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Original Article

Expression status and prognostic value of cancer/testis antigen OY-TES-1 in glioma

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Abstract: Cancer testis (CT) antigens are attractive therapeutic targets for tumor immunotherapy because of their restrictive expression in normal tissues and excessive in majority of tumor types. OY-TES-1 is a member of the cancer/testis (CT) antigen family. Current research about OY-TES-1 expression in glioma is practically at mRNA level. The role of OY-TES-1 protein in glioma has not yet been described. In this study, we detected OY-TES-1 protein expression in 124 samples from patients with glioma by immunohistochemistry and analyzed the correlation between OY-TES-1 expression and clinical indexes. Furthermore, its clinical significance on glioma prognosis was determined by follow-up data. Our results showed that the OY-TES-1 staining was mainly located in the cell cytoplasm and nucleus and the total positive rate of its protein was 69.35% in 124 isolated tissue samples. OY-TES-1 expression was closely related with the WHO glioma grade. Kaplan-Meier analysis revealed a significant negative correlation between OY-TES-1 expression and survival. OY-TES-1 expression may be a candidate biomarker for prediction of glioma progression and survival of patients.

Keywords: Cancer/testis antigen, OY-TES-1, glioma, prognostic

Introduction

Glioma is the most common central nervous system tumor which accounted for about 50% [1]. Among the different types of gliomas, glioblastoma is one of the most malignant tumors whose median survival time is only about 14 months [2]. Currently, the traditional treatment method is surgery combined with adjuvant radiotherapy and chemotherapy [3]. Unfortunately, it is still failed to significantly prolong the survival of patients with glioma. The main causes are the presence of blood brain barrier making chemotherapy drugs difficultly enter the cranio-cerebral, drug resistance of glioma [4] and diffuse infiltrating of tumor cells giving rise to the low total resection rate of glioma. In addition, the presence of cancer stem cell which acting as the source of tumors lead to high relapse rates and high metastasis rate [5]. Therefore, it is urgent to develop a new adjuvant therapy. Immunotherapy is one of such desirable treatment methods. For many years the central nervous system was believed to have immune privilege [6]. But in recent years, the study found the destruction of blood brain barrier would be happened in some malignant glioma patients. Astrocytes and microglia cells have ability of antigen presentation, thus immunocyte could enter the brain tissues. These pathological changes provide the evidence of practicability of brain tumor immunotherapy [7].

All the immunotherapy are premised on the basis of suitable tumor associated antigen [8]. Cancer testis antigen (CTA) is such a group of tumor antigens because they are almost expressed in majority of tumors and restrictively expressed in normal tissues (except for testis and placenta). It has been proved that a part of CTA could cause the humoral and cellular immune response [9]. These properties render

Table 1. Clinical parameters of patients with glioma

Variable	n = 124	
	No.	(%)
Gender		
Male	78	62.90
Female	46	37.10
Age (years)		
<38	62	50.00
≥38	62	50.00
Tumor size (cm)*		
<5	54	43.55
≥5	60	48.39
WHO grade		
I-II	55	44.35
III-IV	69	55.65
KPS#		
<70	52	41.94
≥70	61	49.19

Abbreviations: WHO grade, 2007 World Health Organization Classification of Tumors of the Nervous System; KPS, Karnofsky Performance Scale; *10 cases of specimens without records of tumor size. #11 cases of specimens without KPS score data.

them as attractive targets for cancer immunotherapy. Currently, tumor immunotherapy targeting some of cancer testis antigens, such as MAGE-A1, MAGE-A3 and NY-ESO-1 were already on the stage of clinical trials and got satisfied results [10-12]. However, there are only a few relevant reports of CTA expression in glioma at the present stage [13-15]. The lack of understanding of CTA expression in glioma restricts the further study of CTA application in immunotherapy.

