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NSMF acts as a novel biomarker in glioma

Yancheng Liu[a,b#], Xing Xu[a#], Yang Xie[a,b], Yongchang Yang[a,b], Chunlan Ning[a,b], Wenliang Li[c*], Xun Jin[a*]

Abstract:
Glioma is the most prevalent and deadly tumor of the central nervous system (CNS) in adults. However, Specific markers which are applied to diagnose and track the disease is still undiscovered. We found that the expression of NSMF is lowly expressed in glioma tissues and negative related to tumor grade and survival time, expressing databases by multiple glioma mRNA. Furthermore, we use The Human Protein Atlas discovered that glioma tissue NSMF protein expression is similar to transcriptome level, particularly obvious in the typical glioma pathological structure. Multivariate Cox analysis showed that high NSMF expression (HR value: 0.6, 95% CI 0.443–0.813, P=0.001) were independent predictors of long OS. This work revealed that NSMF may be a protective factor for the occurrence and development of gliomas, and can be used as a reliable biomarker for gliomas to diagnose and prompt prognosis of the disease.
Keywords: NSMF, glioma, biomarker, overall survival

Introduction

Diffuse glioma is one of the most lethal and recalcitrant cancers of all human, accounting for about 80% of overall gliomas. It is characterized by the infiltration of tumor cells into normal brain tissue, resulting in unclear tumor boundaries (Wesseling and Capper,2018). Even for low-grade diffuse gliomas, more than half of patients have early recurrence because surgery is difficult to completely remove glioma cells scattered in normal tissue. The glioblastoma, which is the highest level of diffuse gliomas, shows not only tumor cell highly diffuse but also high heterogeneity, such as tumor vascular hyperplasia, accompanied by edema, hemorrhage and necrosis. In spite of multiple therapies, the median survival time of the glioblastoma is only 15 months (Weller et al.,2013). Recurrence and distal metastasis is the main factor for the failure of diffuse gliomas treatment. When the tumor forms a mass, the diffused glioma cells in the normal brain tissues can be well detected and actively treated, which can effectively prevent glioma recurrence and distal metastasis. Therefore, finding markers to track these tumor cells is particularly crucial in the treatment of diffuse glioma. However, an excellent way to track scattered tumor cells still not to be found.

Clinical tumor markers can be currently divided into four categories, including diagnostic markers, prognostic markers, predictive markers and tracking markers. Diagnostic markers might assist in the tumor diagnosis and improving diagnostic accuracy. For instance, the serum level of CA19-9 is very low in the normal population but extremely high in the serum of patients with digestive system tumors. Therefore, the increase in serum CA19-9 is profitable for the diagnosis of digestive system tumors (Poruk et al.,2013). Prognostic markers provide information on the likely clinical course of the disease in an untreated individual. For example, IDH-mutant and 1p19q co-deleted oligodendroglioma have the longest median overall survival (17.5 years) (Bourne and Schiff,2010). Predictive markers are defined as a hallmark that is used to detect precancerous lesion or early stage of cancer and to identify a subpopulation. It is most likely that patients will go to respond to a certain therapy. The most notable predictive markers is the prostate-specific antigens (PSA), which are applied to the early detect of prostate cancer, making the mortality of prostate cancer steep reductions (Welch and Albertsen,2020). Tracking marker refers to the marker that can be used to track the site of tumor invasion and metastasis. In clinical practice, the pathological or radiological technology is mainly

[a.] Department of Biochemistry and Molecular Biology, Tianjin Medical University Cancer Institute and Hospital. National Clinical Research Center for Cancer, Key Laboratory of Cancer Prevention and Therapy, Tianjin. Tianjin's Clinical Research Center for Cancer, Tianjin 300060, China
[b.] Tianjin Medical University, Tianjin 300060, China
[c.] Department of Neuro-Oncology and Neurosurgery, Tianjin Medical University Cancer Institute and Hospital. National Clinical Research Center for Cancer, Key Laboratory of Cancer Prevention and Therapy, Tianjin. Tianjin's Clinical Research Center for Cancer, Tianjin 300060, China
[#.] The author contributed equally to this article.
**. Address correspondence to: Wenliang Li and Xun Jin*

