Frontiers in Bioscience Landmark

Front. Biosci. (Landmark Ed) **2022**; 28(3): 54
https://doi.org/10.31083/j.fbl2803054

Original Research

Increased Neuromedin B is Associated with a Favorable Prognosis in Glioblastoma

Suqin Li[1,2,†], Shihuan Li[1,3,†], Qingjie Li[1,3], Fei Liu[1,3], Wenli Liao[1,2], Liangzhu Yu[2], Changhan Ouyang[1], Hongli Xia[2,4], Chao Liu[1,*], Mincai Li[1,2,*]

[1]Hubei Key Laboratory of Diabetes and Angiopathy, Medicine Research Institute, Xianning Medical College, Hubei University of Science and Technology, 437100 Xianning, Hubei, China
[2]School of Basic Medical Sciences, Xianning Medical College, Hubei University of Science and Technology, 437100 Xianning, Hubei, China
[3]School of Pharmacy, Xianning Medical College, Hubei University of Science and Technology, 437100 Xianning, Hubei, China
[4]The Central Hospital of Xianning, Hubei University of Science and Technology, 437100 Xianning, Hubei, China
*Correspondence: liu_chao@hbust.edu.cn (Chao Liu); mincaili@163.com (Mincai Li)
[†]These authors contributed equally.
Academic Editor: Maria Pina Concas
Submitted: 21 September 2022 Revised: 14 November 2022 Accepted: 21 November 2022 Published: 16 March 2023

Abstract

Background: Neuromedin B (NMB) is a neuropeptide that plays a key role in many physiological processes and is involved in the pathology of various diseases. Increased levels of NMB have been reported in solid tumors. Therefore, we investigated the prognostic value of NMB in glioblastoma (GBM). **Methods**: Expression profiles of NMB mRNA were investigated in GBM and normal tissues using data from the cancer genome atlas (TCGA). NMB protein expression was obtained using data from the Human Protein Atlas. Receiver operating characteristic (ROC) curves were evaluated in GBM and normal tissues. The survival effect of NMB in GBM patients was evaluated using the Kaplan-Meier method. Protein-protein interaction networks were constructed using STRING, and the functional enrichment analyses were performed. The relationship between NMB expression and tumor-infiltrating lymphocytes was analyzed using the Tumor Immune Estimation Resource (TIMER) and the Tumor-Immune System Interaction database (TISIDB). **Results**: NMB was overexpressed in GBM relative to normal biopsy specimens. The ROC analysis showed that the sensitivity and specificity of NMB in GBM were 96.4% and 96.2%, respectively. Kaplan-Meier survival analysis showed that GBM patients with high NMB expression had a better prognosis than those with low NMB expression (16.3 vs. 12.7 months, $p = 0.002$). Correlation analysis showed that NMB expression was associated with tumor-infiltrating lymphocytes and tumor purity. **Conclusions**: High expression of NMB was associated with increased GBM patient survival. Our study indicated that the NMB expression may be a biomarker for prognosis and that NMB may be an immunotherapy target in GBM.

Keywords: glioblastoma; neuromedin B; prognosis; tumor-infiltrating lymphocytes; ROC; survival analysis

1. Introduction

Glioblastoma (GBM) is the most common primary brain tumor in the world and the most aggressive malignant tumor of the central nervous system [1,2]. GBM still presents with significantly high mortality and morbidity rate, which has increased in recent years [3]. Although many therapeutics have been developed to treat GBM, the 5-year overall survival (OS) rate remains at about 5.6% [2,4]. With the rapid development of high-throughput sequencing technologies, many GBM molecular biomarkers have recently been reported in the literature. Therefore, it is imperative to identify new biomarkers to predict the prognosis of GBM.

NMB is a neuropeptide and growth factor widely distributed in the brain and gastrointestinal tract. As an autocrine growth factor, NMB plays a significant role in many physiological functions, such as energy metabolism and memory. In addition, NMB is a potent mitogen and a proangiogenic factor that regulates diverse physiological processes in normal epithelial cells. It has been reported that high level of NMB is associated with an unfavorable prognosis in colon and prostate cancers [5]. However, the prognostic value of NMB in GBM has not been reported.

The roles of tumor-infiltrating leucocytes in GBM patients remain unknown. The correlation between immune infiltrates in GBM and the prognostic value of NMB is not well understood. Given the upregulated NMB expression in most cancers, we hypothesized that the increased NMB expression was correlated with the survival in GBM. To explore this hypothesis, we assessed the prognostic role of NMB based on data from The Cancer Genome Atlas (TCGA) and analyzed NMB expression and its correlation with increased survival and immune infiltrates in GBM.

2. Materials and Methods

2.1 TCGA Datasets

Transcriptional expression data of NMB and corresponding clinical information were downloaded from

TCGA official website [6]. The 18 enrolled cancer types and the RNA-Seq gene expression data were converted to log2 conversion for further studies. Because all the data were obtained from TCGA, this study was not required to provide the approval from the Ethics Committee.

2.2 RNA-Sequencing Data of NMB in GBM

The RNA-Seq expression data of NMB in GBM was obtained from TCGA. Then 166 GBM and 5 normal brain tissue data were captured for analysis. The mRNA expression data were presented as the mean values with standard deviation (mean $\pm$ SD).

2.3 UALCAN database Analysis

UALCAN (http://ualcan.path.uab.edu/) [7], a publicly available web resource for analyzing cancer data, was used to perform the analysis of NMB protein expression from Clinical Proteomic Tumor Analysis Consortium (CPTAC) in our study.

