Serum immunoreactivity of cancer/testis antigen
OY-TES-1 and its tissues expression in glioma

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Received August 2, 2015; Accepted January 10, 2017

Abstract. OY-TES-1 is a member of the cancer/testis antigen family that is expressed in healthy testis tissue and certain types of cancerous tissue. The present study aimed to analyze the expression pattern of OY-TES-1 and serum anti-OY-TES-1 antibody concentration in patients with glioma. OY-TES-1 mRNA was detected in 28/36 (78%) of glioma cases using conventional reverse transcription polymerase chain reaction (RT-PCR) analysis. RT-quantitative-PCR revealed that OY-TES-1 was expressed at a higher level in glioma tissues compared with normal adult tissues (with the exception of testis tissue). Anti-OY-TES-1 antibodies were present in the serum of 5/36 (14%) of patients with glioma, but absent in all the serum samples from 107 healthy donors. Immunohistochemical analysis demonstrated that OY-TES-1 protein was expressed in all glioma tissues from patients with anti-OY-TES-1 antibody seropositivity. These results suggest that OY-TES-1 is a novel candidate for glioma immunotherapy.

Introduction

Glioma is one of the most common primary intracranial tumors of the central nervous system, with an annual incidence of ~6/100,000 individuals in the USA (1), accounting for 40-50% of brain tumors. The median survival time of patients with glioblastoma multiforme (GBM), the most aggressive subtype of malignant glioma, is 12-16 months (2). Despite the advances made in multimodal treatment, including surgery, radiotherapy and chemotherapy, the prognosis for the majority of patients with malignant glioma remains poor, with a median survival time of 14.6 months (3). Therefore, the development of novel therapeutic strategies is required.

A potential lifespan-prolonging approach for patients with glioma is to administer immunotherapy during the course of treatment (4). This approach requires suitable tumor antigens with specific characteristics, including high expression levels in glioma tissues, restricted expression levels in normal tissues and inherent immunogenicity. The expression of a potential tumor antigen, cancer/testis (CT) antigen, is restricted to adult testicular germ cells under normal conditions, but aberrantly activated and expressed in a range of tumor types such as melanoma, bladder, breast, prostate, liver, ovarian, colon and non-small cell lung cancer (5-9). As the testes do not express major histocompatibility complex class I, cytotoxic T lymphocytes do not target the CT antigens expressed in the testis (10). At present, CT antigens are considered to be novel targets for immunotherapy in various tumor types, including glioma (11,12).

OY-TES-1, a member of the CT antigen family, is the human homolog of proacrosin binding protein sp32, which was originally identified in pigs, guinea pigs and mice (13,14). OY-TES-1 has been the subject of numerous recent studies (15-17) due to its expression in various cancerous tissues and restricted expression in normal tissues. Furthermore, OY-TES-1 is able to increase the humoral immune response across a broad spectrum of cancer types, including that of the bladder, prostate, liver, colon, lung and ovaries (13,15,18). To the best of our knowledge there is no data available at present regarding the expression profile and immunogenicity of OY-TES-1 in patients with brain tumors. Whether OY-TES-1 is expressed in glioma tissue, and induces an immune response in patients with glioma, requires further investigation. Therefore, the present study was performed to examine the expression levels and serum immunoreactivity of OY-TES-1 in human glioma.