used to track the tumor invasion and metastasis site. For example, using tissue staining to observe whether the pathological features of tumor cells or PET/CT exist, or whether the tumor has distant metastasis (Podoloff,2009; Niikura et al.,2013). However, when the lesion is very small, two methods in the above are inadequate to find tumor infiltration and distant metastasis. In conclusion, we have found a molecular marker which can not only make accurate diagnosis of tumors but also guide clinical treatment and reflect the prognosis of patients, what's more, tracking the progress of tumor. The markers can contribute to early screening, diagnosis and treatment of tumors.

As the diffuse gliomas we are studying now, diagnosing the clinical pathological of glioma is mainly to find the characteristic histological structure of malignant tumor cells. Then the differentiation markers of glial cell were used to determine the source of tumor cells and differential diagnosis with other brain tumors. For instance, that glial fibrillary acidic protein (GFAP), oligodendrocyte transcription factor 2 (OLIG2) and S100 calcium binding protein B (S100β) positive can be diagnosed glioma (Ikota et al.,2006). Normal glial cells express these markers, even its expression level is higher than glioblastoma. For the degree of glioma cell differentiation is different, glioblastoma have more glioma stem cells (Prager et al.,2020). Thus, these differentiation markers can only be qualitative, poorly reflecting the malignant degree of the tumor or prompting the patient's prognosis. With the growing popularity of genetic diagnosis, IDH status and CPG island methylation can help prompt the prognosis and guide the treatment of glioma (Jansen, Yip and Louis,2010), but is lack of tracking tumor progress.

Studies have found that a large amount of BDNF can be secreted in normal nerve cells, which promotes and maintains the differentiation of nerve cell (Hu et al.,2010). The continuous secretion of BDNF is regulated by NMDA receptor synaptonuclear signaling and neuronal migration factor (NSMF) (Spilker, Grochowska and Kreutz, 2016). NSMF is under-expressed in gliomas, and its expression level is negatively correlated with the grade of gliomas and has the characteristics as a tracking marker. Therefore, this article mainly discusses whether NSMF can be used as a great marker in diffuse gliomas in many ways or not.

Materials and Methods

Data acquisition

All kinds of gene expression profiles of glioma datasets were downloaded from the GlioVis (https://gliovis.bioinfo.cnio.es/), including TCGA GBMLGG dataset (n = 667), TCGA LGG dataset (n = 513), Rembrandt dataset (n = 472), Grzmil dataset (n = 32), Gorovets dataset (n = 80) and Ivy GAP dataset (n =270). Tissue staining results from the online tool the Human Protein Atlas (HPA) (www.proteinatlas.org), including the nissl stain of the mouse brain tissue, NSMF in situ hybridization (ISH) and immunofluorescence staining in mouse brain tissue and immunohistochemistry (IHC) staining of NSMF expression in normal brain tissues and gliomas.

Statistical analysis

Data were analyzed by SPSS 21.0 and GraphPad Prism 7.0. One-way analysis of variance (ANOVA) was applied to analyze the difference of NSMF expression between groups. The relationship between NSMF expression and clinicopathological variables was assessed by using the $\chi 2$ test. Survival analysis for overall survival (OS) was performed by utilizing the Kaplan-Meier method and log-rank test. Correlation analysis of survival time and various clinical pathological variables was performed by using univariate and multivariate Cox proportional hazard analysis. In the univariate Cox analysis, factors with p value below 0.1 were included in the multivariate Cox analysis. A two-tailed p value below 0.05 was considered statistically significant.

Results

NSMF is mainly expressed in mature nerve cells.