2.4 The Human Protein Atlas Analysis

The Human Protein Atlas (HPA) includes tumor and normal tissues information regarding the acquisition and expression profiles for protein level of human genes [8]. We performed HPA to compare the protein expression of NMB between normal brain and GBM samples in our study.

2.5 STRING Protein-Protein Networks Analysis

The STRING database can retrieve the protein networks online [9] (version 11.0, http://string-db.org/). In this study, we performed STRING to search for co-expression genes and to build the protein-protein interaction (PPI) networks with an interaction score $>$0.4. Gene ontology (GO) enrichment analyses and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses of these co-expression of genes were conducted.

2.6 Tumor Immune Estimation Resource (TIMER) Database Analysis

TIMER database is a user-friendly online resource for systematic analysis of immune infiltrates about amount of cancer types [10]. We conducted TIMER to verify the relationship between NMB expression in GBM and six immune cells ($CD4^+$ T cells, $CD8^+$ T cells, B cells, neutrophils, macrophages, and dendritic cells) infiltration.

2.7 Tumor-Immune System Interaction Database (TISIDB) Analysis

TISIDB is an interactive online web resource integrated repository portal for tumor-immune system interaction [11]. We performed TISIDB to verify the expression of NMB and tumor-infiltrating lymphocytes (TILs) among human cancers. According to the gene expression profile, the relative abundance of TILs was speculated through gene set variation analysis. The correlations of NMB and TILs were calculated by Spearman's test.

2.8 PrognoScan Database Analysis

The PrognoScan is a very powerful online database to assess the correlation between gene expression and survival rates for all types of cancers [12]. We used PrognoScan to analyze the correlation between NMB gene expression and overall survival in two different datasets (GSE4412_GPL96 and GSE4271_GPL96).

2.9 The Cell Proliferation Assay of NMB

The U-87 MG GBM cells were cultured in DMEM containing 10% fetal bovine serum (FBS) and 1% penicillin, and 1% streptomycin in 5% CO_2 at 37 °C in a humidified incubator. Cells were seeded into 96-well tissue culture plates at a density of 1×10^4 cells per well in 100 μL of medium. After overnight incubation, cells were treated with various concentrations of NMB (10 µM, 50 µM, 100 µM) for 24 hours. CCK8 solution was added to the plate and the optical density (OD) was measured at 450 nm using an ELISA plate reader (EL340 Microplate Reader; Winooske, VT, USA). Untreated cells at different times (24 h, 48 h, 72 h) were used as a negative control (100% viability). Each experiment was performed at least three times.

2.10 Statistical Analyses

All statistical analyses were applied with R (V 4.1.0, R Foundation for Statistical Computing, Vienna, Austria) and R package was performed to visualize the expression differences. Paired *t*-test and Mann-Whitney *U*-test were performed to examine the differences between GBM and normal brain tissues. ROC curve was applied to verify the cut-off value of NMB using the pROC package [13]. Kaplan-Meier and log-rank tests were performed to assess the effect of NMB on survival rates.

3. Results

3.1 Pan-Cancer NMB Expression

To assess the mRNA expression of NMB in different cancer types, we obtained 19 cancer types from the analysis datasets that contained at least five samples in the control group. As shown in Fig. 1, NMB mRNA expression was significantly increased in 14 of the 19 cancer types compared with the control group. Compared with the control group, NMB mRNA expression was significantly decreased in breast invasive carcinoma (BRCA), kidney renal clear cell carcinoma (KICH) and lung squamous cell carcinoma (PRAD) cancers. The result showed that NMB mRNA expression was abnormal in all cancer types.

3.2 Increased NMB mRNA and Protein Expression in GBM Patients

To verify NMB mRNA expression in GBM, we analyzed NMB expression in TCGA database. The characteristics of GBM patients were analyzed from TCGA and Ta-

