lower comparing to the OC tissues. Therefore, application of OY-TES-1 immunotherapy for OC patients may not severely affect those tissues with low OY-TES-1 expression.

Nowadays the expression of several CT antigens, such as SPAG9 in 90% [14], SP17 in 68% [15], CT45 in 37% [16], NY-ESO-1 in 30%, MAGE-1 in 28% [17], SCP-1 in 15% [18], SSX4 in 12.5% [19], MAGE-3 in 7% [17], and SSX2 in 2.5% [19], have been reported in OC. Among these CT antigens, NY-ESO-1 has been successfully applied to immunotherapy for OC patients. After primary surgery and chemotherapy, high-risk OC patients in first clinical remission received NY-ESO-1b peptide without serious vaccine-related adverse events. Vaccine-induced CD8+ and CD4+ T cell clones were shown to recognize

NY-ESO-1-expressing tumor targets. Long-lived and functional vaccine-elicited CD8+ and CD4+T cells were detectable in some patients up to 12 months after immunization [20, 21]. As OY-TES-1 has higher frequency in OC demonstrated by the present authors and others, it seems to be potential in the future to develop OY-TES-1 immunotherapy for OC. However, the present result also indicated the majority of cases with immunostaining intensity and heterogeneity of OY-TES-1 protein expression, which may affect OY-TES-1 as an immunotherapeutic target applied at least in OC. It is unknown whether the heterogeneity of OY-TES-1 protein expression may result from DNA melthylation and/or histone acetylation. It has been demonstrated that the epigenetic events influence some of CT gene expression, such as MAGE family is responsible for the gene demethylation [5,22]. Therefore, the mechanism of OY-TES-1 expression should be further investigated. If the expression of OY-TES-1 is regulated by the methylation of CpG islets in its promoter region, utility of combining demethylating agent 5-aza-2'-deoxycytidine (DAC) therapy with OY-TES-1 vaccine therapy may help to improve the heterogenic expression of OY-TES-1 that will greatly increase the efficiency of treatment.

Recently, it was found that OY-TES-1 was both necessary and sufficient for paclitaxel resistance in ovarian cancer cell lines and ovarian tumor explants. Moreover, high expression of OY-TES-1 was correlated with reduced survival time and faster relapse among ovarian cancer patients [10, 23]. Although no correlation between OY-TES-1 expression and clinicopathological parameters in OC was found in the present study, one-third of OC presented moderate (+++) to strong (++++) expression, which may imply immunotherapy suitability for these patients. The high level of OY-TES-1 expression may also be predictive of humoral immune response and cellular immune response presented in OC patients. It has been observed that an OC patient with specific humoral response to OY-TES-1 initially had optimal surgical debulking of Stage IIIC, and was without evidence of disease 40 months after chemotherapy [10]. Moreover, a HLA-A24-bingding peptide of OY-TES-1 can be recognized by CD 8+ T cells and induced cytotoxic reaction against tumor cell line expressing OY-TES-1 [24]. Clearly, further extensive analysis of the immune responses in patients including serum antibody as well as T-cell responses will be necessary for development of OC immunotherapy based on the OY-TES-1 as a target.

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