OY-TES-1, as a member of CTA family, is also called CT23 according to its ranking in CTA database (http://www.cta.lncc.br). It was initially identified as the human homologue of proacrosin binding protein sp32 precursor and first reported by Ono T in 2001 [16]. In human cells, OY-TES-1 is located on chromosome 12p12-p13 and contains ten exons. It has been confirmed that OY-TES-1 mRNA was not expressed in a variety of normal tissues (except for testis) but detected in multiple tumors tissues (mammary carcinoma, hepatoma, colorectal carcinoma and epithelial ovarian cancer) with 15% to 40% positive rate [16, 17]. Some tumor cell lines, such as ovarian cancer cell lines, prostate cancer cell lines, lung cancer cell lines and breast cancer cell lines were also certified expressing OY-TES-1 mRNA. In addition, Tammela et al [17] detected OY-TES-1 protein expression in different pathological types of ovarian cancer and found its positive rate of protein expression was much higher than in normal tissues. In assay of serum antibody, about 3% to 10% of cancer patients have progressed humoral immune response to OY-TES-1 but no corresponding antibody could be detected in healthy human serum [18]. In the identification of antigenic peptides, Okumura [19] identified HLA-A24 restricted antigen peptide, which is located at the carboxyl terminus of OY-TES-1 (TES 401-409). Vitro experiment results showed that Cytotoxic T lymphocyte (CTL), which induced by dendritic cells (DCs) loaded with this antigen peptide could specifically kill OY-TES-1 mRNA positive tumor cell lines. Our previous study had found CTL induced by OY-TES-1 fusion protein sensitized DC could effectively attack and kill hepatoma cells as well. In 2010, Whitehurst [20] found that OY-TES-1 was both necessary and sufficient for paclitaxel resistance in ovarian cancer cell lines and ovarian tumor explants. Moreover, high OY-TES-1 expression indicated shorter survival time and faster relapses among ovarian cancer patients. Recent studies have showed that OY-TES-1 was related with apoptosis, migration and invasion of tumor cells [21-23].

All of the findings above suggested that OY-TES-1 may be used as candidate target gene for tumor immunotherapy. Our group had conformed previously that the expression rate of OY-TES-1 mRNA in glioma tissues was 80.4% (41/51) and the protein expression was also detected in glioma. Besides, normal brain tissues and adjacent non-tumor tissues were not expressed OY-TES-1. It prompted that OY-TES-1 may be an adaptive target for glioma immunotherapy as well. However, our previous study focus attention on mRNA level but the biological effects of gene mainly work on the protein level. Therefore in our present study, we analyzed OY-TES-1 protein in 124 glioma patients and further evaluate associations of OY-TES-1 overexpression with clinical indexes (age, sex, tumor size, WHO grade and KPS) and clinical outcome by follow-up data. Our results will provide novel evidences for glioma immunotherapy possibility.

Table 2. Clinical parameters of follow up patients with glioma

Variable	n = 84	
	No.	(%)
Gender		
Male	56	66.67
Female	28	33.33
Age (years)		
<39	41	48.81
≥39	43	51.19
Tumor size (cm)		
<5	38	45.24
≥5	46	54.76
WHO grade		
I-II	39	46.43
III-IV	45	53.57
KPS		
<70	35	41.67
≥70	49	58.33

Abbreviations: WHO grade, 2007 World Health Organization Classification of Tumors of the Nervous System; KPS, Karnofsky Performance Scale.

Materials and methods

Ethics statement

The study was approved by the Ethics Committee of the Guangxi Medical University. The data were analyzed anonymously after signing informed consents by patients.

Tissue samples

A total of 124 patients with glioma were examined in this study (**Table 1**). Surgically resected tumor tissues from patients with confirmed pathological diagnosis were collected at the First Affiliated Hospital of Guangxi Medical University between September 2009 and June 2013. These patients consisted of 78 males and 46 females, and the age range was from 2 to 78 years (median 38 years). Tumor staging was evaluated based on the 2007 WHO classification of the nervous system tumors [24, 25]. Low grade glioma (WHO I-II grade) and high grade glioma (WHO III-IV grade) were 55 cases and 69 cases, respectively. In addition, five cases of normal brain tissues and one normal testis tissue from Specimens Library of Department of Histology and Embryology, Guangxi Medical University were used as control.

