Surgical Oncology 25 (2016) 405–410

Contents lists available at ScienceDirect

Surgical Oncology

journal homepage: www.elsevier.com/locate/suronc

Slit2 and Robo1 expression as biomarkers for assessing prognosis in brain glioma patients

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ARTICLE INFO

Article history:
Received 12 May 2016
Received in revised form
21 August 2016
Accepted 12 September 2016

Keywords:
Slit2
Robo1
Brain gliomas
Survival proportion and prognosis of gliomas

ABSTRACT

Objectives: This study was conducted to investigate the clinical significance of Slit2 and Robo1 expression in prognosis of patients with brain gliomas.
Methods: Human brain tissue samples were collected from normal glial tissues (control), low- and high-grade glioma tissues. Slit2 and Robo1 expression levels in cells were assessed by an immunohistochemistry (IHC), and population of the Slit2- and Robo1-presenting patients was examined. The Slit2 and Robo1 mRNA expression levels in three types of the brain cells was determined by RT-PCR.
Results: $Slit2^+$ cell counts were decreased with increased $Robo1^+$ cells in the low-grade and high-grade glioma tissues as compared to the control. The percentage of cells expressing Slit2 decreased from the control to the high-grade glioma and the percentage of cells expressing Robo1 in low- and high-grade gliomas was increased as compared to the control ($P < 0.01$). The decrease in the Slit2 mRNA expression was associated with the increase in the Robo1 mRNA expression in the low- and high-grade gliomas ($P < 0.01$ or 0.05). Survival time for patients with $Slit2^-/Robo1^+$ gliomas was shorter than patients with $Slit2^+/Robo1^+$ gliomas in the investigated cohorts ($P < 0.01$).
Conclusion: Slit2 and Robo1 expression levels serve as a biomarker with utility in grading gliomas as well as predicting patient survival. The change in Slit2 expression is more reliable and effective than Robo1 expression in predicting a poor prognosis of brain glioma patients.

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1. Introduction

A glioma is a type of tumor that arises from glial cells and the most common site of glioma is the brain [1]. Incidence of glioma accounts for 30% of all brain tumors and 80% of all malignant brain tumors [2]. The severity of the brain tumor depends variably on its grade. Grades III and IV are the highest grades, with the worst prognosis and greatest need for the most aggressive treatment [3–5]. The exact cause of glioma is unknown and therefore the tumor is rarely curable with a median patient survival of 12–15 months after diagnosis [6,7].

Slit-family proteins (Slits) are widely known as a repulsive axon guidance cue and are ligands of Roundabout (Robo) receptors that repel developing axons in the nervous system [8,9]. Slit2 binds Robo1 in a flexible linkage between its D2 domain and the first two Ig-like domains of Robo1 [10], which have been shown to influence the migration of neurons, glia and leukocytes [11]. Slit2 may mediate the effect of Robo1 inhibition in reduction of tumor mass in malignant melanoma [12]. Furthermore, Slit2 is predicted to play a role in a theoretical molecular network in which Robo1 is thought to be one of 10 factors that fit into the network involved in neuronal migration and neurite outgrowth [13].

This goal of this study was to explore clinical roles of Slit2 and Robo1 protein expressions in evaluating brain glioma progression. Our results indicate that the changes in Slit2 and Robo1 expression are effective indicators in helping to identify the nature of the glial cells. Furthermore, the Slit2 and Robo1 expression levels in glioma cells can be applied as clinical measures for assessing malignant

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http://dx.doi.org/10.1016/j.suronc.2016.09.003
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degrees of brain gliomas and prognosis of the patients with the tumors.

2. Patients and methods

2.1. Patient and inclusion criteria

We randomly recruited 40 patients with glial brain tumors requiring surgical resection in the period from October 2013–January 2014. These patients included 27 males and 13 females aged 35–70 years (median, 55 years). Normal brain tissue samples were obtained from 10 patients who underwent a partial excision of the brain tissue due to traumatic brain injury or intracerebral hemorrhage.

Major inclusion criteria were 1) Histopathological diagnosis as brain gliomas; 2) Glioma grade from I–IV by signs of cell division under the light microscope and categorized as low- (I–II) and high-grade (III–IV) gliomas according to WHO grading system [14]; 3) No radiotherapy, chemotherapy or immunotherapy prior to surgical resection of the brain tumors; 4) All patients received one-year follow-up and survival survey after surgery.

The study has been approved by the Ethics Committee of the Second Affiliated Hospital of Hebei Medical University. The Ethics Committee also approved the related tissue collection from these patients based on the experimental design and analysis of clinical outcome. All subjects signed written informed consent forms for this study.

