

Original Article

Characterization of NFAT expression in human glioma and its correlation with tumor grade

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Abstract

The nuclear factor of activated T cells 1 and 2 (NFAT1 and NFAT2) are transcription factors shown to be upregulated and correlated with the severity of several malignancies. This study aimed to examine the expression of NFAT1 and NFAT2 in grades I-IV glioma and its correlation with tumor grade using immunostaining and Western blot. The samples were glioma tissues (n=52) from Ramathibodi Hospital, Thailand. Both NFAT1 and NFAT2 were overexpressed in the tumor cells of grades I-IV gliomas shown by co-staining with olig2, a marker for glioma cells. High grade gliomas appeared to express more NFAT2 levels compared with low grade tumors. No NFAT was observed in microglia/macrophages illustrated by CD68, a microglial marker. Moreover, there was co-localization of NFAT2 and VEGF, a prominent angiogenic factor in grade IV tumors. The results suggested the roles of NFATs in the progression of glioma and the association with angiogenesis.

Keywords: brain tumor, glioma, NFAT, Olig2, VEGF

1. Introduction

The nuclear factor of activated T cells (NFAT) is a family of transcription factors with 5 members (NFAT1-5) (Macian, 2005). It is present ubiquitously throughout the body and has a wide range of functions physiologically and pathologically (Muller & Rao, 2010). NFAT proteins can be

activated by various stimuli and subsequently lead to pathways involved in the regulation of genes related to cell proliferation, differentiation, apoptosis, and angiogenesis which are important in tumor development. NFATs are overexpressed in many types of cancers such as breast cancer, pancreatic cancer, lung cancer, colorectal cancer, and hematological cancers (Mancini & Toker, 2009). Research has pointed out that NFAT signaling is implicated in all steps of cancer development from tumor cell growth, progression, migration to tumor angiogenesis, and interaction with normal cells (Pan, Xiong, & Chen, 2013). For example, NFAT1 was protumorigenic in colorectal cancer and knock-out of NFAT1 protected

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the mice from developing a colorectal tumor. Such effect might be from roles of NFAT1 in cytokine production, regulation of apoptosis as well as regulation of vascular endothelial growth factor (VEGF)-induced angiogenesis (Daniel, Gerlach, Vath, Neurath, & Weigmann, 2014). NFAT2 was upregulated and could directly induce c-myc oncogene transcription in pancreatic cancer (Koenig *et al.*, 2010). For NFAT3, the results varied across the tumors with all the experiments performed in cell lines. It appeared to inhibit motility of breast cancer cells (Fougere *et al.*, 2010). On the other hand, NFAT3 induced cell transformation in skin cancer cells (Ouyang *et al.*, 2007; Xiao *et al.*, 2017). NFAT4 was the downstream mediator of Fas signaling in gastrointestinal cancer metastasis (Zheng *et al.*, 2014). NFAT5 promoted migration of breast cancer cells through interaction with integrins (Jauliac *et al.*, 2002).

Glioma is the most prevalent and most invasive type of primary brain tumor (Ostrom, Gittleman, Stetson, Virk, & Barnholtz-Sloan, 2015). World Health Organization (WHO) classified astrocytic tumors into four grades on the basis of histopathological features including the presence or absence of nuclear atypia, mitosis, microvascular proliferation, and necrosis (Louis *et al.*, 2007). Grade I or pilocytic astrocytoma is a benign slow-growing tumor. Grade II or low grade diffuse astrocytoma shows increased cellularity with a monomorphic population of cells infiltrating the neuropil. Grade III or anaplastic astrocytoma is a cancerous tumor characterized by dense cellularity, mitotic activity, nuclear atypia, and loss of cellular differentiation. It can quickly grow and spread. Grade IV or glioblastoma multiforme (GBM) is the most aggressive and the most common of these tumors. It is characterized by uncontrolled cellular proliferation, heterogeneous histology, diffuse infiltration, robust angiogenesis, and necrosis (Gladson, Prayson, & Liu, 2010). Recently, WHO updated the classification of glioma to include profiles of *IDH* (isocitrate dehydrogenase) gene mutations into *IDH*-mutant and *IDH*-wildtype tumors (Louis *et al.*, 2016). However, *IDH* profiling cannot differentiate gliomas with 100% accuracy and co-detection of other cancer biomarkers could help guide better diagnostic and therapeutic measures.