Immunohistochemistry (IHC)

Immunostaining with polyclonal anti-human OY-TES-1 antibody were prepared by our laboratory [26, 27]. Continuous sections in 4 μm thick were prepared from each formalin-fixed, paraffin embedded tissue. Subsequently, the sections were heated in citrate buffer (pH 6.0) for high-temperature antigen retrieval. After endogenous peroxidase had been inactivated by 3% hydrogen peroxide, the sections were immunostained with anti-OY-TES-1 polyclonal antibody (1:200 dilution) or pre-immune serum (negative control) overnight at 4°C. Then the treated sections were recovery at room temperature and incubated with the biotinylated second antibodies (ZSGB-BIO, China). Lastly, immunoreactivity was visualized with 3, 3'-diaminobenzidine (DAB) (Maixin Biotechnology, China) followed by hematoxylin counterstain.

The results were recorded and quantitatively analyzed using the pathological image computer analysis system. We found the highest area with the OY-TES-1-positive cancer cell ratio at low magnification (× 100) and the percentage of positive cells was calculated at high magnification (× 400). According to the staining intensity and the percentage of positive cells, the expression of OY-TES-1 proteins can be analyzed semi- quantitatively. Staining localized to the cell cytoplasm and nucleus was graded on a 0 to 3 intensity scale (0 = no staining; 1 = weak staining; 2 = moderate staining; 3 = strong staining). Positivity was defined as more than 5% of the tumor cells stained by the antibody. According to the sum of both points, the final score of each section was graded on 0 to 6 (0~1 = negative; 2~3 = weakly positive; 4~5 = moderate positive; 6 = strong positive). We utilized the receiver operating characteristic curve analysis to determine the threshold of positive protein expression then stipulated low OY-TES-1 expression when the sum score was between 1 to 3, and high OY-TES-1 expression when the sum score was greater than or equal to 4.

Follow-up

Among 124 patients, there were 84 patients (median 39 years) be tracked by postoperative follow-up and telephone interview (**Table 2**). Follow-up period was defined from hospital dis-

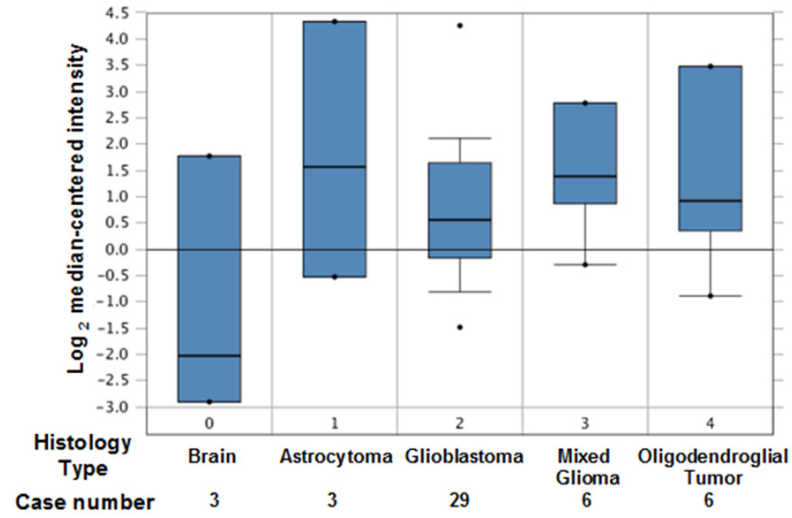

Figure 1. OY-TES-1 was up-regulated in astrocytoma, glioblastoma, mixed glioma and oligodendroglial tumor tissues compared to normal brain tissue samples in Bredel Brain 2 data set. The graph was generated using Oncomine (https://www.oncomine.org/).

charge to the date of patient's death or the last follow-up.

