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ORIGINAL ARTICLE

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Expression of a transcriptional factor, SOX6, in human gliomas

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Abstract By screening a human testis cDNA library with glioma patients' sera, we isolated a transcriptional factor, SOX6. Here, we analyzed SOX6 expression in gliomas having a range of malignancy grades using immunostaining. Murine Sox6 is a transcriptional factor that is specifically expressed in the developing central nervous system and in the early stages of chondrogenesis in mouse embryos. The reverse transcription-polymerase chain reaction (RT-PCR) revealed that the SOX6 gene was more highly expressed in glioma tissues and fetal brain than in normal adult brain and other cancer cells, except melanoma cells. Immunohistochemical analysis with the anti-SOX6 antibody showed that all the glioma tissues analyzed (14 glioblastomas, 14 anaplastic astrocytomas, 3 anaplastic oligoastrocytomas, 5 diffuse astrocytomas, 1 oligodendroglioma, and 1 pilocytic astrocytoma) expressed SOX6 in tumor cells, but only a few SOX6-positive cells were detected in nonneoplastic tissues from the cerebral cortex. These results indicate that the developmentally regulated transcription factor SOX6 may be a potential diagnostic marker for gliomas.

Key words Glioma · Transcriptional factor · SOX6

Introduction

Human malignant gliomas are rapidly progressing brain tumors of unknown etiology. Despite aggressive multimodal therapy, the prognosis for patients with malignant glioma is still poor. To clarify its etiology and develop new therapeutic strategies, such as immunotherapy, identification of tumor antigens for glioma is required.

There are numerous glioma-associated antigens, which are characterized by a differential expression in analyzed glioma versus normal cells. The majority of the glioma-associated antigens, however, have been found to be of minor therapeutic and diagnostic utility.[1] Previously, we reported SOX6 as a new glioma-associated antigen detected by serological screening, using a testis cDNA library and allogenic glioma patients' sera.[2] Now, we have focused our study on SOX6 expression in human gliomas and have investigated its availability as a diagnostic marker for glioma by immunohistochemical analysis of a greater number of cases.

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Materials and methods

Patients and tissues

This study was approved by the local ethical review board of Keio University (No. 12-21-2). Tumor tissues were obtained from the Department of Neurosurgery, Keio University, School of Medicine. The tumors obtained from surgical cases were classified according to the recent World Health Organization (WHO) criteria[3] as: glioblastoma (WHO grade IV), anaplastic astrocytoma and anaplastic oligoastrocytoma (WHO grade III), diffuse astrocytoma and oligodendroglioma (WHO grade II), or pilocytic astrocytoma (WHO grade I). The tissues were frozen in liquid nitrogen immediately after surgery and then stored at −80°C.

RT-PCR analysis.

Total RNAs from normal tissues and cancer cell lines were purchased from Clontech Laboratories, Palo Alto, CA, USA. Total RNAs from glioma tissues were isolated using

Trizol (Gibco BRL). The cDNA preparations used as templates in the reverse transcription-polymerase chain reaction (RT-PCR) reactions were synthesized by incubating total RNA template (5μg), oligo (dT), and avian myeloblastosis virus reverse transcriptase (Takara, Tokyo, Japan) in a total reaction volume of 200μl at 42°C. Primers were designed as follows: forward primer, 5′-GATGCCATCAA CTCCACAGC-3′; reverse primer, 5′-GCTGCAGAGCC ATTCATTGC-3′; and for β-actin, forward primer, 5′-GGCACCCAGCACAATGAAG-3′; reverse primer, 5′-GCCGATCCACACGGAGTACT-3′. PCR was performed with an initial denaturation at 94°C for 10min, followed by 30 cycles of amplification (denaturation at 94°C for 1 min, annealing at 60°C for 1 min, and primer extension for 1 min at 72°C), followed by a 6-min extension at 72°C. The amplification products were analyzed by agarose gel electrophoresis and visualized by ethidium bromide staining.

