การปรากฏร่วมกันของโปรตีน RUNX1 และ NFAT2 ในเนื้องอกสมองกลิโอมาในมนุษย์

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บทคัดย่อ

กลิโอมา (Gliomas) เป็นเนื้องอกสมองที่เกิดจาก glial cells จัดเป็นเนื้องอกในสมองที่พบมากที่สุดในผู้ใหญ่ มีอาการรุนแรงอย่างมาก แต่แทบไม่พบการกระจายออกนอกเนื้อสมอง องค์การอนามัยโลก (WHO) จำแนกเนื้องอกสมองกลิโอมาตามความรุนแรงเป็นเกรด I ถึง IV โดยแบ่งตามลักษณะทางเนื้อเยื่อที่ปรากฏเป็นหลัก ในปี 2016 WHO ได้ปรับปรุงการจำแนกเกรดของเนื้องอกทางระบบประสาทส่วนกลางขึ้นใหม่ โดยได้เพิ่มเทคนิคทาง molecular genetic ร่วมด้วย ทำให้มีความเห็นพ้องกันในเนื้องอกสมองแบบ diffuse-type มากขึ้น เป็นที่ทราบกันดีว่า ปัจจัยสำคัญที่นำไปสู่การเจริญอย่างรวดเร็วของเนื้องอก คือ การเกิดหลอดเลือด (angiogenesis) กระบวนการนี้ทำงานร่วมกับโปรตีน transcription factors มากมาย ในการศึกษานี้ผู้วิจัยได้ตรวจสอบการแสดงออกของ transcription factors จำนวน 2 ชนิด คือ RUNX1 และ NFAT2 ในเนื้องอกสมองกลิโอมาเกรด I-IV ด้วยเทคนิคการย้อมแบบ double immunofluorescence ผลการศึกษาสามารถยืนยันได้ว่ามีการแสดงออกของ RUNX1 และ NFAT2 ในไซโตปลาสซึมของกลิโอมาอย่างมีนัยสำคัญ เมื่อเทียบกับเนื้อเยื่อปกติของสมองมนุษย์ ($p<0.05$ และ $p<0.01$) และพบการปรากฏร่วมกันของ RUNX1 และ NFAT2 ในไซโตปลาสซึมของเซลล์เดียวกัน โดยพบความเข้มของ fluorescent มากขึ้นเป็นลำดับในเนื้อเยื่อกลิโอมา โดยเฉพาะเนื้อเยื่อเกรด II ถึง IV ผลของ immunoreactivity จากการย้อมด้วย GFAP บ่งบอกว่าเนื้อเยื่อกลิโอมานี้เป็น glial cells ชนิด astrocytes ดังนั้น การแสดงออกของ RUNX1 และ NFAT2 อาจบอกถึงหน้าที่ของโปรตีนทั้งสองในการควบคุมการเปลี่ยนแปลงของการเกิดหลอดเลือดในผู้ป่วยเนื้องอกสมองกลิโอมา

คำสำคัญ: glioma, RUNX1, NFAT2, brain tumor, transcription factor, double immunofluorescence

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Co-existence of RUNX1 and NFAT2 in the human glioma tissues

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Abstract

Glioma is a type of CNS tumors that arises from glial cells. It is the most common primary brain tumors in adults. This type of brain tumor is extremely aggressive, but rarely expanded outside the brain parenchyma. Previously, the World Health Organization (WHO) classified gliomas into 4 grades, i.e. grade I-IV, which depend largely on the conventional histological appearances. More recently, the 2016 WHO classification of CNS tumors was released. Since then, the correlation of histology and molecular genetic features approval has facilitated some diffuse-type tumors. It has been well established that the major aspects contributing to the rapidly growth of tumors is angiogenesis. This is a highly cooperative process involving multiple transcription factors. In this study, we investigated expression of RUNX1 and NFAT2 transcription factor proteins in human glioma specimens, WHO grade I-IV, by double immunofluorescence staining. Our findings confirmed the distribution of RUNX1 and NFAT2 that was confined to the cytoplasm of glioma tissues compared with human normal brain control ($p<0.05$ and $p<0.01$). The co-existence was significantly observed in the same tumor cells with higher fluorescent extent in the gliomas with higher grade, especially in grade II-IV. The GFAP immunoreactivity indicated that all neuronal-glia tumors were astrocytes in current finding. Therefore, involvement of RUNX1 and NFAT2 in each grade of gliomas may suggest their functions in controlling differentiation and transformation of angiogenesis in human glioma patients.