Regarding the roles of NFATs in glioma, NFAT2 was present in rat C6 glioma cell line and it was found that cyclosporine A, an immunosuppressant drug, induced C6 cell apoptosis (Pyrzynska, Lis, Mosieniak, & Kaminska, 2001). Wang *et al.* found that NFAT2 promoted invasion of U251 human glioma cell line via activation of COX-2 and that knocking down NFAT2 expression could prevent invasion of U251 cells (Wang *et al.*, 2015). Similar to NFAT2, NFAT1 was also found to regulate invasion of human glioma cell line (Tie, Han, Meng, Wang, & Wu, 2013). Importantly, administration of NFAT antagonist reduced NFAT1 expression and invasive activity of tumor cells but not their proliferation (Tie *et al.*, 2013). However, Gopinath *et al.* (2009) found NFAT to be protective against glioma progression and demonstrated that doxorubicin, a chemotherapeutic drug used in the treatment of glioblastoma, required NFAT3 to mediate apoptotic effect in the U87 glioma cell line.

The examination of NFAT1 and NFAT2 expression is scarce and has not been performed in Thai patients. In this study, glioma tissues of various grades were used to examine the levels of NFAT expression and the correlation with tumor grade. Also, VEGF levels and their correlation with NFAT in

four grades of glioma were determined. The information gained from this study could help define the significance of NFAT in glioma progression, especially in Thai patients.

2. Materials and Methods

2.1 Human tissue

All specimens of human tissue were obtained from the Department of Pathology, Faculty of Medicine, Ramathibodi Hospital. The tissues were biopsy specimens from patients between 1997 and 2013. The diagnosis was confirmed by a pathologist. The samples included 9 cases of grade I pilocytic astrocytoma, 10 cases of grade II diffuse astrocytoma, 16 cases of grade III anaplastic astrocytoma, and 17 cases of grade IV GBM. The mean ages (\pm SEM) of patients were 7 ± 1.17 , 26.89 ± 5.37 , 35.07 ± 5.38 , and 49.12 ± 4.04 years in grades I-IV glioma, respectively. Forty-five percent were male. The study was approved by the committee on human rights related to research involving human subjects based on the Declaration of Helsinki (Institutional Review Board, Ramathibodi Hospital, Mahidol University).

2.2 Immunohistochemistry and immunofluorescence

Formalin-fixed and paraffin-embedded tissue sections at 10-15 μ m thickness were deparaffinized in xylene, rehydrated through ethanol gradient and washed with phosphate buffered saline (PBS). Antigen retrieval was performed in pH 8.0 EDTA buffer 95 °C for 40 min. Endogenous peroxidase was blocked with 3% H₂O₂ for 15 min. The slides were treated with normal goat serum blocking solution for 1 h and incubated with primary antibody overnight at 4 °C. The primary antibodies employed were NFAT1 (1:500, clone G1-D10, Santa Cruz Biotechnology, CA, USA and clone D43B1, Cell Signaling Technology, Beverly, MA, USA) and NFAT2 (1:500, clone 7A6, Abcam Inc., Cambridge, MA, USA and clone D15F1, Cell Signaling Technology). On the next day, the slides were incubated with biotinylated secondary antibody for 30 min and peroxidase-labeled avidin-peroxidase-complex for 1 h. The peroxidase reaction was visualized using H₂O₂ as substrate and 3,3'-diaminobenzidine as chromogen, yielding a brown staining. Finally, sections were counterstained with Mayer's hematoxylin, dehydrated and coverslipped. For negative controls, the primary antibodies were omitted. Normal brain tissues were used for the control. The sections were observed under a microscope (BX53, Olympus, Japan) with cellSens software (Olympus).