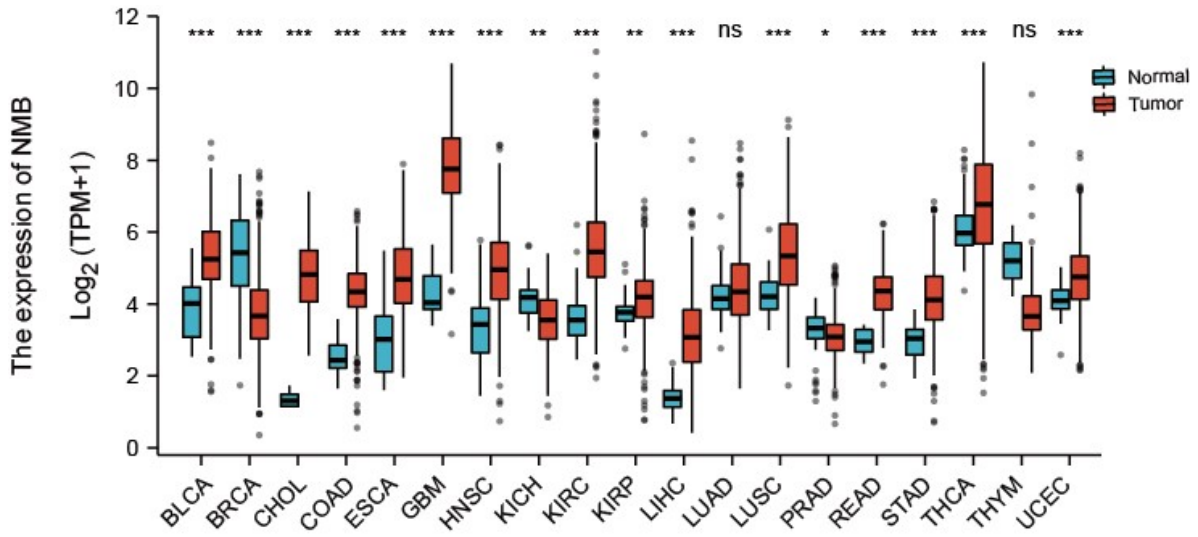

Fig. 1. Pan-cancer expression pattern of NMB. The mRNA expression of NMB was increased in 14 of 19 cancer types compared with normal tissues. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. ns, not significance; BLCA, bladder urothelial carcinoma; BRCA, breast invasive carcinoma; CHOL, cholangiocarcinoma; COAD, colon adenocarcinoma; ESCA, esophageal carcinoma; GBM, glioblastoma; HNSC, head and neck squamous cell carcinoma; KICH, kidney chromophobe; KIRC, kidney renal clear cell carcinoma; KIRP, kidney renal papillary cell carcinoma; LIHC, liver hepatocellular carcinoma; LUAD, lung adenocarcinoma; LUSC, lung squamous cell carcinoma; PRAD, prostate adenocarcinoma; READ, rectum adenocarcinoma; STAD, stomach adenocarcinoma; THCA, thyroid carcinoma; UCEC, uterine corpus endometrial carcinoma.

Table 1. Clinical Characteristics of the GBM Patients (Taken from TCGA).

Characteristic	Total	Low expression of NMB	High expression of NMB	*p*-value
	N (%)	*N (%)*	*N (%)*	
Gender				0.747
Female	59 (35.2%)	31 (18.5%)	28 (16.7%)	
Male	109 (64.8%)	53 (31.5%)	56 (33.3%)	
Race, n (%)				1.000
Asian	5 (3%)	2 (1.2%)	3 (1.8%)	
Black or African American	11 (6.6%)	6 (3.6%)	5 (3%)	
White	150 (90.8%)	75 (45.2%)	75 (45.2%)	
Age, n (%)				0.757
≤60	84 (51.8%)	45 (26.8%)	42 (25%)	
>60	81 (48.2%)	39 (23.2%)	42 (25%)	

ble 1 lists the baseline characteristics. Unpaired data analyses demonstrated that NMB mRNA expression in GBM (n = 169) significantly increased compared with control tissues (n = 5) (as shown in Fig. 2A, 2.565 ± 0.993 vs. 6.534 ± 1.293, Mann-Whitney *U*-test, $p < 0.001$).

Next, we examined NMB protein expression in GBM using the HPA database. As shown in Fig. 2B,C, NMB expression, evaluated with immunohistochemical staining, was enhanced in GBM (Fig. 2C) compared with normal brain (Fig. 2B). NMB protein expression located in the cytoplasm and membrane of U-251 MG cells (Fig. 2D) using immunofluorescence staining. These results showed that both NMB mRNA and protein expression were increased in GBM patients.

3.3 NMB Expression Levels as a Prospective Biomarker in GBM Samples

To obtain a NMB value for distinguishing GBM from normal brain samples, we conducted ROC curve analysis. As shown in Fig. 3A, by analyzing the ROC curve was determined that the AUC value of NMB was 0.984 (95% CI: 0.971–0.997). At the 4.490 cutoff, the specificity and sensitivity of NMB expression were 96.2% and 96.4%, respectively. The positive and negative predictive values were 78.4% and 99.5%, respectively. The 4.490 cutoff was beneficial in distinguishing high NMB expression from low NMB expression in GBM patients. These results demonstrated that NMB expression could be a prospective biomarker for differentiating GBM patients from healthy individuals.