Keywords: glioma, RUNX1, NFAT2, brain tumor, transcription factor, double immunofluorescence

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Introduction

There are more than 120 known types of brain and central nervous system (CNS) tumors. Of all the types of brain tumors, glioma, which arises within the brain parenchyma, is the most common. To standardize the classification and grading of the CNS tumors, which has to be worldwide accepted and conducted beyond institutional confine, the World Health Organization (WHO) has classified human CNS tumors into four grading systems[1]. In accordance with the name, glioma is generally referred to tumors arising from a group of glial cells differentiation. Typically, there are three types of normal glial cells that can produce tumors, i.e. astrocytes, oligodendrocytes, and ependymal cells[1-2]. Obviously, gliomas account for 28 percent of all primary brain tumors, which approximately 54 percent of all gliomas are malignant[3-4]. The key indications for invasive growth and aggressiveness of malignant gliomas, which contribute to uniformly fatal outcomes, are the formation of new blood vessels through the process of angiogenesis, and the invasion in glioma cells along white matter tracts[3,5].

Grading system has been implemented to a spectrum of brain tumors, including gliomas. According to the 2007 CNS WHO classification, gliomas are separated into grades I-IV[1] as follows. The gliomas, WHO grade I, typically grow very slowly and occur frequently in children. The tumors are relatively benign and associated with long-term survival. The gliomas, WHO grade II, show with cytological atypia alone, whereas the WHO grade III and IV are those showing anaplasia and mitotic activity with histologic evidences of malignant lesions. The gliomas, WHO grade IV, also known as glioblastoma multiforme (GBMs), are the most common and extremely aggressive tumors in adults. They exhibit additionally microvascular proliferation, so called angiogenesis, and/or necrosis[1,2,6]. However, grading of tumor only under microscopic examination has not given sufficient information. Therefore, after 2007 version, WHO grading system has been updated and released in 2016. The new classification is successfully combined histologic appearances with genetic or molecular information[2]. More recently, an alternative possibility has been conducted on immunohistochemical studies of constricted diagnostic material with certain markers that may result in more applicable due to enhanced identification.

RUNXs (Runt-related transcription factors) belong to a family of heterodimeric transcription factors that encode the DNA-binding protein α-chain of Runt domain partners of the non-DNA-binding core-binding factor (CBFβ) complex, which can either activate or repress transcription of lineage-specific essential regulators in proliferation and differentiation during development[7]. The mammalian RUNX gene family comprises three members, namely RUNX1, RUNX2 and RUNX3, which have pivotal roles and display different gene expressions in both normal and abnormal developments[8].

Studies in area of molecular genetics and developments discovered that RUNX1 (also known as AML1/CBFA2/PEBP2aB) is essential for definitive hematopoiesis, differentiation of T- and B-cell lineages, and neuronal development[9]. RUNX2 (also known as AML3/CBFA1/PEBP2aA) is required for osteogenesis by controlling chondro-osteoblast differentiation[10]. RUNX3 (also known as AML2/CBFA3/PEBP2aC) is involved in gut development, neurogenesis, thymopoiesis, and lung alveolar differentiation growth[11].

In fact, all three RUNX family members also play important roles in carcinogenesis. In cancers, RUNXs act as either oncogenes or tumor suppressor genes in different cancers depending on cellular context[8,12]. The involvement of RUNX genes in the regulation of carcinogenesis was first discovered in 1991, in human RUNX1, as a gene involved in the chromosome rearrangement associated with acute myeloid leukemia[13]. So far, many studies have confirmed that in addition to participating in hematopoiesis or angiogenesis, RUNX1 is also related to embryonic development, tumorigenesis, immune response, and inflammatory response[14]. Besides, RUNX1 is involved in regulation of epithelial cell adhesion, migration, and epithelial-mesenchymal cross talk[15]. RUNX1 overexpression has been found to be correlated with tumor pathogenesis that reported in acute lymphoblastic leukemia[16], astrocytomas[17], breast cancer[18], endometrial cancer[19], epithelial cancer[20], neuroblastoma[21], ovarian cancer[22] and glioblastoma[23].