Statistical analysis

Statistical analyses were conducted by SPSS software (version 13. 0) for Windows. The OY-TES-1 protein expression correlated with clinical parameters were analyzed by χ^2 test (Chi-square test); Overall survival rates of patients was calculated by Kaplan-Meier method and differences in survival curves were compared using the log-rank test; To detect the prognostic factors of glioma survival, Multivariate analysis using Cox proportional hazards regression analysis. The *P* value lower than 0.05 ($P<0.05$) was considered as statistically significant.

Results

Oncomine analyses and tissue-microarray-immunohistochemistry

ONCOMINE is a cancer microarray database and web-based data-mining platform aimed at facilitating discovery from genome-wide expression analyses (https://www.oncomine.org) [28]. Searching this database we can understand the expression of OY-TES-1 mRNA in different types of gliomas. Compare with the normal brain tissue, Bredel Brain 2 dataset query results (**Figure 1**) showed the expression of OY-TES-1 mRNA in astrocytoma, glioblastoma, mixed glioma and oligodendroglial tumor tissues were all increased significantly ($P<0.05$). But the OY-TES-1 proteins were not detected in 23 cases of glioma specimens in Human Protein Atlas database (http://www.proteinatlas.org).

OY-TES-1 expression in glioma tissue

We analyzed OY-TES-1 expression in 124 glioma tissues by IHC performed with OY-TES-1-specific antibody. The results showed staining was mainly located in the cell cytoplasm and nucleus and the total positive rate of OY-TES-1 protein was 69.35% (86/124); In low-grade glioma (WHO I-II) and high-grade glioma (WHO III-IV), the positive rate was 21.77% (27/55) and 47.58% (59/69), respectively (**Table 3**). According to observation of staining intensity, high OY-TES-1 protein expression (**Figure 2A** and **2B**) was demonstrated in 70 of 124 patients (56.45%) and low (**Figure 2C**) in the other 54 patients (43.55%). Meanwhile, we detected 5 normal brain tissues and 1 normal testis tissue. OY-TES-1 was almost not expressed in normal brain tissues (**Figure 2D**). Testis germinal epithelium and sperm cell (**Figure 2E**) showed protein positive reaction. Negative control section was immunostained by pre-immune serum which substitute for anti-OY-TES-1 polyclonal antibody (**Figure 2F**).

Association of OY-TES-1 expression with clinical index in glioma

OY-TES-1 expression was significantly associated with the WHO classification, P53 and Ki67 expression ($P<0.05$). However, it was not associated with age, gender, tumor size and KPS score ($P>0.05$) (**Table 3**).

Relationship between OY-TES-1 expression and prognosis in patients with glioma

To provide a powerful explanation of the prognostic role of OY-TES-1, we assessed the effects

Table 3. Correlation between the OY-TES-1 protein and clinical charateristic of glioma patients

Characteristics	Positive/Total test (%)	Positive/total (%)		χ^2	*P* value
		High	Low		
Gender				0.720	0.869
Male	55/124 (44.35)	46/124 (37.10)	32/124 (25.81)		
Female	31/124 (25.00)	24/124 (19.35)	22/124 (17.74)		
Age (years)				4.722	0.193
<39	38/124 (30.65)	31/124 (25.00)	31/124 (25.00)		
≥39	48/124 (38.71)	39/124 (31.45)	23/124 (18.55)		
Tumor size (cm)				1.473	0.689
<5	36/124 (29.03)	30/124 (24.19)	24/124 (19.35)		
≥5	44/124 (35.48)	34/124 (27.42)	26/124 (20.97)		
WHO grade				24.531	0.000***
I-II	27/124 (21.77)	18/124 (14.52)	37/124 (29.84)		
III-IV	59/124 (47.58)	52/124 (41.94)	17/124 (13.71)		
Ki-67 (%)				18.021	0.000***
<10%	40/124 (32.26)	28/124 (22.58)	42/124 (33.87)		
≥10%	46/124 (37.10)	42/124 (33.87)	12/124 (9.68)		
P53 (%)				17.744	0.000***
<10%	35/124 (28.23)	24/124 (19.35)	39/124 (31.45)		
≥10%	51/124 (41.13)	46/124 (37.10)	15/124 (12.10)		
KPS				0.800	0.850
<70	40/124 (32.26)	28/124 (22.58)	42/124 (33.87)		
≥70	43/124 (34.68)	35/124 (28.23)	26/124 (20.97)		
Total	86/124 (69.35)	70/124 (56.45)	54/124 (43.55)	-	-