Western blot analysis for blocking experiment with polyclonal antibodies against SOX6

To confirm the specificity of the anti-SOX6 antibody used, we performed a Western blot analysis after absorbing the antibody with recombinant SOX6 protein. SOX6 protein exists in the nuclei of mammalian cells.[4] To isolate nuclear extracts, 293T cells transfected with the full-length of SOX6 cDNA[5] were homogenized in 0.25M sucrose and centrifuged for 10min. The pellets were resuspended in 0.25M sucrose supplemented with a protease inhibitor cocktail (Sigma Aldrich). Twenty micrograms of total protein were loaded on each lane, and the proteins were transferred onto a nitrocellulose membrane. The first antibody used was a rabbit antihuman SOX6 polyclonal antibody (10μg/ml, Chemicon International, Temecula, CA, USA), followed by 1:2000-diluted goat antirabbit IgG (Fc) antibody conjugated with alkaline phosphatase (Cappel, Aurora, OH, USA). To evaluate the specificity of the anti-SOX6 antibody, the antibody was incubated with 293T cells expressing SOX6 at a final concentration of 10μg/ml in 1% PBS, then applied to a Western blot as described above.

Immunohistochemical staining

Paraffin-embedded tissue sections (7μm) were deparaffinized in xylene and rehydrated. The sections were treated with a heat-based antigen retrieval method, using a citrate solution (pH 6.0, 10mM). Endogenous peroxidase was blocked by incubation in 0.3% hydrogen peroxide in methanol, and nonspecific binding of antibodies was blocked by incubation in 5% BSA (bovine serum albumin) in 0.02M PBS and 0.1% Triton X-100 in phoshate-buffered saline (PBS) for 1h. The slides were then incubated with antihuman SOX6 antibody (1μg/ml) diluted in the same blocking solution overnight at 4°C. The slides were incubated with a second antibody (Universal Immuno-peroxidase Polymer, Anti–Rabbit; Histofine Simple Stain MAX PO, Nichirei, Tokyo, Japan) for 30min at 37°C, and the horseradish peroxidase (HRP) labeling was visualized using diaminobenzidine (DAB). The sections were lightly counterstained with hematoxylin. Each step was followed by three washes in PBS.

Results

Analysis of SOX6 mRNA expression in glioma

To evaluate the expression of SOX6 mRNA in glioma tissues, normal central nervous tissues, and other cancer cells, we performed an RT-PCR analysis. SOX6 mRNA was highly expressed in fetal brain, glioma tissues, and melanoma cells and was faintly expressed in the adult brain, but it was not detectable in other tissues (Fig. 1).

Blocking experiment with polyclonal antibodies against SOX6

To confirm the specificity of the anti-SOX6 antibody used, we performed a Western blot analysis after absorbing the antibody with recombinant SOX6 protein. The 90-kDa

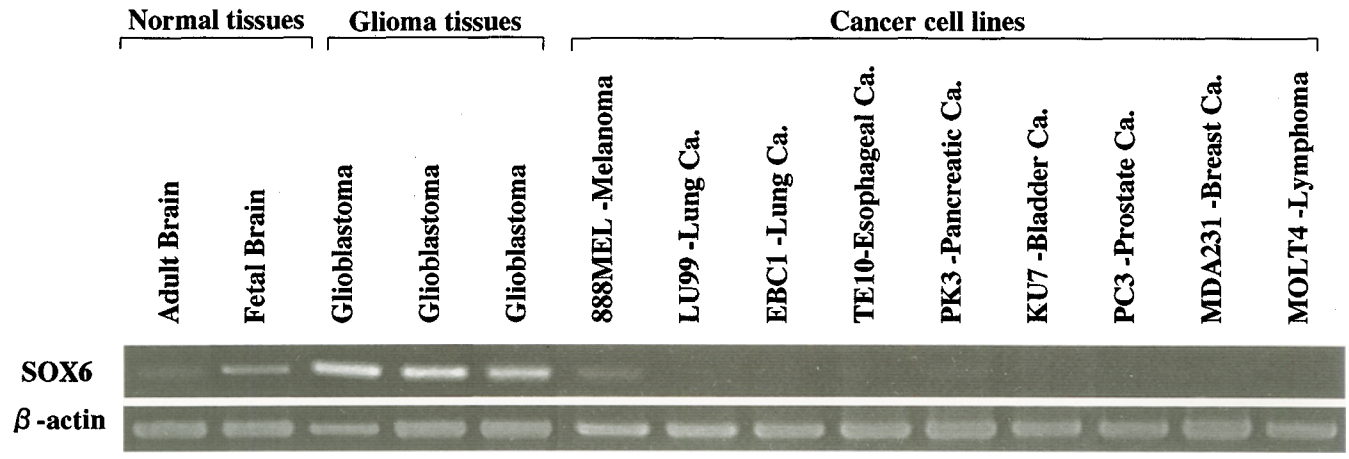

Fig. 1. Reverse transcription-polymerase chain reaction (RT-PCR) analysis of human SOX6 mRNA. SOX6 mRNA was expressed in fetal brain, glioma tissues, and melanoma cells. The adult brain showed faint expression