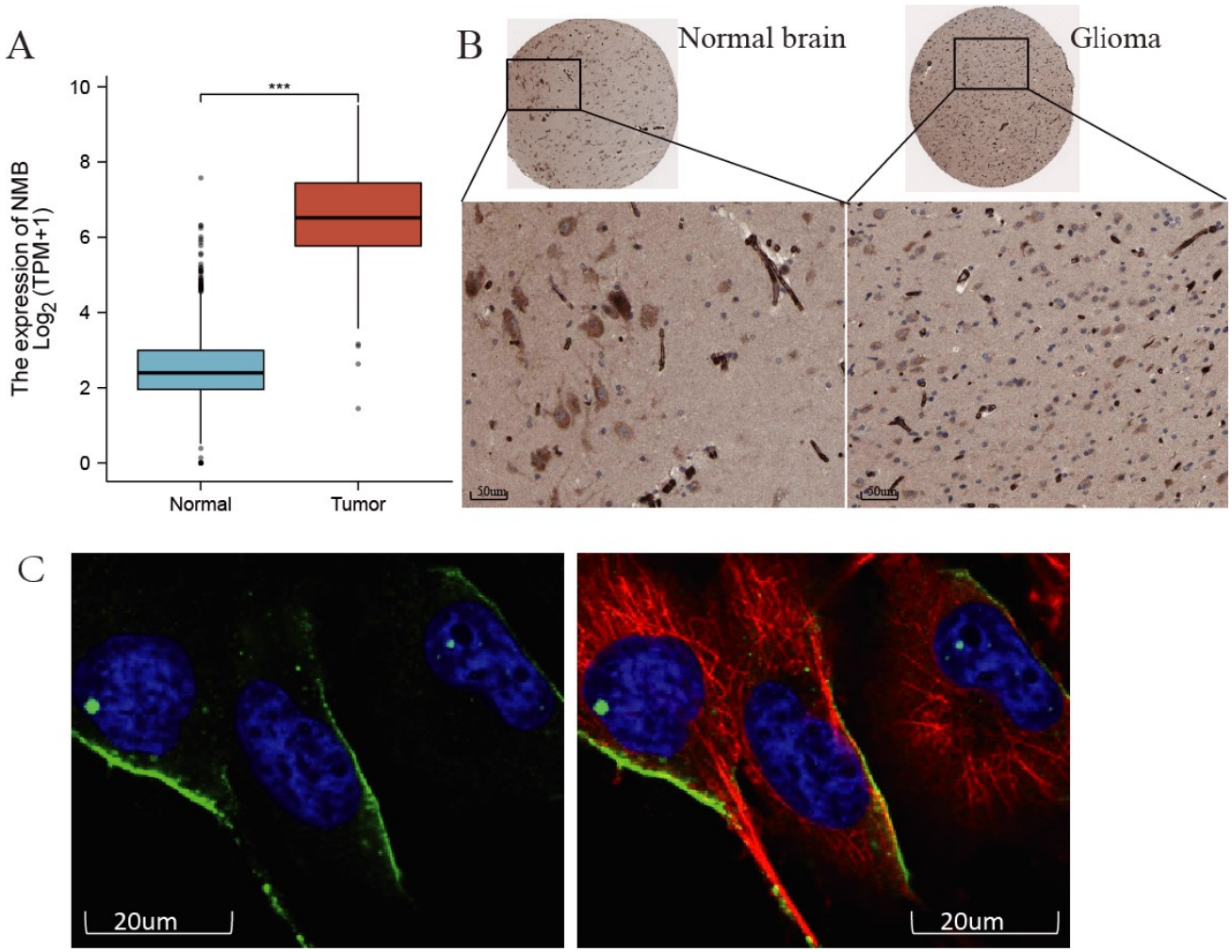

Fig. 2. The mRNA and protein expression of NMB in GBM. (A) The mRNA expression levels of NMB in 169 GBM samples and five normal samples. ***$p < 0.001$, compared with normal tissues. (B) The NMB protein expressed in normal brain tissue and GBM. The NMB expression sourced from the Human Protein Atlas. (C) The NMB protein is expressed and located in U-251 MG cells based on the Human Protein Atlas. The subcellular location of NMB protein expression was shown in green, the nucleus in blue, and the microtubules in red.

3.4 Long OS Associated with High NMB Expression

To determine the relationship between NMB expression and relative OS in GBM patients, Kaplan-Meier curves were constructed, and the PrognoScan database was applied to the analysis OS. As shown in Fig. 3B, the OS in GBM patients with higher expression levels of NMB was significantly longer than the OS in those with lower expression levels (12.7 vs. 16.3 months, $p = 0.002$). These results shown in Fig. 3C,D, also demonstrated that high NMB expression was associated with longer OS in GBM patients using two different datasets (GSE4412 and GSE4271). These results indicated that low NMB expression is one of the biomarkers of poor prognosis in GBM patients.

3.5 PPI Networks and Functional Annotations

To construct the PPI networks of NMB and the relative functional annotations, we used the STRING database to construct the PPI network, and performed GO analysis and KEGG pathway analysis. As shown in Fig. 4A, the NMB PPI network was constructed including its 10 co-expression genes. Fig. 4B shows that these biological processes of NMB were associated with neuroactive ligand-receptor interactions, receptor ligand activity, and neuropeptide signaling pathways. Functional annotations showed that these genes participated in the axon initial segment and neuronal cell body, as well as in hormone activity. As shown in Fig. 4C,D, the correlation coefficients were significant between NMB expression and co-expressed genes in GBM patients.

3.6 Correlation Analysis of Tumor-Infiltrating Lymphocytes (TILs) and NMB Expression in GBM Patients

We performed a correlation analysis between NMB expression and TILs using the TIMER database. Fig. 5A shows that NMB expression was correlated with tumor pu-