NFAT, which is a component of the nuclear factor of activated T cells DNA-binding transcription complex, was originally discovered as a rapidly transcriptional factor that could bind to human interleukin 2 (IL-2) promoter during activation of T cells[24]. In humans, there are five distinct members in the NFAT gene family. Four of the five primary structures of the NFAT family are regulated by calcium signaling those are named: NFAT1 (also known as NFATc2 and NFATp); NFAT2 (also known as NFATc1 and NFATc); NFAT3 (also known as NFATc4); NFAT4 (also known as NFATc3 and NFATx). In contrast to the classic NFATc proteins, NFAT5, also known as tonicity enhancer binding protein (TonEBP) or osmotic response element-binding protein (OREBP), does not require a calcium-binding site and thus is calcium and calcineurin-insensitive. NFATs have been characterized as cytosolic proteins constitutively expressed in resting cells[25]. Regardless their names, extensive studies have indicated that NFATs have not been related to only the immune system, but also implicated in the expression of different genes that regulate cell cycle progression and apoptosis related proteins, cell development and differentiation, signaling proteins, cell surface receptors, angiogenesis, and possibly tumorigenesis[26,27].

Besides indirect evidence, there is plenty of data demonstrating the effective participation of NFAT proteins in tumor-related processes, particularly NFAT2. The first studies in mouse 3T3-L1 preadipocyte cell line implicated regulatory role of NFAT2

in proliferation. This indicated that NFAT2 was able to induce cell transformation and inhibit cell differentiation, thus revealed its oncogenic potential[28]. In addition, active nuclear NFAT2 was found in cases of Burkitt lymphoma, diffuse large B cell lymphoma and in aggressive T cell lymphoma[29]. Studies on the role of NFAT transcription factors in cancer progression to date have largely been restricted to *in vitro* or cell-based assays, whereas a number of studies by using immunohistochemistry techniques in NFAT2 are still limited.

Objectives

In this study, therefore, we investigated the existence of RUNX1 and NFAT2 in human glioma specimens, WHO grade I-IV, compared with human normal brain tissues by double indirect immunofluorescence technique.

Materials and Methods

Materials and reagents

All glioma specimens and normal human brain in paraffin blocks were obtained from Department of Pathology, Faculty of Medicine, Ramathibodi Hospital, Mahidol University. Primary antibodies used in this study were rabbit anti-RUNX1 antibody (Santa Cruz, USA), mouse anti-NFAT2 antibody (abcam, USA). List of antibodies that purchased from Southern Biotech (USA) were Alexa fluor 488 goat anti-rabbit IgG, goat anti-mouse IgG TRITC. Mouse anti-GFAP antibody conjugated with Alexa fluor 647 was purchased from Cell Signaling Technology, USA. List of chemical were purchased as follows Silane (3-aminopropyltriethoxylane) (Sigma Aldrich, USA), bovine serum albumin (BSA), Tween 20 (Bio-Rad Laboratories, USA), normal horse serum (Cell Signaling Technology, USA), DAPI nuclear staining (Vector, USA). Other chemicals used were of analytical grade.

Glioma specimens

Twenty human glioma specimens, WHO grade I-IV (n = 5 each), were kindly provided by Assoc. Prof. Dr. Noppadol Larbcharoensub, M.D., Ramathibodi Hospital, Mahidol University. All glioma specimens were surgically resected from patients and all histologic examines were made on formalin-fixed, paraffin-embedded, and were confirmed to original diagnosis as stated in the 2007 WHO classification[1] by the neuropathologist (Assoc. Prof. Dr. Noppadol Larbcharoensub, M.D.). Considering the glioma immunostaining confirmation, five additional normal adult human brain specimens were also obtained from Ramathibodi Hospital, Mahidol University, and served as a control for the experiment.