Based on The Brain Atlas, we analyzed the expression of NSMF in normal brain tissue. According to the nissl stain of the mouse brain tissue, it is found that Subventricular Zone (SVZ), hypothalamus and cerebellum was junior than cortex (Figure 1A), indicating the fewer mature nerve cell in these areas. Combined with previous studies, it is found that neural stem cells are mainly distributed in these areas. Analysis of NSMF in situ hybridization (ISH) and immunofluorescence staining in mouse brain tissue showed that the SVZ, hypothalamus and cerebellum staining was obvious shallow (Figure 1B, C), indicating that NSMF mainly exists in differentiated

nerve cells at the nucleic acid and protein levels. Then we analyze the expression of NSMF gene in different areas of mouse brain tissue, it is found that

NSMF is low expressed in the anatomical area corresponding to SVZ (Figure 1D). According to the above, NSMF is mainly expressed in mature nerve cells.

NSMF is low expressed in glioma tissue and

characteristic structures of glioblastoma

In glioma, we analyzed the expression datasets from The Cancer Genome Atlas (TCGA) GBMLGG cohort and found that NSMF was significantly down-regulated in glioblastoma compared to low grade glioma (P<0.001, Figure1E). Furthermore, analysis of expression dataset from Rembrandt showed that NSMF was down-regulated in different subtype of glioma tissues compared to normal brain tissues (P<0.001, Figure 1F). More specifically, analysis of Ivy_GAP dataset indicated that the expression of NSMF getting lower and lower as it closer to the cellular tumor (Figure 1G).

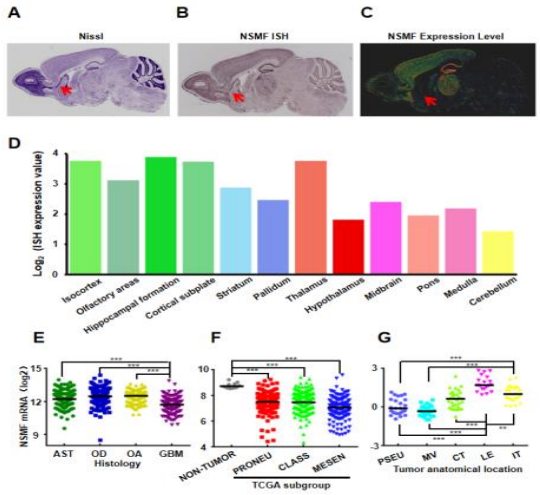
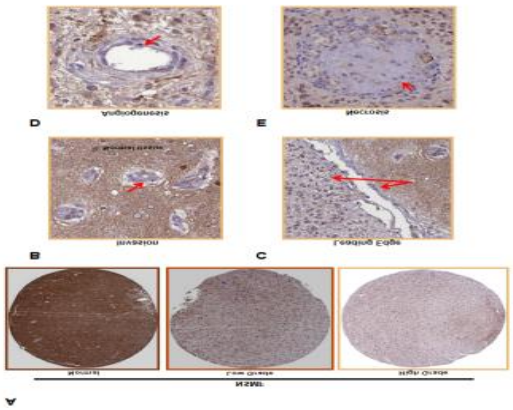


Figure 1.

(A) The nissl stain of the mouse brain tissue; the red arrow points to SVZ. (B) NSMF in situ hybridization staining of the mouse brain tissue. (C) NSMF immunofluorescence staining of the mouse brain tissue. (D) NSMF expression value in different areas of mouse brain tissue. (E) NSMF expression in gliomas of the TCGA GBMLGG dataset. NSMF expression in GBM was significantly lower than low grade gliomas (AST, OD and OA). (F) NSMF expression in gliomas of the Rembrandt dataset. NSMF expression in various molecular subtypes of gliomas was significantly lower than normal brain tissue. (G) NSMF expression in gliomas of the Ivy_GAP dataset. The lower expression of NSMF near the core of the tumor. Abbreviations: AST, Astrocytoma; OD, Oligodendroglioma; OA, Oligoastrocytoma; GBM, Glioblastoma; PSEU: Pseudopalisading cells; MV: Microvascular proliferation; CT: Cellular Tumor; LE: Leading Edge; IT: Infiltrating Tumor. ** P < 0.01; *** P < 0.001.