Abbreviations: High, OY-TES-1 protein expression (++/+++); Low, OY-TES-1 protein expression (-/+); KPS, Karnofsky Performance Scale; ***, P<0.001.

of OY-TES-1 expression and glioma grade by Kaplan-Meier survival analysis. Of the 124 glioma patients who were followed, 84 had complete records with an average follow-up time of 24.8 months (range, 1-69 months). Combined with these clinical data of patients, we found that there was a significant negative correlation between OY-TES-1 expression and overall survival in patients with glioma (P = 0.003). Higher OY-TES-1 expression means shorter survival (**Figure 3A**). In low grades of glioma, we got the similar result (P = 0.024) (**Figure 3B**). However, in all of high grade glioma patients, the OY-TES-1 expression showed no correlation with overall survival (P>0.05) (**Figure 3C**). Taking into account the survival of patients was affected by many factors, it was necessary to perform a multivariate COX analysis. The results showed that the survival of patients with glioma was not only influenced by age and WHO grade. The expression of OY-TES-1 also had an effect on the survival. The high expression of OY-TES-1 protein was negatively correlated with the prognosis of glioma patients. We inferred that the expression of OY-TES-1 protein could be used as an independent prognostic factor for patients with glioma (**Table 4**).

Discussion

Immunotherapy is an attractive adjuvant therapy which is regarded as an important method of anti-tumor therapy after surgical resection and chemoradiotherapy. The aim of tumor immunotherapy is to improve anti-tumor immunity so as to control tumor growth or kill tumor cells. Currently, Strategies for tumor immunotherapy mainly includes tumor vaccines, non-specific immune stimulants, adoptive cellular immunotherapy and so on. Among them, tumor vaccine is one of the hot spots in recent years. Its principle is activation of the immune system by exogenous tumor antigen. According to the use of tumor vaccines, it can be divided into two types: one is prophylactic vaccines, namely preparing tumor-related gene vaccines to inoc-