Materials and methods

Patients and specimens. The use of human tissue specimens in the present retrospective study was approved by the Ethics
Committee of the First Affiliated Hospital of Guangxi Medical University (Nanning, China) and the written informed consent was obtained from all patients included in this study. A total of 36 tumor tissue samples (11 GBM, 7 anaplastic astrocytoma, 11 diffuse astrocytoma, 4 pilocytic astrocytoma, 1 anaplastic ependymoma, 1 pleomorphic xanthoastrocytoma and 1 ependymoma) and 20 preoperative serum samples were obtained from in-patients at the Department of Neurosurgery at the First Affiliated Hospital of Guangxi Medical University between March 2013 and March 2014. Their clinical data were retrospectively reviewed. All patients underwent resection of the primary tumor and did not receive radiotherapy or chemotherapy prior to surgery. The mean patient age was 36.4 years (range, 3-65) and the gender distribution was 21 males and 15 females. All tumor specimens were classified according to the World Health Organization (WHO) criteria (19), with 17 cases of WHO grade I-II (low-grade) and 19 cases of WHO grade III-IV (high-grade) identified. Serum samples from 107 healthy donors (54 male; 53 female) and 7 types of normal tissue samples (3 testis, 3 kidney, 3 thyroid, 3 appendix, spleen, tonsil and normal brain) were collected between May 2013 and March 2014 and were used as controls.

Reverse transcription polymerase chain reaction (RT-PCR) analysis. Total RNA was isolated from frozen tumor tissues using the RNeasy Total RNA kit (TianGen Biotech Co., Ltd., Beijing, China), according to the protocol of the manufacturer. The concentration of isolated RNA was determined by spectrophotometer (SmartSpec™ plus; Bio-Rad Laboratories, Inc., Hercules, CA, USA), then 4 µg total RNA was reverse-transcribed into single-stranded cDNA with PrimeScript II First Strand cDNA Synthesis kit (Takara Biotechnology Co., Dalian, China). The integrity of obtained cDNA was tested by amplification of p53 transcripts in a 30-cycle PCR reaction as previously described (20). The OY-TES-1 gene was amplified using established primers (21). The PCR reactions were in a total volume of 25 µl containing 0.125 µl of Takara Ex Taq HS (Takara Biotechnology Co., Japan). The PCR cycling parameters were as follows: Initial denaturation at 94˚C, 5 min; denaturation at 94˚C, 1 min; annealing at 58˚C, 1 min; extension at 72˚C, 2 min, for 35 cycles; and final extension at 72˚C, 8 min. Tumor protein (p)53 served as an internal control for normalization, as previously described (20). The quality of the RNAs and the PCR product was examined by electrophoresis on 1% agarose gel and observed under Gel Documentation and Analysis system (Uvipro 7600Z; UVItec Ltd., Cambridge, UK).

Reverse transcription quantitative PCR (RT-qPCR). The presence of OY-TES-1 mRNA was quantitatively detected using the iCycler iQ™ Multi-Color Real-Time PCR Detection system (Bio-Rad Laboratories, Inc.) with the following primer sequences: Sense, 5'-GCG ACA CCT CCC ACA AGAC-3' and antisense, 5'-GCCACCTCCCAACAAGAC-3'. The following 6-carboxyfluorescin (FAM)-labeled TaqMan probe was also used: 5'-FAM CAAACCAGTAGGGTCC TAMRA-3'. The amplified product consisted of a 123 bp segment of the OY-TES-1 gene. The PCR reactions were in a total volume of 25 µl containing 2.5 µl 10xbuffer, 5 µl 25 mM MgCl₂, 1 µl 2.5 mM dNTP, 0.75 µl 10 µM forward primer, 0.75 µl 10 µM reverse primer, 1 µl 10 µM probe primer, 12 µl RNase-free H₂O and 2 µl cDNA template. Thermocycling conditions were as follows: 95˚C for 2 min, followed by 40 cycles of 95˚C for 5 sec and 60˚C for 20 sec. The target OY-TES-1 mRNA was quantified by measuring the Cq value (22). The Cq value was defined as the threshold cycle number at which the fluorescence generated by cleavage of the probe passed above the baseline value. The value of the target OY-TES-1 mRNA in each sample was normalized to hypoxanthine phosphoribosyl transferase (HPRT) amplification (23). All samples were run in triplicate.