2.2. Preparation of brain specimens

Brain tissue samples were collected from patients with traumatic brain injury (control) or glial brain tumors after surgical treatment. Each sample was placed in a wide-mouth bottle filled up in 4% (v/v) formalin for the IHC examination. Some samples prepared for RT-PCR (qPCR) were immediately snap-frozen in liquid nitrogen and then stored at −70 °C until processed.

For immunohistochemical detection, cross-sections of glial brain tissues were embedded into paraffin and then sliced on a microtome at 4 μm. The slices were mounted on slides, dehydrated using alcohol washes of increasing concentration (75%, 80%, 90%, 95%, and 100%) for different time periods, and cleared using a detergent like xylene before being imaged under a microscope. A two-step indirect IHC staining method was employed in this study. Briefly, the primary rabbit antibodies (Epitomics, Burlingame, CA, USA) directed against human Slit2 and Robo1 were diluted to 1:200 and 1:250, respectively. The slides from individual samples were stained with the antibodies at 4 C overnight. A horseradish peroxidase-conjugated secondary antibody reacting with a 3,3′-Diaminobenzidine (DAB) substrate-chromogen was intended for use on the formalin-fixed, paraffin-embedded tissue sections. Reaction with DAB on the sections can produce a brown product at the site of the target antigen that is insoluble in alcohol and xylene. Slides were washed three times with PBS before being analyzed using a light microscope (Olympus, Japan) at a 400-fold magnification for visualizing expression of the targets in the investigated tissues during the disease process.

2.3. Application of immunohistochemistry

5 circular areas from 200 high power fields on each slide were randomly selected and viewed by a pathologist under a light microscope. The relative proportions of the glial cells and glioma cells from a total 200 cells/slide were determined in each specimen based on the number of the stained cells and their scores according to immunoreactive remmele scores (IRS) [15]. Positive staining for the Slit2 staining was indicated by color response seen in both cell cytosol and nuclear membrane inside the cell. A positive staining for Robo1 was indicated by color in the cytosol.

There are two types of brain cells, neurons and glial cells. The Robo1 protein was mainly expressed in glioma cells, whereas the Slit2 protein was detected in the neurons (nerve cells) and glial cells (glioma cells). In this study, immunostaining of the glial brain cells was observed under a light microscope. According to IRS, a standard for negative (−) or a positive (+) effect of Slit2- and Robo1-staining cells is defined as cells of ≤4% or > 4% in the total cell counts in each microscopic area, respectively. Population proportion for $Slit2^+$ and $Robo1^+$ patients was calculated according to the numbers of the patients with the positive cell staining in their brain tissue specimens.

2.4. Determination of Slit2 and Robo1 mRNA levels

Slit2 and Robo1 mRNA expression levels were assessed by using SYBR Premix Ex TaqTM (Takara, Japan). The following forward primers for the Slit2 and Robo1 mRNA were used sequences in the 5′-TGTATTCCTCCTCGCACCTTT-3′ and 5′-GAAACAGCGACAGCAACCT-3′. Reverse primers for Slit2 and Robo1 were 5′-TTCACCCAGTCGGATAACCAC-3′ and 5′-TGACAAAACGCCCATCCT-3′. Briefly, the total RNA from glial and glioma tissues in brain was extracted using a tissue homogenizer in lysis buffer and purification of RNA was performed with RNeasy minicolumns following the manufacturer's protocol (CWbio Co., Ltd., Beijing, China). RNA was quantified using the NanoDrop ND-1000 spectrophotometer and amplified and biotin-labeled with Nugen's Ovation System. The yield of total RNA per replicate varied from 0.6 μg to 2.0 μg 50 ng of the RNA was added in a SYBR qPCR master mix for real-time qPCR. Contents of Slit2 and Robo1 mRNA were shown with a fold-change as compared to the expression level of the control sample.

2.5. Survival function of patients with gliomas

The Kaplan-Meier estimate is the simplest way of computing the survival over time in spite of all these difficulties associated with subjects or situations [16]. The survival curve can be created assuming the patient's prognosis in a statistical graph showing the percentage surviving in the investigated cohort. Points on the curve indicate the proportion or percentage survival at a particular time (month) after the start of this observation.

2.6. Statistical analyses

Data were expressed as a percentage of population proportion in the study cohort and a sample median of an interquartile range (IQR). Statistical analysis was performed using Statistical Package for the Social Science (SPSS, version 16.0). One-way analysis of variance (ANOVA) was implemented for comparison of an independent variables. Student's paired *t*-test was used to compare measurements of cells stained by antibodies. The Chi-square test ($\chi 2$) was conducted to analyze the significance of population distribution for the Slit2- and Robo1-presenting patients. A p value of <0.05 was considered significant.

3. Results

3.1. Identification of glial brain cells

Glial cells from brain specimens were observed in absence and presence of specific antibodies to Slit2 and Robo1. The results are shown in Fig. 1. Types of brain glioma were categorized by appearance as oligodendrogliomas according to the features of

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