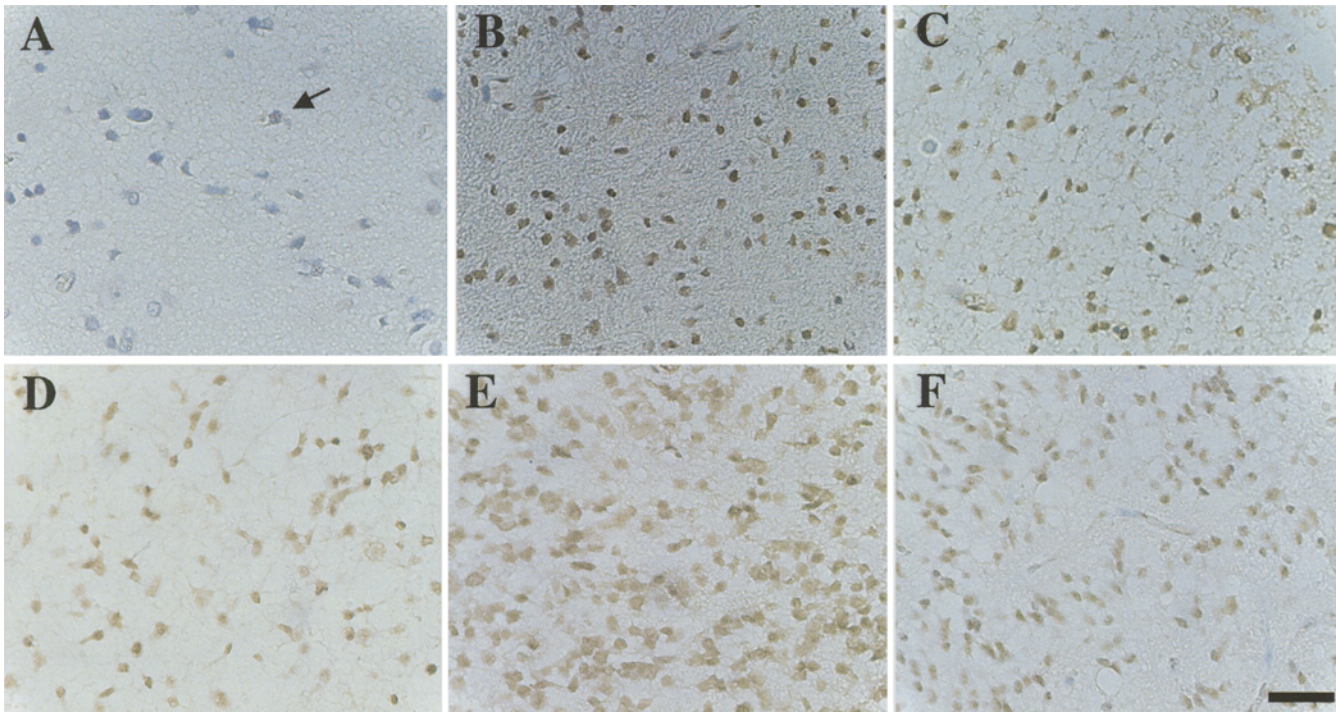

Fig. 2. Immunohistochemical analysis of SOX6 expression in gliomas and normal brain tissue. Only a few SOX6-positive cells (*arrow*) were detected in nonneoplastic tissue from the cerebral cortex (**A**). In a diffuse astrocytoma (**B**), an anaplastic astrocytoma (**C**), an anaplastic astrocytoma (**D**), a glioblastoma (**E**), and a glioblastoma (**F**), SOX6 was expressed in the nucleus of most tumor cells. *Bar* = 25 μm

bands in the 293T cells transfected with the full-length SOX6 cDNA disappeared in this blocking experiment (data not shown), indicating that the antibody used specifically recognized SOX6, and confirming the expression of SOX6 in glioma.