ELISA analysis. Serum antibody against OY-TES-1 was detected by ELISA, as described previously (15). The recombinant OY-TES-1 protein (15) and maltose binding protein (MBP; blank control) (15) were diluted serially from 1:100 to 1:3,200, coated onto 96-well plates and incubated at 4˚C overnight. Subsequently, the plates were blocked with 5% nonfat milk and incubated with serum (1:400; 100 µl/well) at 37˚C for 1 h, followed by incubation with horseradish peroxidase (HRP)-conjugated sheep anti human IgG (cat. no. 109-035-003; dilution, 1:5,000; Jackson ImmunoResearch, West Grove, PA, USA). Finally, the plates were incubated with 3,3',5,5'-tetramethylbenzidine at room temperature for 20 min, and 2 mol/l sulfuric acid was added to terminate the reaction. The absorbance was measured at a wavelength of 450 nm using a microplate reader. The healthy donor serum samples were used as negative controls. All the serum samples were evaluated ≥2 times. A positive reaction was defined as an optical density (OD) value that exceeded the mean OD of the healthy donor sera by three standard deviations.

Immunohistochemistry (IHC). IHC was performed using the tissue samples from patients with glioma who were anti-OY-TES-1 antibody seropositive. The testis and normal brain tissues were used as positive and negative controls, respectively. The IHC procedure was performed according to a previous protocol (15). In brief, deparaffinized tissue sections underwent heat-based antigen retrieval in citrate buffer (pH 6.0, 10 mM). Following the inactivation of endogenous peroxidase, the tissue sections were incubated with an anti-OY-TES-1 primary antibody (cat. no. ab64809; dilution, 1:1,000; Abcam, Cambridge, UK) or rabbit pre-immune serum (negative control) (15) at 4˚C overnight. Subsequently, the tissue sections were washed and incubated with a HRP-labeled goat anti-rabbit IgG (cat. no. D-3004; dilution, 1:500; Shanghai Long Island Biotec, Shanghai, China) at room temperature for 1 h, labeled with 3,3'-diaminobenzidine and counterstained with hematoxylin. Then they were viewed under an optical microscopy (Olympus BX53; Olympus Corporation, Tokyo, Japan).

Sequencing analysis. The open reading frame (ORF) of OY-TES-1 was amplified from the cDNA of tumor tissues using PCR with specific primers as follows: Sense, 5'-GGCGGC GGATCTTTCGCCGCCATG-3' and antisense, 5'-ACCGGAT CTTATCATTTGGGCGTGGTGT-3'. A total of 35 PCR amplification cycles were performed, each consisting of
denaturation at 98°C for 10 sec, followed by annealing at 63°C for 15 sec and extension at 72°C for 2 min. The final extension step was performed at 72°C for 10 min. PCR products were purified and ligated into pMD8-T vectors (Takara Biotechnology Co.), which were transformed into DH5α competent cells (Beijing TransGen Biotech Co., Ltd., Beijing, China) (24). The transformed cells were smeared on LB-ampicillin agar plates containing X-gal. White colonies were screened and then inoculated into 5 ml bacterial culture medium overnight. Plasmid was extracted by EZ Spine Column Plasmid Mini-Preps kit (Sangon Biotechnology Co., Shanghai, China) and verified by PCR, as previously described (21). Clones with the correct insertion were identified via Sanger sequencing in 3730XL DNA Analyzer (Sino Genomax Co., Ltd., Beijing, China).

**Statistical analysis.** Results are presented as the mean ± (SD). All statistical analyses were performed using SPSS version 15.0 (SPSS Inc., Chicago, IL, USA). The association between gene expression levels, the presence of antibodies in the sera and the clinicopathological characteristics of patients with glioma was evaluated using Fisher’s exact test. The expression levels of OY-TES-1, relative to HPRT, in glioma samples of different WHO grades and normal tissues were compared using the Mann-Whitney U test. P<0.05 was considered to indicate a statistically significant difference.