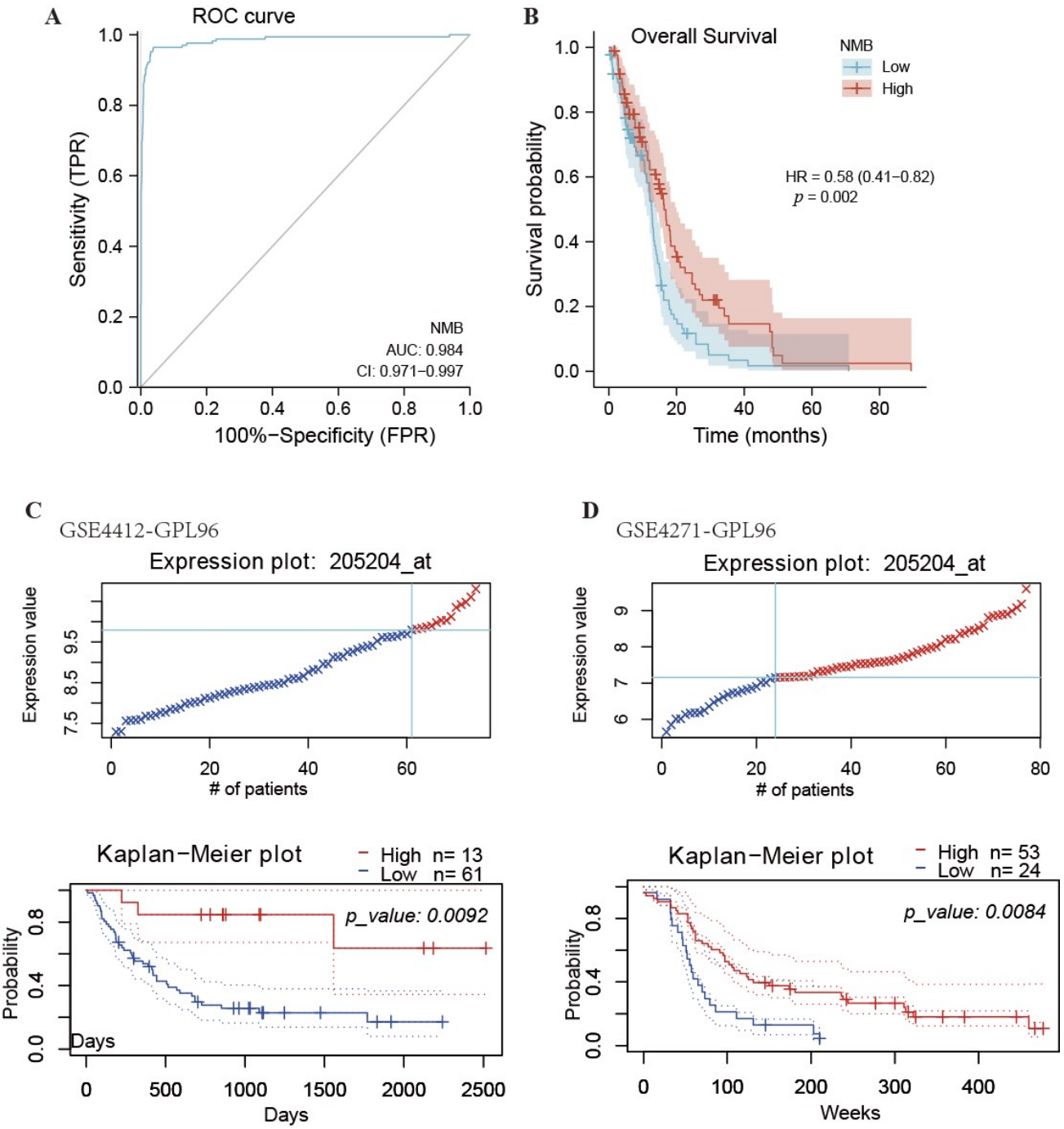

Fig. 3. ROC and Kaplan-Meier curves for NMB. (A) ROC curve showed that NMB had an AUC value of 0.984 to discriminate GBM from healthy control tissues. With a cutoff of 4.490, the sensitivity and specificity were 96.4% and 96.2%, respectively. (B) Kaplan-Meier survival curves indicated that GBM patients with a high level of NMB mRNA expression had a long OS compared with those with low-level of NMB expression (16.3 vs. 12.7 months, $p = 0.002$). Low expression of NMB was associated with poor overall survival in datasets GSE4412_GPL96 (C) and GSE4271_GPL96 (D) analyzed using PrognoScan. HR, Hazard Ratio.

rity ($r = 0.105$, $p = 3.1 \times 10^{-2}$), B cells ($r = 0.143$, $p = 2.89 \times 10^{-5}$), $CD8^+$ T cells ($r = 0.16$, $p = 1.02 \times 10^{-3}$), macrophages ($r = 0.136$, $p = 5.50 \times 10^{-3}$), neutrophils ($r = 0.135$, $p = 5.62 \times 10^{-3}$), and dendritic cells ($r = –0.112$, $p = 2.24 \times 10^{-2}$). Next we determined the correlation between NMB expression and TILs in the TISIDB. Fig. 5B shows the relation between NMB expression and TILs in 28 human cancer types. Fig. 5C shows that NMB expression was associated with abundance of $CD4^+$ T cells ($r = –0.286$, $p = 2.03 \times 10^{-4}$), $CD8^+$ T cells ($r = –0.194$, $p = 1.25 \times 10^{-2}$), Th1 cells ($r = –0.168$, $p = 3.09 \times 10^{-2}$), Treg cells ($r = –0.256$, $p = 8.93 \times 10^{-4}$), memory B cells