Double immunofluorescence staining

Section of 5 μm thickness were cut and mounted on silane-coated slides. The sections were deparaffinized in xylene, rehydrated with 100% and 95% alcohol, and rinsed in distilled water for 3 min each, then immersed in 0.01 M phosphate-buffered

saline with 0.1% Tween 20 for 10 min. After that the sections were incubated with blocking buffer containing 4% BSA and 20% normal horse serum in 0.01 M PBS for 30 min at room temperature. The primary antibody mixture which contained rabbit anti-RUNX1 (1:200 dilution) and mouse anti-NFAT2 antibody (1:500 dilution) in a blocking buffer was prepared just prior to use. Then, the mixture of primary antibodies was overlaid on the sections in a humidified chamber for overnight at 4°C. After that the sections were washed with agitation in 0.01 M PBS 3 times, for 5 min. The secondary antibodies were prepared by mixing between Alexa fluor 488 goat anti-rabbit IgG and goat anti-mouse IgG TRITC in 0.01 M PBS (1:1000 dilution) shortly before use. The sections were treated with secondary antibody mixture for 1 h at room temperature. After three washes in PBS, the sections were incubated with mouse anti-GFAP antibody conjugated with Alexa fluor 647 in 0.01 M PBS (1:500 dilution) for 1 h. Then the sections were washed in 0.01 M PBS for 3 times with agitation, 5 min each. Finally, all sections were counter-stained with DAPI nuclear staining. The immunoreactivity was visualized and photographed under Olympus FV10i confocal laser scanning microscope (Olympus Life Science, USA).

In our protocol, controls were carried out on human normal adult brain specimens. All the control sections were performed in the same way as described above.

Quantitative and statistical analysis

RUNX1 and NFAT2 in glioma tissues, WHO grade I-IV, immunofluorescently positive was analyzed each by five immunofluorescence pictures taken under the same magnification. To quantify the fluorescent intensity level, briefly, each image was segmented into 5 regions, i.e. upper right and left comers, lower right and left comers, and the center. The analysis was done with ImageJ software (https://imagej.nih.gov/ij/download.html). The same procedure as described above was performed in normal brain control pictures. Relative immunofluorescence intensity were determined as mean ± SEM for each group. The difference between control and each glioma grade was determined by one-way ANOVA with Dunnett's post hoc test using GraphPad Prism statistical analysis software (GraphPad Software Inc, USA). $p<0.01$ or $p<0.05$ was considered significant.

Results

Expression of RUX1 and NFAT2 transcription factors in human gliomas

The coimmunofluorescence study was carried out to explore the expression profile of RUNX1 and NFAT2 in human glioma tissues grade I-IV, according to WHO 2007 classification[1]. At histological level, it was observed that gliomas displayed massive progression from grade I through IV (Figure 1). Certain manners of critical tumor

proliferation, such as increased cellularity and mitotic activity, were clearly identifiable particularly in grade III and IV. The immunoreactivity revealed highly expression of RUNX1 represented in green (Figure 1, panel RUNX1), and NFAT2 represented in red (Figure 1, panel NFAT2), which was confined in the cytoplasm of glioma ranging from grade I to IV, when compared with human normal brain tissues (Figure 1 A-D). Moreover, current finding demonstrated the co-expression of RUNX1 with NFAT2 that was significantly observed in cytoplasm of the same tumor cells (Figure 1, panel merged). The colocalization was higher in high grade glioma, especially in grade II-IV. Additionally, the appearance of nuclear atypia in tumor cells was increased in high grade glioma as shown in the DAPI-stained nuclei presented in light blue (Figure 1, panel DAPI and merged).