For immunofluorescence, two primary antibodies were applied. The antibodies employed were NFAT1/2, CD68 for microglia (Abcam Inc.), Olig2 for glioma cells (Abcam Inc.), and VEGF (Abcam Inc.). The secondary antibodies were Alexa 488 fluor goat anti-mouse and Alexa 647 fluor goat anti-rabbit (both from Abcam Inc.). The sections were observed using FV10i-DOC Confocal Laser Scanning Microscope (Olympus) and FluoView software (Olympus).

2.3 Quantification with H-Score

The immunohistochemistry results were presented by using H-Score which is a semi-quantitative method as described previously (Detre, Saclani Jotti, & Dowsett, 1995).

This method takes both the percentage of cells stained and the intensity of the staining into account. The intensity of staining was scored as 0, 1, 2, or 3, corresponding to negative, weak, intermediate, or strong immunoreactivity, respectively. The H-score (H) was calculated according to this formula: $H = (\% \text{ of cells that stained intensity category } 1 \times 1) + (\% \text{ of cells that stained at intensity category } 2 \times 2) + (\% \text{ of cells that stained at intensity category } 3 \times 3)$. This score ranges from 0 to 300.

2.4 Western blot analysis

After deparaffinization, the proteins were extracted using EXB plus (Qiagen, Germany). The protein concentration was defined using Lowry's method (Geiger & Bessman, 1972). Then 40 μg of proteins were loaded onto 9% SDS polyacrylamide gel electrophoresis (SDS-PAGE). The current of electrophoresis was at 80V for 1.5 h. Proteins were transferred to a nitrocellulose membrane, blocked with PBST + 3% BSA for 1 h, and incubated at 4 °C overnight with primary antibodies (NFAT1; 1:100, NFAT2; 1:500). Blots were then exposed to horseradish peroxidase-conjugated secondary antibodies (1:3000). To ensure equal protein loading, we stripped the blots and reprobed with an anti- β -actin antibody. Membranes were visualized by enhanced chemiluminescence using ECL plus TM western blotting detection reagents (Amersham Biosciences, Piscataway, NJ, USA).

2.5 Statistical analysis

The results are represented as mean \pm SEM. The statistical significance of H-score differences among tumor grades was evaluated with one-way analysis of variance (ANOVA) followed by Dunnett's T3 post hoc test using SPSS program version 16 (IBM Corporation, Armonk, NY, USA). The assumptions of ANOVA were tested. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1 Expression of NFAT1 and NFAT2 in gliomas

Glioma tissues from 52 patients that consisted of grade I pilocytic astrocytoma (n= 9), grade II diffuse astrocytoma (n=10), grade III anaplastic astrocytoma (n=16), and grade IV GBM (n= 17) were immunostained with NFAT1 antibody and compared with normal brain tissue (n=5). Figure 1A demonstrates the representative staining pattern of NFAT1 in glioma grades I-IV and normal brain. Normal brain tissue did not show NFAT1 expression (data not shown).

H-score was used to quantify the levels of NFAT1 staining in all grades of glioma. The average H-scores were 64.67 \pm 10.45 for grade I pilocytic astrocytoma, 57.45 \pm 10.48 for grade II diffuse astrocytoma, 58.63 \pm 11.72 for grade III anaplastic astrocytoma, 91.40 \pm 13.57 for grade IV GBM, and 4.67 \pm 1.91 for normal brain tissue (Figure 1B). H-scores of gliomas of all grades were significantly different from the normal brain tissue. Grade IV GBM tended to express a higher level of NFAT1 but it was not significantly different from low grade gliomas.

Similar to NFAT 1 staining, immunohistochemistry of NFAT2 was evaluated in 52 patients of all grades of glioma

compared with normal brain tissue. NFAT2 staining in grades I-III glioma showed mainly cytoplasmic localization while in grade IV GBM, NFAT2 existed in both the nuclei and the cytoplasm (Figure 2A). Normal brain tissue did not express NFAT2 (data not shown). The levels of NFAT2 staining were determined using the H-score. The average H-scores were 54.20 \pm 9.69 for grade I pilocytic astrocytoma, 79.47 \pm 13.55 for grade II diffuse astrocytoma, 87.29 \pm 19.04 for grade III anaplastic astrocytoma, 122.63 \pm 17.85 for grade IV GBM, and 4.73 \pm 1.94 for normal brain tissue (Figure 2B). The H-scores of gliomas of all grades were significantly different from normal brain tissue. Grade IV GBM expressed a significantly higher level of NFAT2 than grade I pilocytic astrocytoma and there was a non-significant positive correlation between NFAT2 expression and glioma grades.