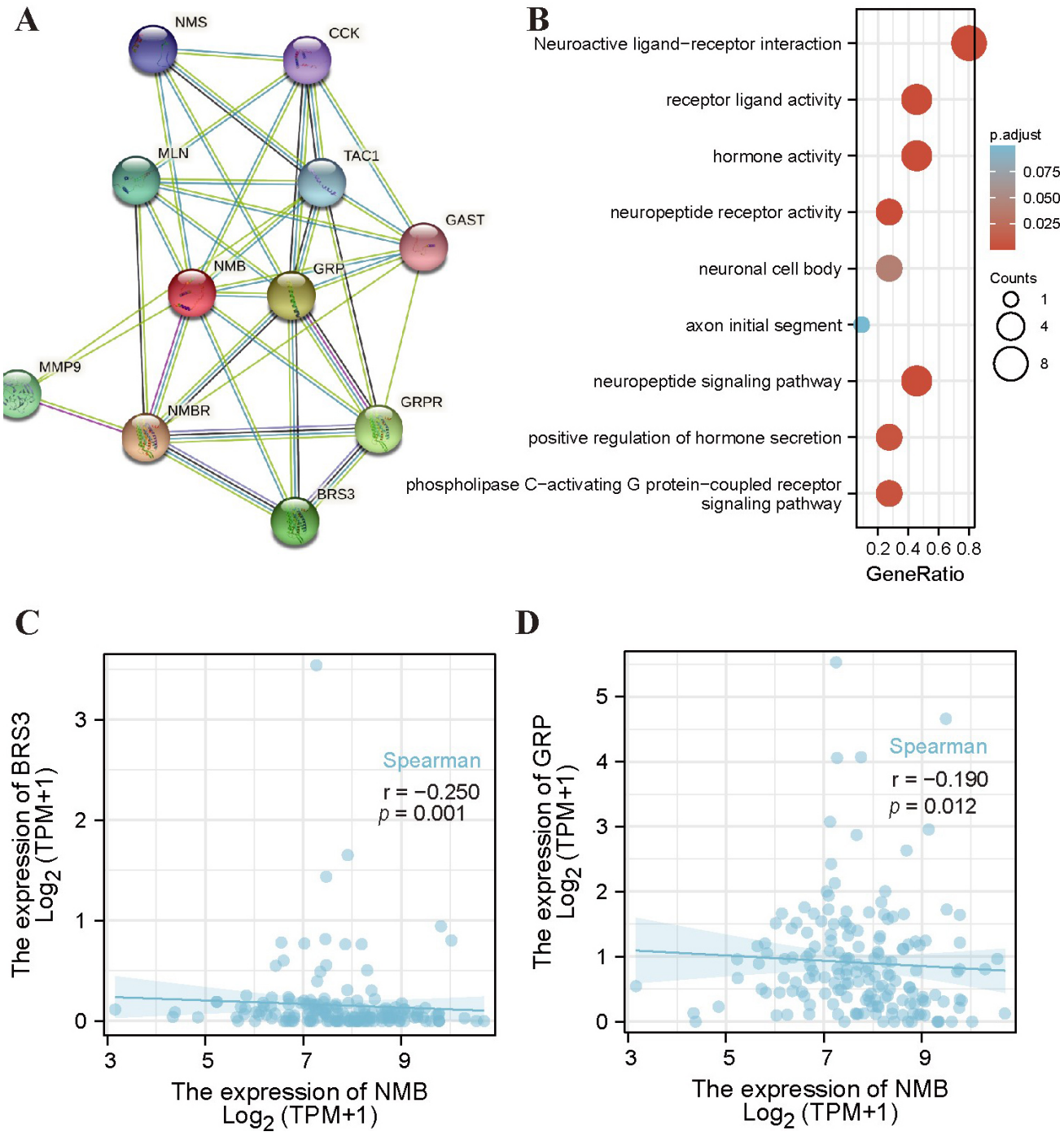

Fig. 4. PPI networks and functional enrichment analyses of NMB. (A) A network of NMB and its co-expression genes. (B) Functional enrichment analyses of 11 involved genes. NMB was associated with actin filament organization, regulation of actin filament-based process, and actin cytoskeleton organization. These genes were involved in purine ribonucleoside binding, GTP Binding, and GTPase Activity. (C,D) The correlation analyses between the expression of NMB and co-expressed genes in GBM. BRS3, bombesin receptor subtype 3; GRP, gastrin releasing peptide.

(r = –0.278, $p = 2.97 \times 10^{-4}$), NK cells (r = –0.191, $p = 1.38 \times 10^{-2}$), CD56^{bright} cells ($r = 0.185$, $p = 1.71 \times 10^{-2}$), and mast cells (r = –0.165, $p = 3.41 \times 10^{-2}$). These data demonstrated that NMB expression may play an important role in immune cell infiltration in GBM patients.

3.7 NMB Increased Glioblastoma Cell Proliferation

We measured the proliferation effect of NMB on glioblastoma using the CCK8 assay. U-87 MG cells are treated with 0-, 10-, 50-, and 100 μM NMB for 24-, 48-, and 72-h. The morphology of the glioblastoma cells becomes large and shows cell stretch out due to the increasing