Then, based on The Human protein Atlas we analyzed that the expression of NSMF in human glioma tissue and found that the degree of tissue staining was negatively correlated with pathological grade (Figure 2A). The most distinctive feature is that, at the site of glioma infiltration and invasion, the NSMF staining of tumor cells is significantly shallower than that of normal brain tissue cells, and the tissue staining of the invasion and leading edge is clearly distinguishes from the normal brain tissue (Figure 2B, C). The angiogenesis and necrosis regions are characteristic structures of high level gliomas. The tumor cells in these two regions have strong stemness and proliferation ability, which are important areas for glioma cells infiltrate into normal brain tissue. We found that NSMF staining of tumor cells in agiogenesis and necrosis areas



were also significantly shallower than that of surrounding normal brain tissue (Figure 2D, E).

Figure 2. **Lower NSMF expression correlates with glioma malignancy.**

(A) Lower protein expression of NSMF was found in High grade gliomas than in Low grade gliomas and normal brain tissues in the TCGA dataset. (B) Low expression of NSMF at the site of tumor invasion. (C) Low expression of NSMF in the tumor leading edge. (D) Low expression of NSMF in tumor angiogenesis. (E) Low expression of NSMF in tumor necrosis.

High NSMF expression in patients with better prognosis indicators

Kaplan-Meier analysis indicated that high NSMF expression was associated with better OS in all kind of datasets, including TCGA GBMLGG dataset (HR = 2.259, P < 0.0001; Figure 3A), Rembrandt dataset (HR = 1.391, P = 0.0067; Figure 3B), Gramil dataset (HR = 6.077, Figure 3C), Gorovets dataset (HR = 3.971, P = 0.0031; Figure 3D) and TCGA LGG dataset (HR = 1.544, P = 0.019; Figure 3E). Univariate Cox analysis identified the potential OS-related variables, including the age, histopathology, WHO classification, IDH mutation status, 1p/19q col-deletion status, NSMF expression and so on (Table1).

Table 1. **Using TCGA GBMLGG dataset for Univariate Cox analysis. Abbreviations: CI, confidence interval; HR, hazard ratio.**

Univariate analysis

	HR	95% CI of Ratio	P-Value
NSMF	0.496	0.415-0.592	<0.0001
Age at Diagnosis	4.507	3.161-6.427	<0.0001
<40			
>=40			
Histology Type	8.967	6.795-11.832	<0.0001
NON-GBM			
GBM			
WHO Grade	9.563	7.157-12.777	<0.0001
II-III			
IV			
IDH.status	9.995	7.478-13.359	<0.0001
Mutant			
Wild Type			
Chr.1p-19q.codeletion	4.598	2.906-7.276	<0.0001
codeletion			
Non-codeletionl			
IDH-codeletionl.subtype	0.217	0.137-0.344	<0.0001
IDHmut-codeletion			
IDH-non-codeletion			
MGMT.promoter.status	3.301	2.432-4.373	<0.0001
Methylatedl			
Unmethylated			
Chr.7.gain_Chr.10.loss	0.123	0.092-0.164	<0.0001
Gain chr 7 & loss chr 10			
No combined CNA			
Chr.19_20.co_gain	0.292	0.189-0.453	<0.0001
Gain chr 19/20			
No chr 19/20 gain			
ATRX.status	2.521	1.826-3.481	<0.0001
Mutant			
Wild Type			
TERT.promoter.status	0.446	0.292-0.68	<0.0001
Mutant			
Wild Type			
TERT.expression.status	0.413	0.316-0.541	<0.0001
Expressed			
Not expressed			
DAXX.status	2.657	0.362-19.482	0.336
Mutant			
Wild Type			
Gender	1.168	0.891-1.532	0.261
Female			
Male			

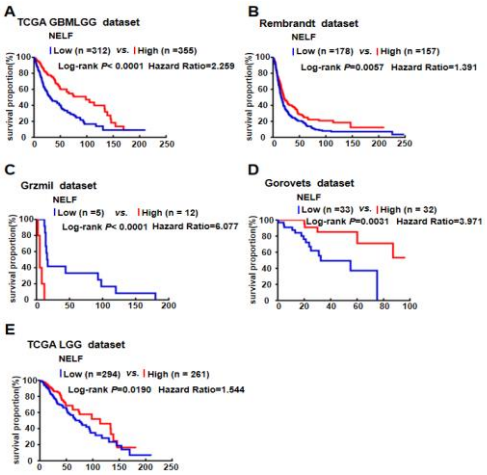


Figure 3. **Kaplan–Meier curves for OS according to NSMF expression in all kind of datasets.**

(A) TCGA GBMLGG dataset, (B) Rembrandt dataset, (C) CGGA dataset, (D) Grovets dataset, (E) TCGA LGG dataset.