**Results**

**OY-TES-1 mRNA expression is upregulated in glioma.** To examine the presence of OY-TES-1 mRNA in glioma, conventional RT-PCR was initially performed to detect OY-TES-1 transcription. A total of 78% (28/36) of the glioma tissue samples were OY-TES-1 mRNA positive (Fig. 1A), with 76% (13/17) of low-grade tumors and 79% (15/19) of high-grade tumors positive for OY-TES-1 mRNA. Subsequently, the association between OY-TES-1 mRNA expression and the clinicopathological characteristic of patients with glioma, including gender, age and WHO tumor grade, were examined. As presented in Table I, no significant association was

| Clinicopathological characteristic | No. of mRNA positive patients/total (%) | P-value<sup>a</sup> | No. of serum anti-OY-TES-1 antibody positive patients/total (%) | P-value<sup>a</sup> |
|-----------------------------------|----------------------------------------|---------------------|---------------------------------------------------------------|---------------------|
| Gender                            |                                        |                     |                                                               |                     |
| Male                              | 16/21 (76.2)                           | 1.00                | 3/21 (14.3)                                                   | 1.00                |
| Female                            | 12/15 (80.0)                           |                     | 2/15 (13.3)                                                   |                     |
| Age, years                        |                                        |                     |                                                               |                     |
| <30                                | 9/13 (69.2)                            | 0.42                | 3/13 (23.1)                                                   | 0.34                |
| ≥30                                | 19/23 (82.6)                           |                     | 2/23 (8.67)                                                   |                     |
| WHO tumor grade                   |                                        |                     |                                                               |                     |
| I-II                              | 13/17 (76.5)                           | 1.00                | 3/17 (17.6)                                                   | 0.65                |
| III-IV                            | 15/19 (78.9)                           |                     | 2/19 (10.5)                                                   |                     |

<sup>a</sup>P-values were calculated using Fisher's exact test. WHO, World Health Organization.

Figure 1. Expression of OY-TES-1 mRNA in glioma tissue. (A) Representative 12 RT-PCR results of OY-TES-1 expression in glioma (lanes 1-12), positive control testis samples (lane P) and negative controls with no cDNA (lane N). p53 was amplified as the internal control. (B) RT-qPCR analysis of OY-TES-1 mRNA. OY-TES-1 mRNA was elevated in glioma tissues compared with normal adult tissues, with the exception of testis tissue. The levels of OY-TES-1 mRNA in glioma tissues were significantly higher than in normal brain tissues (P=0.0015). (C) Comparison of OY-TES-1 mRNA between WHO grade I-II and III-IV glioma tissues, as examined by RT-qPCR. The level of OY-TES-1 mRNA was significantly higher in high-grade compared with low-grade glioma tissues (P=0.0002 vs. WHO I-II). Results are presented as the mean ± standard deviation. RT-PCR, reverse transcription-polymerase chain reaction; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; p53, tumor protein 53; WHO, World Health Organization; HPRT, hypoxanthine phosphoribosyl transferase.
observed between the expression of OY-TES-1 mRNA and the clinicopathological parameters.

As a high frequency of OY-TES-1 mRNA expression was present in the glioma tumor samples, the expression pattern of OY-TES-1 mRNA was further examined. RT-qPCR analysis demonstrated that the OY-TES-1 mRNA expression levels in glioma tissues were markedly elevated compared with normal tissues (with the exception of testis tissue); but significantly higher than normal brain tissues (P=0.0015; Fig. 1B). The RT-qPCR results also revealed that high-grade tumors expressed significantly higher levels of OY-TES-1 mRNA compared with low-grade tumors (P=0.0002; Fig. 1C).

Anti-OY-TES-1 antibody is present in the patient serum samples. The expression of serum antibodies directed against OY-TES-1 was analyzed in 36 patients with glioma and 107 healthy donors by ELISA analysis. The OY-TES-1 antibody was detected in the serum of 5/36 (14%) patients with glioma, whereas the sera of all healthy donors were negative for the anti-OY-TES-1 antibody (Fig. 2A). Titration curves were produced for anti-OY-TES-1 antibody-positive
and -negative sera from representative patients with glioma, 
in addition to a healthy donor control, using the recombinant 
OY-TES-1 protein (Fig. 2B). The possibility of an association 
between the presence of anti-OY-TES-1 antibodies in the sera 
and the clinicopathological characteristics of patients with 
glioma was evaluated, but no statistically significant associa-
tion was identified (Table I).