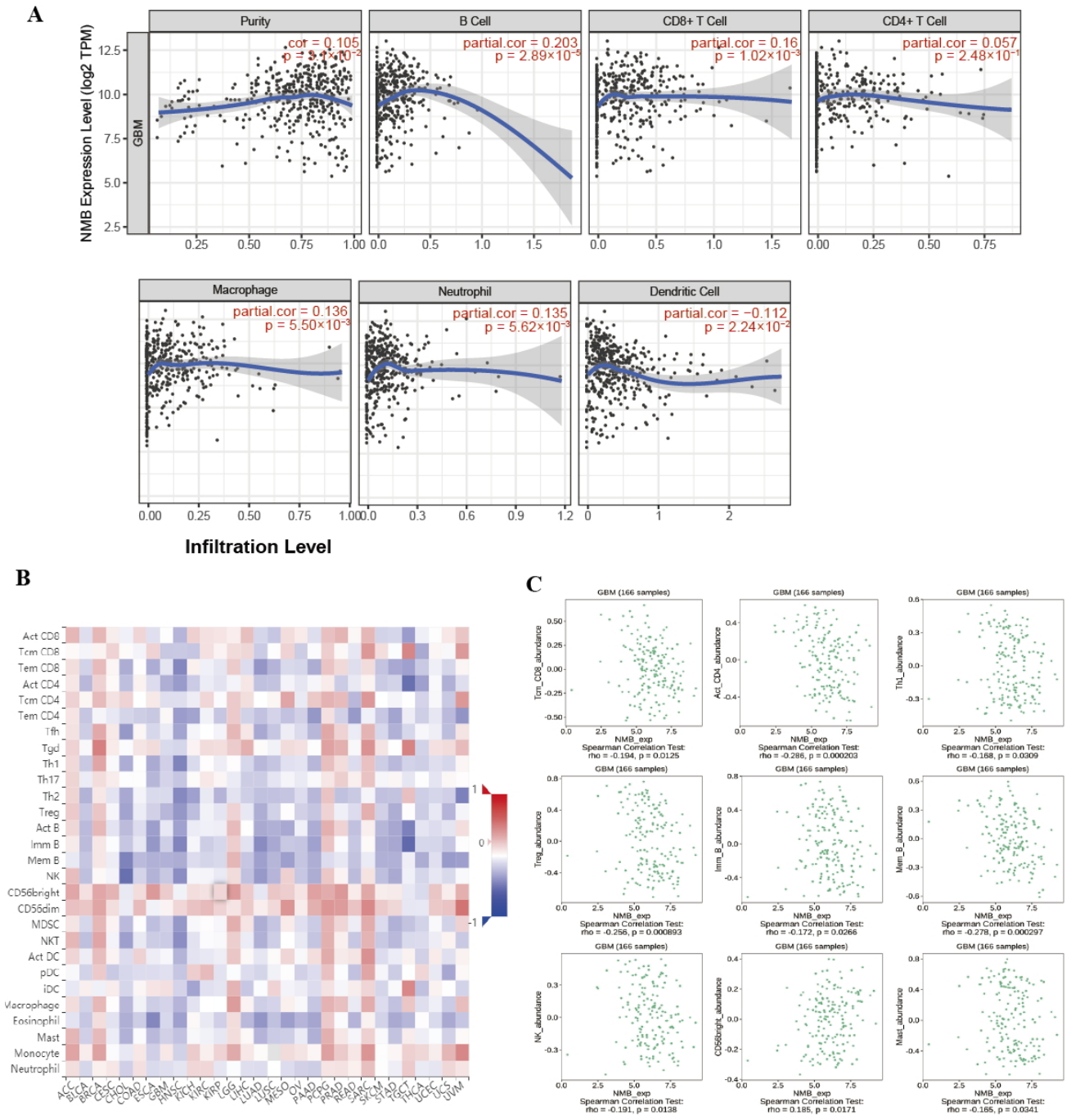

Fig. 5. Correlation of NMB expression with TILs. (A) NMB expression is negatively related to tumor purity and is correlated with B cells, $CD8^+$ T cells, neutrophils, macrophages, and dendritic cells in GBM. (B) Relationships between the NMB expression and TILs across 28 types of human cancers. (C) NMB was associated with an abundance of $CD8^+$ T cells, $CD4^+$ T cells, Th1 cells, Treg cells, B cells, NK cells, $CD56^{bright}$ cells, and mast cells.

concentrations of NMB. After treatments with 10- and 50-μM NMB for 48 h to U-87 MG cells, the proliferation rates are 1.28 $\pm$ 0.27 and 1.59 $\pm$ 0.34. After treatments with 10- and 50-μM NMB for 72 h to U-87 MG cells, the proliferations are 1.40 $\pm$ 0.37 and 1.67 $\pm$ 0.45. Compared to the 24 h group, there was a significant increase in U-87 MG cell proliferations in the 48 h group and 72 h group (Fig. 6). These results show that NMB increased glioblastoma proliferation in a time-dependent manner.

4. Discussion

In this study, we proved that NMB expression increased in GBM compared with normal brain tissues. The analysis of ROC curve indicated that NMB might be used to distinguish GBM from healthy brain tissue. According to the Kaplan-Meier curves and the univariate analysis, we

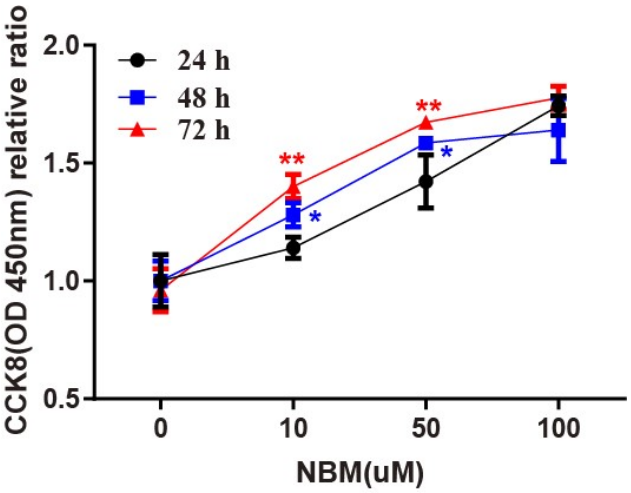

Fig. 6. The proliferation effects of NMB on glioblastoma cells. The proliferative effects of varying concentrations of NMB (10 uM, 50 uM, 100 uM) in U-87 MG cell lines were performed by the CCK8 assay. Untreated cells at 24-, 48-, and 72-h were used as a negative control (100% viability). The relative ratios of cell viability are given as mean ± SD. of three independent experiments. *$p < 0.05$ and **$p < 0.01$, compared to 24 h group.

found that low expression of NMB mRNA was correlated with poor OS and that NMB expression could be regarded as a potential biomarker for poor prognosis in GBM patients. Furthermore, NMB expression plays an important role in TILs of GBM patients.

NMB is a member of bombesin (BN)-like peptide family in mammals that has various autocrine and paracrine functions. Several studies have reported that NMB regulated tumor cell proliferation in several cancer cells [14–16]. According to our pan-cancer analysis, the expression of NMB mRNA was abnormal in various cancers. Our results are consistent with those studies. We demonstrated that NMB expression was significantly upregulated in GBM patients.