Multivariate Cox analyses showed that high NSMF expression (HR value: 0.6, 95% CI 0.443–0.813, P=0.001), age of diagnosis before 40 (HR value: 0.41, 95% CI 0.227–0.743, p=0.003), IDH mutation status (HR value: 0.073, 95% CI 0.021–0.246, p＜0.0001) were independent predictors of long OS (Table 2).

Table 2. **Using TCGA GBMLGG dataset for Multivariate Cox analysis. Abbreviations: CI, confidence interval; HR, hazard ratio.**

Multivariate analysis

	HR	95% CI of Ratio	P-Value
NSMF	0.6	0.443-0.813	0.001
Age at Diagnosis	0.41	0.227-0.743	0.003
<40			
>=40			
Histology Type	0.579	0.277-1.214	0.148
Non-GBM			
GBM			
WHO Grade	--	--	--
II-III			
IV			
IDH.status	0.073	0.021-0.246	<0.0001
Mutant			
Wild Type			
Chr.1p-19q.codeletion	1.35	0.31-5.884	0.69
Codeletion			
Non-codeletion			
IDH-codeletion.subtype	--	--	--
IDHmut-codeletionl			
IDH-non-codeletionl			
MGMT.promoter.status	1.108	0.618-1.986	0.73
Methylatedl			
Unmethylated			
Chr.7.gain_Chr.10.loss	1.413	0.587-3.398	0.441
Gain chr 7 & loss chr 10			
No combined CNA			
Chr.19_20.co_gain	0.459	0.167-1.266	0.133
Gain chr 19/20			
No chr 19/20 gain			
ATRX.status	1.345	0.457-3.957	0.59
Mutant			
Wild Type			
TERT.promoter.status	0.711	0.223-2.27	0.565
Mutant			
Wild Type			
TERT.expression.status	1.102	0.418-2.907	0.844
Expressed			
Not expressed			

The low expression of NSMF in patients with glioma in the older age group suggested that the poor prognosis of patients with low NSMF expression may be related to the old age of the patients (Table 3).

Table 3. **χ2 test was used to assess the associations between NSMF expression and clinicopathological variables.NSMF expression levels and clinical characteristics**

Characteristics	Percentage of patients, n (%)		P-Value
	NSMF high (156)	NSMF low (151)	
Age at Diagnosis			
<40	53 (83)	38 (57)	0.0083
>=40	47 (73)	62 (94)	
Histology Type			
Non-GBM	99 (154)	87 (131)	< 0.0001
GBM	1 (2)	13 (20)	
WHO Grade			
II-III	99 (154)	87 (131)	< 0.0001
IV	1 (2)	13 (20)	
IDH.status			
Mutant	81 (126)	72 (108)	0.0616
WT	19 (30)	28 (43)	
Chr.1p-19q.codeletion			
codeletion	36 (56)	21 (32)	0.0054
non-codeletion	64 (100)	79 (119)	
IDH-codel.subtype			
IDHmut-codeletion	36 (56)	21 (32)	0.0054
IDH-non-codeletion	64 (100)	79 (119)	
MGMT.promoter.status			
Methylated	81 (126)	77 (117)	0.4864
Unmethylated	19 (30)	23 (34)	
Chr.7.gain_Chr.10.loss			
Gain chr 7 & loss chr 10	7 (11)	20 (30)	0.0012
No combined CNA	93 (145)	80 (121)	
Chr.19_20.co_gain			
Gain chr 19/20	1 (1)	7 (10)	0.0049
No chr 19/20 gain	99 (155)	93 (141)	
ATRX.status			
Mutant	36 (56)	40 (60)	0.5562
Wild Type	64 (100)	60 (91)	
TERT.promoter.status			
Mutant	47 (74)	48 (73)	0.9093
Wild Type	53 (82)	52 (78)	
TERT.expression.status			
Expressed	40 (62)	46 (69)	0.2918
Not expressed	60 (94)	54 (82)	