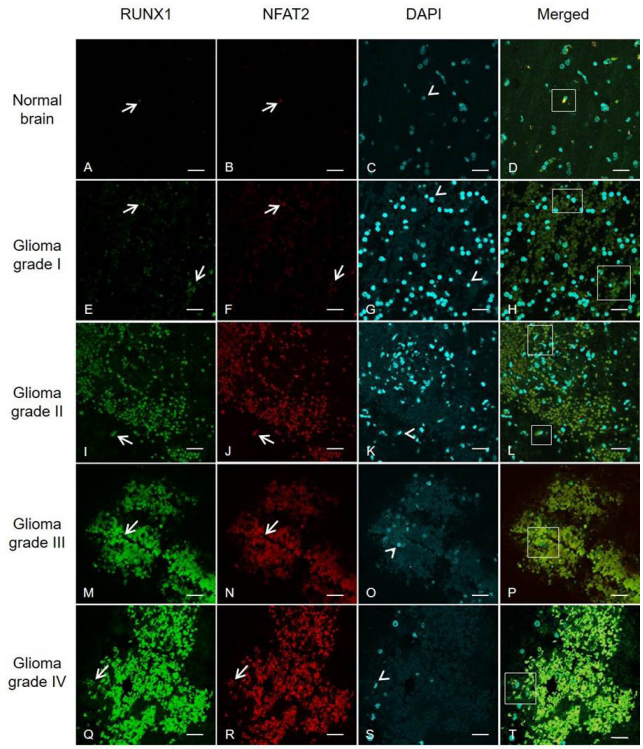

Figure 1 Double immunofluorescence staining of RUNX1 and NFAT2 in human glioma specimens, WHO grade I-IV. Immunoreactivity of RUNX1 (in panel RUNX1, represented in green) and NFAT2 (in panel NFAT2, represented in red) was observed in the cytoplasm of glioma grade I (E-H), grade II (I-L), grade III (M-P), and grade IV (Q-T), respectively, as compared with human normal brain control (A-D). Arrows indicate tumor cell with immunoreactivity. Arrowheads indicate DAPI-labelled nuclei of the corresponding cells (in panel DAPI, presented in light blue). Colocalization with DAPI (in panel merged) was presented in the cytoplasm of each grade. Boxes indicate a cell with colocalized in the area stained with the combined colors. The co-existence of RUNX1 and NFAT2 was found higher in gliomas with higher grade. (scale bar = 20 μm).

Semi-quantitative analysis of RUX1 and NFAT2 expression

In order to validate and provide significance to the obtained results, the semi-quantitative analysis for the relative fluorescent intensity was performed in the study. Expression of RUNX1 and NFAT2 immunoreactivity were markedly increased ranging from grade I to IV relative to normal brain control ($p<0.05$ in glioma grade I-III; and $p<0.01$ in glioma grade IV) (Figure 2). In addition, the immunostaining in the cytoplasm for NFAT2 displayed a slightly more intense than that for RUNX1 in each glioma grade (Figure 2).

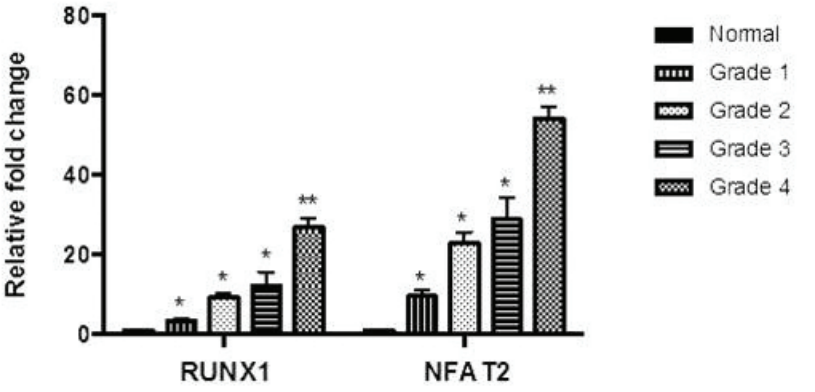

Figure 2 The semi-quantitative analysis of immunofluorescence intensity of RUNX1 and NFAT2 in human glioma, WHO grade I-IV. Evaluation of gliomas each grade with altered RUNX1 and NFAT2 degree was compared as fold change in expression relative to human normal brain control. * $p<0.05$, ** $p<0.01$ (n = 5).

GFAP staining

Astrocytomas express glial fibrillary acidic protein (GFAP) uniquely found in astrocytic tumors in general. Positive reaction to GFAP has been regularly demonstrated to identify a glioma as an astrocytoma. In such manner, an immunofluorescent labeling using antibody to GFAP was also performed in this study. It was found that glioma sections, grade I-IV, corresponding to the experimental groups, showed strongly staining intensity for GFAP which represented in blue (Figure 3 A-D). Thus, this finding revealed their immunoreactivity astrocytes-specific expressions of astrocytoma tumor.