3.2 NFAT1 and NFAT2 localization

NFAT1 and NFAT2 double immunofluorescences were performed to determine if they co-expressed. The tissues from grade IV GBM were used to ensure adequate positive staining. The results demonstrated that NFAT1 and NFAT2 were co-localized (Figure 3).

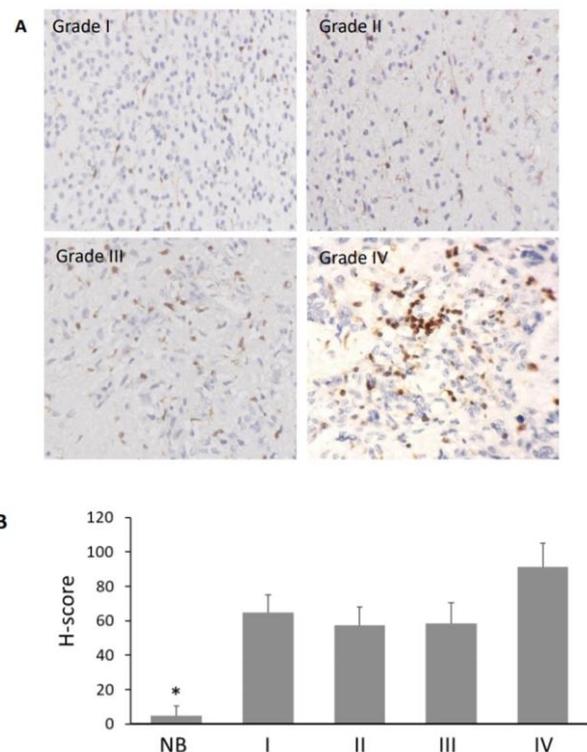


Figure 1. NFAT1 expression in gliomas. A) Representative staining of NFAT1 in Grade I – IV gliomas. The cells showing positive staining are brown. Hematoxylin was employed as a counterstain (blue). B) H-score of NFAT1 staining. The samples are glioma grade I (n=9), grade II (n= 10), grade III (n= 16), grade IV (n= 17), and NB (normal brain tissue; n= 5). Values are expressed as mean \pm SEM; * $P < 0.01$ compared with grades I-IV gliomas.

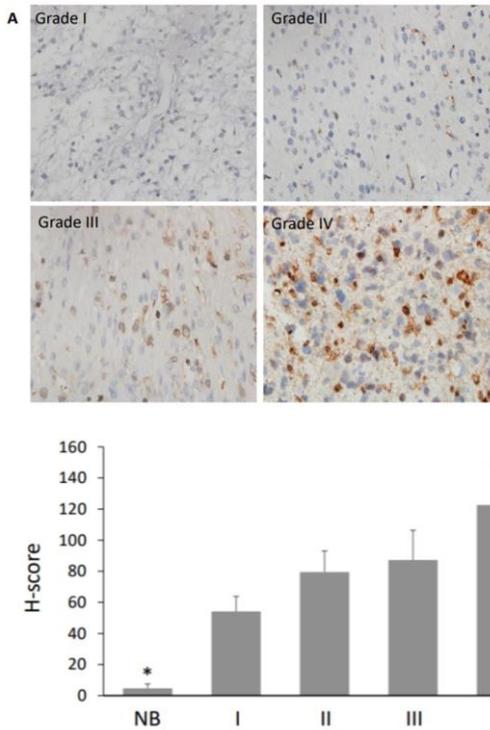


Figure 2. NFAT2 expression in gliomas. A) Representative staining of NFAT2 in Grades I-IV glioma and normal brain tissue. The cells showing positive staining are brown. Hematoxylin was employed as a counterstain (blue). B) H-Score of NFAT2 staining. The samples are glioma grade I (n=9), grade II (n=10), grade III (n=14), grade IV (n=15), and NB (normal brain tissue, n=5). Values are expressed as mean±SEM; *P<0.01 compared with grades I-IV gliomas. ** P<0.05 compared with grade I glioma.