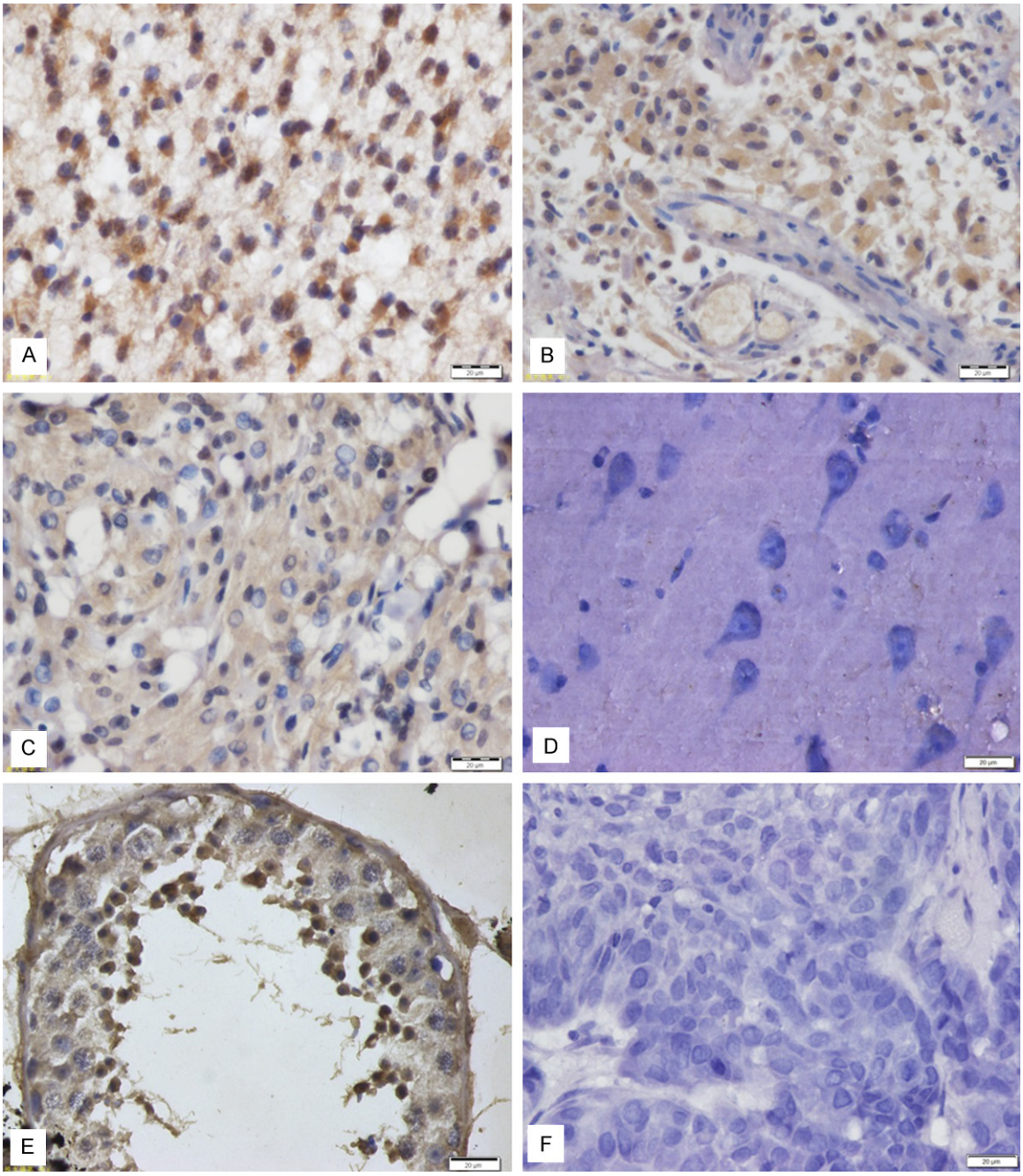

Figure 2. Immunohistochemical staining of OY-TES-1 protein in glioma tissues (A-C), normal brain tissue (D) and testis tissues (E). Staining was mainly located in the cell cytoplasm and nucleus. Testis germinal epithelium and sperm cell with strong staining in normal testis (E). Strong, moderate, weak immunoreactivity of OY-TES-1 protein immunostaining were shown in (A-C) respectively by using polyclonal OY-TES-1 antibody. Normal brain tissue was almost not detected OY-TES-1 protein staining (D). Paired glioma tissues probed with pre-immune serum as negative control (F). Bar, 20 μm.

ulate the susceptible population and finally control tumorigenesis. Another is therapeutic vaccines, these are based on tumor-associated antigen and mainly used for adjuvant therapy after chemotherapy.

No matter which kind of immunotherapy, suitable tumor-associated phase specific antigen is the major premise. We considered that overexpression in tumor tissues and almost no expression in normal tissues was the desirable