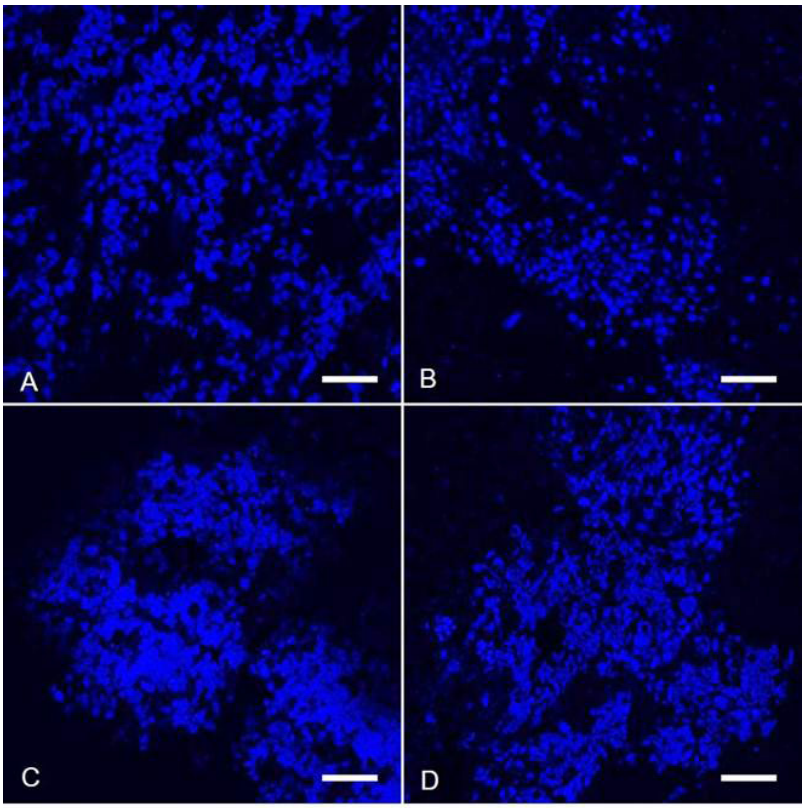

Figure 3 Immunofluorescence staining of GFAP in human glioma specimens, WHO grade I (A), grade II (B), grade III (C), and grade IV (D), corresponding to the experimental groups in each figure. The strongly immunoreactivity in the cytoplasm, which represented in blue, indicated tumors are derived from astrocytes (scale bar = 20 μm).

Discussion

The WHO has classified CNS tumors into grade I-IV by tracing the cellular origin of cancer and how the cells behave abnormally, from the least aggressive (benign/nonmalignant) to the most aggressive (malignant)[4]. Previously, WHO classified brain tumors mainly on the basis of distinctive features microscopically. This resulted in confused among pathologists even if the same criteria were performed. Since 2016, WHO CNS classification was revised by incorporating the genetic and molecular basis of tumorigenesis into the conventional histopathological approach. Thus, this multilayer diagnosis could lead to a more specific grading scheme and increase the possibility of inconsistent results[2]. In this study, gliomas were classified into grade I-IV according to WHO CNS version of 2007[1]. All specimens were shown to be astrocytic cells types of origin

as confirmed by GFAP immunoreactivity. Glial-fibrillary acidic protein is known as type III intermediate filament proteins. Routinely, GFAP has been employed as traditional immunohistochemical marker for glial cells. It is found most typically in astrocytes, with more variable for detection of oligodendroglial cells[30]. In addition, the evidence of massive proliferation and invasion was considerably observed ranging from grade I to IV. This is due to the formation of abnormal tumor vasculature and glioma cell invasion, those of which are believed to be the major factors responsible for the resistance of these tumors to treatment[31]. Consequently, regulation of angiogenesis and invasion in human malignant gliomas are challenging to study in the experimental settings for development of a curative therapy.