Immunohistochemical analysis of SOX6 in glioma

Paraffin-embedded tissues from formalin-fixed tumors were analyzed using the antibody against SOX6. Immunohistochemical analysis with the anti-SOX6 antibody revealed that all the glioma tissues analyzed (14 glioblastomas, 14 anaplastic astrocytomas, 3 anaplastic oligoastrocytomas, 5 diffuse astrocytomas, 1 oligodendroglioma, and 1 pilocytic astrocytoma) expressed SOX6 in the nuclei of the tumor cells, but only a few SOX6-positive cells were detected in nonneoplastic tissues from the cerebral cortex (Fig. 2). The intensity of SOX6 staining and the histological malignancy are summarized in Table 1. Although we evaluated the possibility of a relationship between SOX6 expression and histological malignancy in gliomas, no apparent correlation was observed.

Table 1. Summary of SOX6 immunostaining in gliomas

WHO grade	Histology	*n*	Intensity of SOX6 staining			
			0	1+	2+	3+
IV	Glioblastoma	14	0	2	4	8
III	Anaplastic astrocytoma	14	0	3	5	6
	Anaplastic oligoastrocytoma	3	0	0	2	1
II	Diffuse astrocytoma	5	0	1	2	2
	Oligodendroglioma	1	0	0	0	1
I	Pilocytic astrocytoma	1	0	0	1	0

The patterns of staining are described as 0, negative or faint staining; 1+, positive in less than 30% of tumor cells; 2+, positive in 30% to less than 70% of tumor cells; 3+, positive in 70% or more of tumor cells

Discussion

Analysis of brain tumor gene expression is needed to design potential new therapies and to aid in diagnosis. We previously demonstrated that the developmentally regulated transcription factor SOX6 is aberrantly expressed in glioma and specifically recognized by IgGs from glioma patients' sera.[2] In this previous study, the SOX6 gene was more highly expressed in glioma tissues than in normal adult tissues except for testis; therefore, we performed further immunohistochemical examinations of SOX6 expression in gliomas.

Since Sox genes were first identified in 1990, over 30 members of this family of transcription factors have been described. Only about 20, however, have been studied in detail in mammals, and only 15 have been studied in humans.[6–8] Among those studied, most are expressed during embryonic development, and many are expressed in the

nervous system. These genes are classified into subgroups according to sequence, and the members of a subgroup often share similar tissue-specific expression patterns. Expression is also often related to the stage of maturation of cells, so that cells alter their Sox gene expression as they mature and finally differentiate. Sox6 has been suggested to regulate embryonic development and determine cell fate. Mouse Sox6 is expressed in the central nervous system (CNS) during embryogenesis, but the expression significantly decreases in the adult CNS.[9] At 9.5 days post coitum (dpc), Sox6 expression is observed in the CNS, with highest levels in the forebrain and gradually decreasing in a rostrocaudal gradient to a region posterior to the forelimb bud.[9] This pattern of expression is maintained at 10.5 dpc, but expression is extinguished in the CNS by 12.5 dpc. Sox6, Sox5, and Sox9 are expressed simultaneously and at high levels from early stages of chondrogenesis in all cartilaginous sites in mouse embryos.[10] In this study, we showed that human SOX6 was expressed in fetal brain and gliomas, but faintly in the adult brain. Developmentally regulated proteins in the CNS, such as Nestin[11] and Musashi,[12] have also been shown to be expressed in undifferentiated neural stem cells and glioma, but not in mature neurons. These results suggest that glioma may exhibit a gene expression pattern similar to that of the cells in the developing CNS.

We have studied the correlation between *SOX6* expression and the histological malignancy of human gliomas by immunohistochemistry. No significant relationship was observed between SOX6 expression and the grade of malignancy in gliomas (Table 1). This indicates that SOX6 may not promote proliferation, and aberrant expression of SOX6 may induce transformation activity in the early stage of oncogenesis in gliomas.

In conclusion, although further investigations are required to elucidate the role of SOX6 in oncogenesis and SOX6 expression in other human CNS tumors and undifferentiated cells, SOX6 is predominantly expressed in human gliomas, suggesting that SOX6 can be used as a molecular marker for the diagnosis of glioma.

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