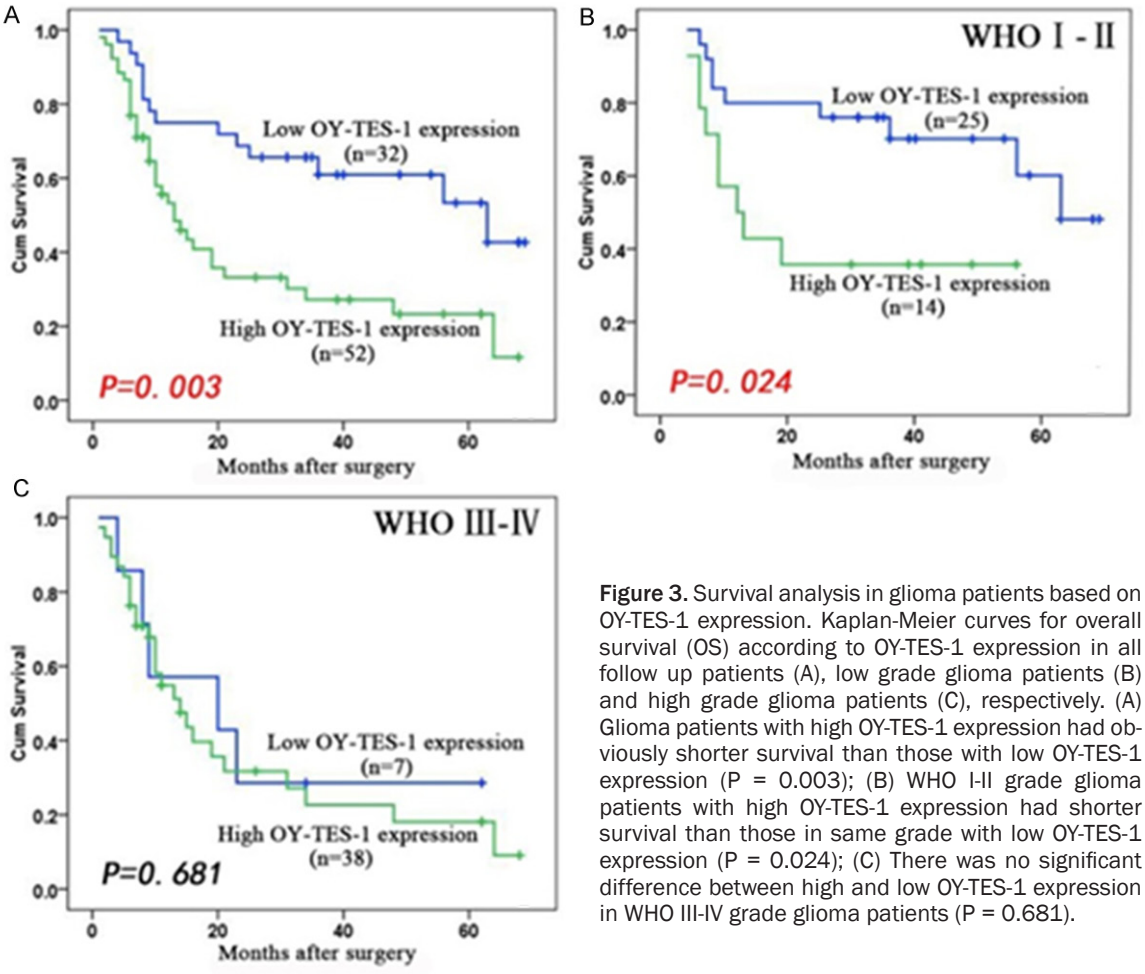

Figure 3. Survival analysis in glioma patients based on OY-TES-1 expression. Kaplan-Meier curves for overall survival (OS) according to OY-TES-1 expression in all follow up patients (A), low grade glioma patients (B) and high grade glioma patients (C), respectively. (A) Glioma patients with high OY-TES-1 expression had obviously shorter survival than those with low OY-TES-1 expression (P = 0.003); (B) WHO I-II grade glioma patients with high OY-TES-1 expression had shorter survival than those in same grade with low OY-TES-1 expression (P = 0.024); (C) There was no significant difference between high and low OY-TES-1 expression in WHO III-IV grade glioma patients (P = 0.681).

tumor-associated antigen for immunotherapy. OY-TES-1 is such an ideal tumor-associated antigen which restrictedly expressed in normal tissues and overexpressed in various tumor types [21, 22, 26, 29]. Recent research indicated that it was related with apoptosis, migration and invasion of tumor cells [23]. Currently, the research of OY-TES-1 was mainly focused on the levels of mRNA but the protein level was less reported, Tammela and other researchers [17] detected the positive rate of OY-TES-1 protein expression in different pathological types of ovarian cancer tissues and found clear-cell carcinoma proportion was 75% (3/4), endometroid carcinoma was 100% (1/1) and papillary serous carcinoma was 76% (26/43), but other normal tissues (except for testis) such as brain, heart, lung, skeletal muscle, kidney, ovary and stomach were not detected. Our early study showed OY-TES-1 mRNA was expressed on 80.39% (41/51) of glioma tissues. However, the expression of genes involved in many regulatory mechanisms, therefore, the expression of mRNA cannot completely represent the expression of its protein.