Transcription factors (TFs) are proteins that bind to short specific DNA-regulatory sequences, usually 8-21 bp, which are called transcription factor binding sites (TFBS), also called *cis*-acting elements. They work as to modulate the regulation of transcription on their target genes. This may lead to up- or down-regulated gene transcription, protein synthesis, and following change of cell function[32]. Moreover, TFs may interact with each other to form homodimers or heterodimers that resulting in activating or repressing of transcriptional activity. Generally, it is necessary to have coincident activation of several TFs in order to provide maximal gene expression[33]. Previous studies showed a trend of greater prediction of synergistic TFs in order to identify the biological context in which the couples are involved in and the process they should contribute to regulate in the transcription regulator network that performed mainly by computational based approach[32,34]. This includes RUNXs and NFATs families. For instance, Runx1-NFAT was found to be overrepresented as TFBS-pairs transcriptional network in the group of regulatory T (Treg) cells[35]. Bioinformatics analyses of global gene expression data elucidated that Runx1-NFAT regulated Treg cell differentiation and maintenance. Until recently, the newly identified NFAT partners (e.g. Raptor, CHEK1, RUNX1, and Ikaros) with NFAT proteins by CoIP-immunoblotting was also confirmed the association between RUNX and NFAT families[36].

Angiogenesis is the formation of new blood vessels via spouting or remodeling of pre-existing capillary beds, which involves proliferation, migration, and degradation of endothelial cells. Similarly, it is well recognized and believed to be the fundamental step of neovascularization that proceeds through the malignant tumors, including gliomas. In tumors, upregulation of angiogenesis is a key process in sustained tumor growth and metastasis. Tumors need independently blood supply, which

is obtained by the expression of growth factors[5,37]. Many studies have revealed that TFs trigger and maintain tumor vasculature via angiogenic growth factors, such as basic fibroblast growth factor (bFGF), and vascular endothelial growth factor (VEFG). The VEGF, a potent angiogenic inducer, plays an indispensable role in regulation of angiogenesis, vasculogenesis and vascular permeability in the developing brain and different types of brain tumors[38]. Thus, inhibition of VEGF-A in myeloid cells attenuates glioma progression and prolongs survival in mouse GL261 glioma cell line[39].

As yet, many on-going researches have focused on the existence of RUNXs and NFATs transcription factors particularly during development of tumors. It was found that VEGF-calcineurin-NFAT regulated tumor angiogenesis and metastasis. In the suppression of two newly identified NFATc1 (aka NFAT2), it was demonstrated that by using patient samples CXCR7 abrogated VEGF-mediated cell migration and tube formation, as well as RND1 could impair vascular barrier function. This suggested that dynamic NFATc1 binding to angiogenesis-related genes was critical for VEGF-mediated endothelial cell activation[40]. Likewise, a number of studies concerning RUNX1 were found to be related to the activation of VEGF and FGF in tumorigenesis. Previous study in mice lacking of RUNX1 exhibited a defect in hematopoiesis and died in utero. In contrast, the expression of RUNX1 was increased by angiogenic growth factors, bFGF and VEGF, which was confirmed at the site of angiogenesis *in vivo*. Besides, VEGF regulated endothelial cell tubulogenesis and sprouting events by controlling tip-cell behavior was indicated in mouse MSS31 endothelial cell line[41]. More recently, suppression of RUNX1 mRNA in human glioblastoma U-87 MG cell line could disrupt the vascular tube morphogenesis and sprouting in human umbilical vein endothelial cells. It was claimed that RUNX1 may be one of the molecular targeted against glioma metastasis and angiogenesis[23]. All the evidences support the findings of this study that we found the expression of RUNX1 and NFAT2 transcription factors in human glioma samples by coimmunofluorescence staining. Interestingly, both RUNX1 and NFAT2 were significantly observed with higher fluorescent extent in the gliomas with higher grade. Accordingly, the extensive involvement of RUNX1 and NFAT2 found in glioma tissues during tumorigenic processes strengthen the oncogenic potential of both transcription factor proteins. In particular, this may profoundly explain their function as transcriptional co-activators regarding the mechanism of angiogenesis.

Conclusion

Human glioma specimens, WHO grade I-IV, were immunostained by dual immunofluorescence with RUNX1 and NFAT2 proteins. The distribution of RUNX1 and NFAT2 was confined in the cytoplasm of glioma as compared with human normal brain control significantly. The co-existence was observed in the same tumor cells with higher fluorescent extent in gliomas with higher grade. The GFAP immunoreactivity indicated all glioma samples were derived from astrocytes. Therefore, involvement of RUNX1 and NFAT2 transcription factors during tumorigenic process may support their functions in controlling differentiation and transformation of angiogenesis in gliomas.

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