The functions of NMB in tumors have been previously reported. Previous studies suggested that NMB may induce proliferation of porcine Leydig cells and ATDC5 cells [17,18]. Neurotensin-like peptides were a target for suppressing the proliferation and growth of colon and prostate cancer cells [5]. NMB could regulate IL-6 and COX-2 expression via the NF-κB/P65 pathway [19]. The underlying mechanism of these studies indicated that NMB and its receptor-mediated NF-κB/P65 pathway played a key role in tumor cells [20]. Based on the conditions that NMB mRNA expression was significantly increased in GBM tumors compared with normal brain tissues, we suggested that the increased NMB mRNA expression could be used to differentiate GBM from normal controls. To verify the diagnostic value of NMB in GBM patients, we performed ROC curve analysis, which showed that the NMB expression had a high AUC value in GBM patients, with 96.4% in sensitivity and 96.2% in specificity. Based on these findings, we concluded that the increased NMB mRNA expression could serve as a key biomarker for differentiating GBM patients from healthy individuals

NMB interacts with its receptor NMBR, which was a G-protein coupled receptor with seven trans-membrane regions, and through that interaction all its potent biological actions are mediated. Some studies reported that NMBR was expressed in various normal tissues and overexpressed in some solid tumors [14–16]. In breast cancer, an NMBR antagonist inhibited tumor angiogenesis and tumor growth [21]. The NMBR antagonist PD168368 inhibited the growth of MDA-MB-231 breast cancer cells by inducing cell cycle arrest and apoptosis [21]. Yang reported that BIM-23127, an NMBR antagonist, could potentially inhibit cellular proliferation [22] and induce the death of the medulloblastoma cell line DAOY [23].

Given that NMBR antagonist suppresses tumor angiogenesis and tumor growth [21], we hypothesized that NMB was involved in the GBM proliferation. According to Kaplan-Meier curves and log-rank test, GBM patients with low NMB mRNA expression had lower survival rates than those with high NMB expression levels. We hypothesized that the reduced NMB is a potential biomarker for determining poor prognosis of GBM.

The relationship between immune cell infiltrates and the prognosis of GBM patients remains debatable. The increased levels of immune cell infiltration are present in GBM patients. Ge reported that the overexpression level of CD163+macrophages was found in GBM, but not in normal brain specimens [24]. Tumor-associated macrophage activation is involved in promoting tumor aggressiveness and is associated with outcome. González-Tablas [25] reported that the overall survival of GBM patients with mixed myeloid and T-lymphoid infiltrates was significantly shorter. M Hajj [26] reported that GBM patients with high expression of activated natural killer cells and M2 macrophages excluded long survivors. The infiltration of immune cells has attracted attention as a potential novel immunotherapeutic option for GBM.

Many reports about the possible role of NMB in tumor cell infiltration have emerged in recent years. Yang reported that NMB and NMBR may be involved in the spontaneous actin polarization of transformed CD8 T cells [22]. However, the correlation analysis of NMB expression and TILs has not been reported in GBM patients. By TIMER analysis, we demonstrated that some TILs (such as macrophages, B cells, $CD8^+$ T cells, neutrophils, dendritic cells) were correlated to NMB expression in GBM patients. We also found a positive correlation between NMB expression and $CD8^+$ T cells, $CD4^+$ T cells, Th1 cells, Treg cells, immature B cells, memory B cells, NK cells, CD56dim cells, and mast cells. Our findings indicated that there was a potential correlation between NMB and TILs in GBM patients. However, further research should be performed to verify

these correlations.

NMB is structurally related to the gastrin-releasing peptide (GRP), which is a member of the BN-like peptide family in mammals. Three types of related receptors make up the BN receptor family: the 390-amino acid NMB-preferring receptor (NMBR), the 384-amino acid GRP-preferring receptor (GRPR) and the 399-amino acid orphan receptor named BN-receptor subtype 3 (BRS3 receptor). The co-expression analyses demonstrated that NMB expression was significantly associated with GRP and BRS3. NMBR regulates EGF receptor transactivation by a mechanism dependent on the activation of metalloprotease and the production of reactive oxygen species [23]. Given these findings, we considered that the upregulation of NMB expression would involve these pathways, and that NMBR antagonism may be an alternative treatment for GBM. However, this hypothesis should be investigated in future experiments.

Our study had some limitations. First, NMB expression and prognostic analysis were performed using online public databases; further research using clinical samples is required to validate these findings. Secondly, additional experiments should be conducted to investigate the potential mechanisms related to the effect of NMB on TILs in GBM patients.

5. Conclusions

In the current study, our findings showed that the expression of NMB mRNA was increased in GBM patients. The increased NMB expression might be regarded as a potential biomarker that could be used to identify GBM patients with good prognosis. Furthermore, NMB expression was correlated to TILs and played an important role in patients with GBM.

Availability of Data and Materials

All data generated or analyzed during this study are included in this published article. The experimental data analyzed and displayed in the present manuscript are available from the corresponding author upon reasonable request.

Author Contributions

ML and CL designed the study. SuqL, QL, and ShiL analyzed the data. FL and WL performed the experiments. HX, LY, and CO contributed to the writing of the manuscript. All authors read and approved the final manuscript.

Ethics Approval and Consent to Participate

Not applicable.

Acknowledgment

Not applicable.

Funding

This work was supported by a grant from the National Natural Science Foundation of China (No. 82073852), the fund of the Science and Technology Department of Hubei Province (ZX2021F007) and the found of Hubei University of Science and Technology (2018xzy02, 2019xz01, 2019-21GP12).

Conflict of Interest

The authors declare no conflict of interest.

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