**OY-TES-1 protein is detectable in glioma tissues from** 
**anti-OY-TES-1 antibody seropositive patients.** As the glioma 
tissues from the 5 anti-OY-TES-1 antibody seropositive 
patients were all positive for the presence of OY-TES-1 mRNA, 
the expression of the OY-TES-1 protein in glioma tissues was 
examined (Fig. 3). IHC detected the OY-TES-1 protein in all 
the glioma tissue. Furthermore, it was observed that OY-TES-1 
protein was primarily localized in the cytoplasm of the tumor 
cells, with occasional positive staining in the nuclei. In certain 
cases, heterogeneity of OY-TES-1 protein expression was 
observed in tumor tissues.

**No aberration is observed in the ORF of OY-TES-1 in glioma** 
tissues from anti-OY-TES-1 antibody seropositive patients. 
To determine whether the humoral immune response against 
OY-TES-1 in the patients with glioma was due to an aberrance 
in this gene, the full-length ORF of the OY-TES-1 gene was 
amplified from the tumor tissues of anti-OY-TES-1 antibody 
seropositive patients and sequenced. No aberrant changes, 
including mutations, deletions or insertions, were detected in 
the ORF of the OY-TES-1 gene (data not shown).

**Discussion**

As a member of the CT antigen family, OY-TES-1 is listed 
in the database of the Ludwig Institute for Cancer Research 
(Brussels, Belgium) (25), in which it is also named as CT23. 
OY-TES-1 is frequently expressed at the mRNA level in various 
types of cancer (13,15,18). OY-TES-1 protein is expressed in 
~60% of ovarian (15) and ~43% of colorectal (18) tumors. To 
the best of our knowledge, OY-TES-1 expression patterns at 
the mRNA and protein level, and its immunogenicity in brain 
tumors, including glioma, have yet to be elucidated.

The present study demonstrated that 78% of the glioma 
tissue samples expressed OY-TES-1 mRNA, which was 
detected via conventional RT-PCR. The levels of OY-TES-1 
expression were high compared with those observed in 
previous studies of OY-TES-1 mRNA expression using the 
same primers in other types of cancer, including bladder 
(11/39, 28%), breast (2/5, 40%), colon (2/13, 15%), liver (2/5, 
40%), ovarian (23/100, 23%) and colorectal (44/60, 73%) 
cancer (13,15,18). Due to the high proportion of glioma 
tissue samples in the present study that expressed OY-TES-1, the 
quantity of OY-TES-1 mRNA was investigated using RT-qPCR. 
The results revealed that OY-TES-1 mRNA expression was elevated 
in glioma, compared with a panel of normal tissues (with the 
exception of the testis) but significantly higher than normal 
brain tissues (P=0.0015). The data suggest that OY-TES-1 is 
a novel target for the treatment of glioma, from an immuno-
therapeutic standpoint.

The present study investigated the association between 
OY-TES-1 mRNA expression and the clinicopathological 
characteristics of patients with glioma. The data suggested 
that there is no significant association between the presence 
of OY-TES-1 mRNA and clinicopathological characteristics 
in glioma; however, the level of OY-TES-1 mRNA was 
significantly higher in high-grade compared with low-grade 
glioma samples. It has been established that a higher grade of 
glioma is correlated with greater malignancy and a poorer 
prognosis (26). Therefore, the expression of OY-TES-1 mRNA 
may be used as a novel prognostic marker for glioma. 
Follow-up studies are required to further investigate the associ-
ation between the quantity of OY-TES-1 mRNA and patient 
outcome.