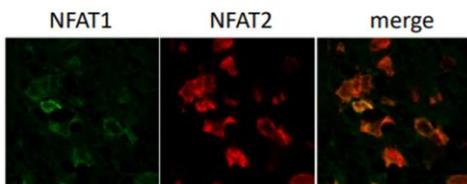


Figure 3. NFAT1 and NFAT2 double staining. Representative double immunofluorescence of NFAT1 and NFAT2 in grade IV GBM tissues. The magnification is at x180.

To determine the types of cells that express NFAT1, double immunofluorescence was performed with NFAT1 or NFAT2 and Olig2, a marker for glioma cells. As expected, normal brain tissue did not express either NFATs or Olig2 (data not shown). Glioma of all grades expressed Olig2. The pattern of Olig2 staining varied with the tumor grades. Grade I pilocytic astrocytoma showed homogeneous Olig2 staining while grade IV GBM showed a heterogeneous abnormal Olig2 pattern suggesting the more pleomorphism of cells (Figures 4 and 5). The percentages of cells expressing Olig2 were 75.05±9.94% in grade I pilocytic astrocytoma, 89.71±3.23% in grade II diffuse astrocytoma, 92.05±4.00% in grade III anaplastic astrocytoma, and 95.63±3.78% in grade IV GBM.

NFAT1 almost always co-localized with Olig2 which suggested that tumor cells expressed this transcription factor (Figure 4). The percentages of Olig2-positive cells that were also stained with NFAT1 were 31.82±8.35% in grade I pilocytic astrocytoma, 39.10±8.33% in grade II diffuse astrocytoma, 64.07±4.37% in grade III anaplastic astrocytoma, and 80.89±5.91% in grade IV GBM.

Similar to NFAT1, NFAT2 also co-localized with Olig2 (Figure 5). The percentages of Olig2-positive cells that were also stained with NFAT2 were 28.79±6.12% in grade I pilocytic astrocytoma, 34.44±8.68% in grade II diffuse astrocytoma, 66.86±14.68% in grade III anaplastic astrocytoma, and 82.22±17.78% in grade IV GBM.

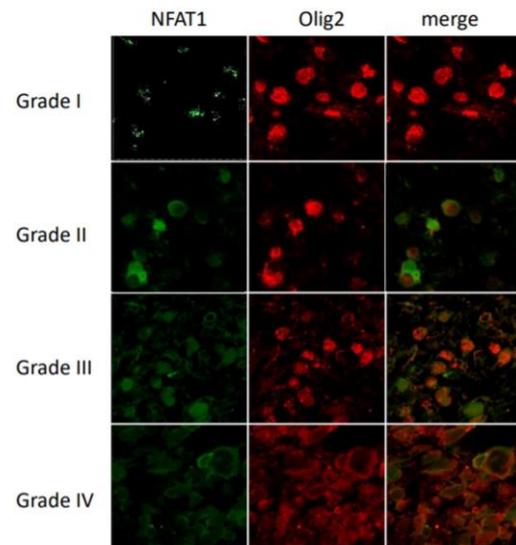


Figure 4. NFAT1 and Olig2. Representative double immunofluorescence of NFAT1 and Olig2 in grades I-IV gliomas. The magnification is x180.

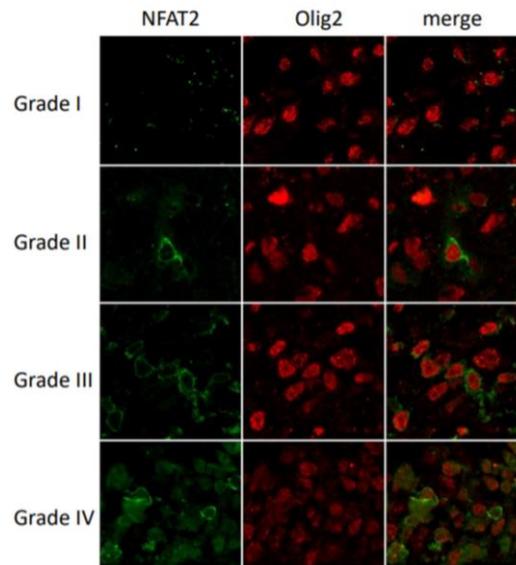


Figure 5. NFAT2 and Olig2. Representative double immunofluorescence of NFAT2 and Olig2 in grades I-IV gliomas. The magnification is x180.