Discussion

For gliomas, the four types of markers are used in clinical practice. And finding a marker to meet these requirements will greatly improve the diagnosis and give a more reasonable treatment. The role of diagnostic markers is to accurately diagnose tumors. A good diagnostic marker requires high sensitivity and strong specificity (Tan et al.,2018). At present, the definite diagnosis of gliomas still depends on the postoperative histopathological results. Finding the morphological structure of malignant tumor cells in the pathological section, and determine the origin of the tumor cells by the markers of glial cells, such as GFAP and S100β (Ikota et al.,2006). In clinical work, a variety of glial differentiation markers and markers of other tissue types are often needed to cooperate with the diagnosis. A clear differentiation marker that is specific to all gliomas is still not to be found. However, according to our analysis, NSMF, a glial cell specific differentiation marker, is generally under-expressed in various histological classifications of gliomas. Therefore, NSMF can be used as a diagnostic marker for the diagnosis of gliomas.

Prognostic and predictive markers need to be able to respond well to the prognosis of patients and the effect of therapy (Coate et al.,2009). The problem with many markers of gliomas is that the judgment is inaccurate. Some patients have a good prognostic marker but the survival time is still very short (Brander et al.,2015). Based on our research on various glioma databases, the prognosis of patients with high expression of NSMF significantly become better, which has a good consistency in multiple databases. Univariate cox analysis and multivariate cox analysis showed that high NSMF expression were independent predictors of long OS. Therefore, NSMF can be used as a prognostic marker in clinical work. The expression level of NSMF is correlated with IDH-mutant and 1p19q co-deleted glioma patients.

Clinical studies have shown that surgical resection is still considered as the first choice for the treatment of glioma. However, due to the biological behavior of the aggressive growth of tumors, it is difficult to determine the boundary between tumor and normal brain tissue during surgery, thus, it is more difficult to completely remove the tumors (Weller et al.,2015). Therefore, how to determine the tumor tissue more intuitively during the surgery and remove as much tumor as possible are the vital factors to ameliorate the glioma surgery. At present, the application of fluorescent dyes can better improve the total resection rate of glioma, such as fluorescein sodium and indocyanine green (Mansouri et al.,2016). In combination with previous studies, we found that NSMF is almost non-expressed in glioma cells which specifically enhances CREB phosphorylation in the nucleus (Spilker, Grochowska and Kreutz, 2016). So, we can design a fluorescent reporter system to combine CREB phosphorylation sites with proteins which can penetrate the blood-brain barrier and be taken up by brain cells. Normal brain tissue cells with NSMF expression can phosphorylate CREB to inhibit the exposure of luciferin protein epitope, and surgery using luciferase in surgery can specifically track glioma cells. Hence, NSMF can be used as a good marker to track glioma cells during surgery to enable more glioma patients and benefit to surgery.

BDNF is mainly secreted by astrocytes and can be used as a differentiation marker for astrocytes. The secretion of BDNF helps maintain the normal brain microenvironment. Some studies demonstrated that BDNF enriched environment reduces glioma growth (Garofalo et al.,2015). We found that BDNF is significantly lower expressed than normal brain tissues expression databases in glioma. The cellular function of Jacob, the protein encoded by the NSMF gene, is involved in NMDAR signaling to the nucleus. Some studies

show that BDNF induces the nuclear translocation of Jacob in an NMDAR-dependent manner in early neurodevelopment, which results in increased phosphorylation of cAMP response element-binding protein (CREB) and enhances CREB-dependent BDNF gene transcription. Therefore, Jacob is a key factor for a positive feedback loop involved in BDNF synthesis (Spilker, Grochowska and Kreutz, 2016). In summary, we can upregulate the expression of NSMF gene to promote the feedback loop of BDNF expression, which plays a role in treating glioma.

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