In this experiment, we collected a total of 124 cases of glioma tissues for IHC detection. The expression rate of OY-TES-1 protein was 69.35% (86/124). There was a positive correlation between antigen expression and WHO classification and Ki-67 /P53 expression (P<0.05). Survival analysis with clinical data of patients showed that the prognosis of the high expression of OY-TES-1 protein (++/+++) group is poorer than the protein (-/+) group in patients with glioma. Combined with our previous reports, the positive rate of OY-TES-1 antibody was 15.68% (8/51) in serum of patients with glioma, but the corresponding antibody was not detected in healthy persons. All of the findings above indicated that OY-TES-1 high expressed

Table 4. Univariate and Multivariate analysis of different prognostic parameters

Variable	n	Univariate		Multivariable	
		HR (95% CI)	*P* value	HR (95% CI)	*P* value
Gender (Male)	56	0.849 (0.470-1.533)	0.587	-	-
Age ≥39 (years)	44	2.564 (1.419-4.630)	0.002**	2.103 (1.138-3.886)	0.018*
Tumor size (≥5 cm)	45	0.820 (0.465-1.446)	0.492	-	-
KPS score (<70)	33	0.686 (0.381-1.235)	0.209	-	-
WHO grade (III + IV)	45	2.316 (1.286-4.170)	0.005**	1.253 (0.558-2.814)	0.584
Ki-67 expression (≥10%)	36	1.671 (0.957-2.944)	0.071	0.855 (0.434-1.684)	0.650
p53 expression (≥10%)	44	1.747 (0.984-3.102)	0.057	1.504 (0.815-2.775)	0.191
OY-TES-1 expression (High)	52	2.549 (1.359-4.781)	0.004**	2.035 (1.062-3.899)	0.032*

Univariate analysis was performed using the log-rank test. Multivariate analysis was performed using the Cox proportional hazards model. Abbreviations: HR, Harzard ratio; 95 percent CI, 95 percent confidence interval for relative risk; *P<0.05; **P<0.01.

specifically in glioma but almost not expressed in normal tissues, and it could stimulate the humoral immune response. These suggested that OY-TES-1 may be one of the candidate target genes for immune therapy of glioma.

Comparing with our previous study, we found the positive rate of OY-TES-1 protein was lower than mRNA levels. The reasons of this inconsistent phenomenon might be mainly by three possibilities below. Firstly, perhaps PCR was more sensitive than immunohistochemistry. The second was the protein translation pathway that had an obstacle so leading to parts of mRNA was unable to translate into proteins. The third was antigenic loss occurred during tumor progression potentially.

Our result suggested that preparing OY-TES-1 antigen-related tumor vaccine might enhance therapy effect of glioma in future. However, from the view point of our research or pertinent published literatures, this treatment concept was still in initial stage and needed further discuss. In addition, from our study alone, the quantity of samples was needed to be increased and the classification of glioma patients was needed to balance as well. Moreover, we should detect the mRNA levels of corresponding samples in order to further understand the relationship between OY-TES-1 and its protein expression. Detecting the relevant antibody in the serums of patients was also essential. Our ultimate goal was through induction of cytotoxic T cells in vitro to observe its lethal effect of OY-TES-1 expressive tumor cells, than established a foundation of immune therapy based on OY-TES-1 for the future.

Conclusions

OY-TES-1 protein was expressed in glioma at a high frequency and it was also significantly correlated with glioma grade. The patients with higher OY-TES-1 expression have poorer prognosis than those with low expression of its protein. These results indicate OY-TES-1 is probably a novel therapeutic target for tumor immunotherapy in future.

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Disclosure of conflict of interest

None.

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