3.3 NFAT2 expression in microglia/macrophages

To explore whether NFAT2 were also expressed in other types of cells, we used CD68 antibody, a marker for microglia and macrophages, double-stained with NFAT2. CD68 was much more prominent in high-grade gliomas than in low-grade ones suggesting more microglia and tumor-associated macrophages involvement in these gliomas. CD68-positive cells did not show NFAT2 expression which indicated that NFAT2 was not expressed in these immune cells (Figure 6).

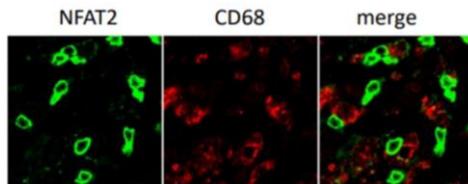


Figure 6. NFAT2 and CD68. Representative double immunofluorescence of NFAT2 and CD68 in grade IV glioma. The magnification is x180.

3.4 NFAT2 and VEGF expression

To determine whether NFAT2 expression co-localized with VEGF, anti-NFAT2 antibody was used for double staining with anti-VEGF antibody. The results revealed that VEGF expression correlated with glioma grades with most prominent staining in grade IV GBM and no staining in low grade gliomas (Figure 7). NFAT2 and VEGF were co-localized in high grade gliomas.

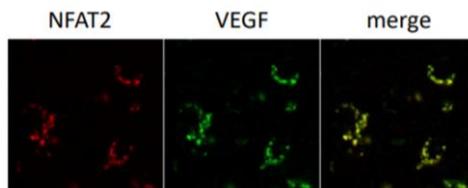


Figure 7. NFAT2 and VEGF. Representative double immunofluorescence of NFAT2 and VEGF in grade IV glioma. The magnification is x180.

3.5 NFATs protein expression

Western blot was also performed to confirm the expression of NFAT1 and NFAT2 proteins. Paraffin-embedded tissue of tonsil and grade IV GBM were employed for this experiment. Tonsil was used as a positive control. Both tonsil and grade IV GBM tissues expressed NFAT1 and NFAT2 (Figure 8).

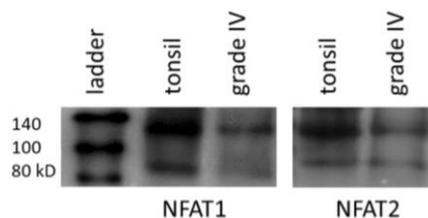


Figure 8. Expression of NFAT1 and NFAT2 proteins in tonsil and grade IV GBM.

4. Discussion

The current study aimed to explore the expression of NFAT1 and NFAT2 in grades I-IV human glioma tissues. NFAT1 and NFAT2 were expressed in all grades of gliomas (Figures 1 and 2) and normal brain tissue did not express either NFAT1 or NFAT2. The levels of NFAT1 expression were not correlated with the tumor grades. It is possible that the number of cases in our study was too low to have statistically significant values. NFAT1 was mainly present in the nucleus suggesting that the active form was at work. Of note was the presence of nuclear pleomorphism in grade IV glioblastoma. Our findings are in line with a recent study which also demonstrated nuclear expression of NFAT1 in human glioblastoma and astrocytoma samples (Tie *et al.*, 2013). However, the levels of NFAT1 in these samples were not determined. Contrary to NFAT1 staining, NFAT2 had a positive correlation with the tumor grades with grade IV tumor showing significantly higher expression than grade I as determined by the H-scores. Another recent study similarly found increased levels of NFAT2 expression in grade II-IV gliomas and none in non-neoplastic brain tissue but the investigation was not performed in grade I samples (Wang *et al.*, 2015). In grade IV GBM, NFAT2 was present in the nucleus suggesting that the active form was prevalent. While in grades I-III, NFAT2 was more prevalent in the cytoplasm which suggested that the inactive form was predominant (Figure 2). Studies on the roles of NFATs in glioma are scarce. Recent studies have demonstrated that NFAT1 and NFAT2 promoted invasion of human glioma cell lines *in vitro* and that inhibition of NFAT activity could block the invasiveness of the cells (Tie *et al.*, 2013; Wang *et al.*, 2015). Examination of NFAT1 and NFAT2 expressions could be utilized especially together with other cancer biomarkers such as *IDH* genes to improve the diagnostic accuracy, and finally the therapeutic options. For example, a tumor with histology of grade IV glioblastoma with high NFAT2 expression and wildtype *IDH* gene might suggest poorer prognosis.