Although the brain is located in an immune-privileged anatomical site, a humoral immune response to several tumor antigens has been detected in patients with glioma (27-29), 
suggesting that the brain is not completely immunoprivileged. 
The presence of antibodies against a number of tumor antigens 
has been examined in association with the survival of patients 
with glioma (30). The current study examined OY-TES-1 seroreactivity in patients with glioma, in addition to healthy 
individuals. The results demonstrated that 5/36 (14%) of 
patients with glioma had the anti-OY-TES-1 antibody present 
in their serum, whereas this antibody was not expressed in 
any of the serum samples from healthy donors 0/107 (0%). The 
anti-OY-TES-1 antibody has previously been observed in 
patients with other types of cancer, with a frequency of 
4.8-40% (13,15). A previous study detected the presence of the 
anti-OY-TES-1 antibody in 9.6% of patients with colorectal 
cancer (15), a result concordant with data from the current 
study in patients with glioma. However, no significant associa-
tion was identified between the presence of the anti-OY-TES-1 
antibody in the serum and the clinicopathological characteris-
tics of patients with glioma in the present study.

The molecular mechanisms underlying the immune 
response against OY-TES-1 in patients with glioma requires 
further investigation, as our serum immunoreactive result 
demonstrated that OY-TES-1 may possess immunogenic 
potential in patients with glioma. The present study utilized 
IHC to examine glioma tissues from anti-OY-TES-1 antibody 
seropositive patients. The results revealed that the OY-TES-1 
protein was detectable in all the glioma tissue samples, and 
was primarily localized to the cytoplasm of tumor cells. As 
OY-TES-1 is not localized to the cell surface, it may be 
hypothesized that a mutation in the gene encoding OY-TES-1 
may trigger a humoral immune response against OY-TES-1 in 
patients with brain tumors, in a similar manner to the mutated 
version of the p53 tumor suppressor gene detected in colon 
cancer and glioma, which raises the levels of the corresponding 
antibodies in patient's sera (31-32). However, the sequence 
analysis performed in the present study revealed no significant 
variation in the ORF of the OY-TES-1 gene, providing no 
support for this hypothesis.

Therefore, enhanced levels of OY-TES-1 expression may 
occur as a result of the immune response to cancer, as in 
the case of human epidermal growth receptor 2, an oncogene 
that is amplified in certain types of cancer (33). A previous 
study suggested that large quantities of immunocompetent 
cells, including B lymphocytes, are able to invade the tissue of 
malignant gliomas with a large necrotic area (34), increasing 
the possibility of an interaction between immunocompetent
cells and amplified gene products, which are able to function as antigens. An alternative hypothesis is that the humoral response against OY-TES-1 may be induced by the products of post-translational modifications of the OY-TES-1 protein. Previous studies have reported that sumoylated or hyperphosphorylated proteins may serve as autoimmunogenic targets, including sumoylated heat shock protein 90 (35) and hyperphosphorylated paratarg-7 (36). Thus, the presence of a humoral response against OY-TES-1 in patients with glioma may be a predictor of the cellular immune response, as is the case with NY-ESO-1, a CT antigen defined using serological analysis of recombinant expression libraries, in esophageal cancer (37).

In conclusion, the results of the present study indicated that OY-TES-1 mRNA is expressed in a high proportion of glioma tissues and possesses inherent immunogenicity. Therefore, it may present a novel target for specific immunotherapy in the treatment of brain tumors, particularly glioma. At present, determination of the prognosis using follow-up data and the cell-mediated immune response to OY-TES-1 is under investigation, which may aid understanding of the role of OY-TES-1 in tumorigenesis.

Acknowledgements

The present was supported by the National Natural Science Foundation of China (grant nos. 81360371 and 81360374), Guangxi Nature Science Foundation (grant nos. 0728148, 0832144 and 2011GXNSFA 018275) and the Guangxi Educational Bureau (grant no. BGZX2007010). The authors would like to thank Ms. Fang Chen for her technical assistance.

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