Since NFATs are generally expressed in immune cells which are present in a great proportion in gliomas, we next examined whether NFAT1 and NFAT2 were present in the tumor cells or in innate cells such as microglia and tumor-associated macrophages. To determine the types of cells that express NFAT1 and NFAT2 we performed double immunofluorescence with Olig2 (a marker for glioma cells) and CD68 (a marker for microglia/macrophages). Olig2 was not found in normal brain tissue but was present in all grades of gliomas. Of note was the characterization of Olig2 staining in different grades of gliomas. More abnormalities from Olig2 staining were seen in the higher grades of tumor. Both NFAT1 and NFAT2 were mainly co-localized with Olig2. CD68 was present in high grade tumors but scarce in low grade tumors and not present in normal brain tissue. This is in line with previous studies that showed the correlation of microglial staining with the tumor grades (Graeber, Scheithauer, & Kreutzberg, 2002). NFAT2 staining was not co-localized with CD68. Since NFAT1 and NFAT2 always co-expressed, it could be implicated that NFAT1 would not co-localize with CD68 like NFAT2 as well. In summary, these results suggested that NFAT1 and NFAT2 were expressed in the tumor cells and not in microglia/macrophages.

Angiogenesis is an essential process in glioma growth and progression and one of the most important factors in angiogenesis is VEGF (Sia, Alsinet, Newell, & Villanueva, 2014). Tumor cells (including glioma cells) can secrete VEGF (Goel & Mercurio, 2013). It was shown that VEGF correlated with tumor progression in pancreatic cancer, gastrointestinal stromal tumor, and brain tumor (Jain *et al.*, 2007). NFAT2 activation was identified as a critical component of VEGF-induced angiogenesis (Suehiro *et al.*, 2014). Previous studies have demonstrated that VEGF induced angiogenesis through activation of NFAT signaling, which further transcribed COX2 and subsequently, PGE2 (Yang *et al.*, 2012). PGE2 also plays important roles in endothelial cell proliferation, migration, and eventually vessel formation (Hernandez *et al.*, 2001). VEGF has been shown to be up-regulated in a portion of grade III anaplastic astrocytoma and most of grade IV GBM. The level of expression was correlated with the vascularity of glioma (Takano *et al.*, 1996). The association of VEGF and NFAT has not been explored in human glioma. Our study found that NFAT2 and VEGF were co-localized and VEGF was more prominent in grade IV glioblastoma and was hardly detectable in low grade tumors which suggested the associated role of NFAT and VEGF in angiogenesis, especially in grade IV GBM. More studies that examine the correlation of new blood vessels or tumor vascularity and NFAT or using NFAT inhibitor in an experimental model of glioma would further consolidate the role of NFAT in angiogenesis.

This study suggested the characteristic expression of NFAT1 and NFAT2 in human glioma grade I-IV using immunohistochemistry, immunofluorescence and Western blot. NFAT1 and NFAT2 levels were analyzed by the H-score which showed a trend to increase from low to high grades. Moreover, most of the tumor cells in grade IV GBM contained NFAT1 and NFAT2 but were hardly found in microglial cells. NFAT2 expression was correlated with VEGF, the hallmark of new blood vessel formation. It is likely that NFATs play important roles in tumor growth, development, and invasion. Further studies are needed to confirm the roles of NFATs in gliomas.

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