Research Article

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Bioinformatics analysis of the expression of inducible nitric oxide synthases (iNOS/NOS2) in human glioma and its correlation with patients' prognoses

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Abstract: Objective To evaluate the expression of inducible nitric oxide synthases (iNOS/NOS2) in human glioma and its correlation with patients' prognoses.

Methods iNOS/NOS2 expression in tumor and corresponding normal tissues of glioma patients was analyzed using the TCGA database and the online analysis tool GEPIA. The mutation statuses of iNOS/NOS2 genes were also explored in the TCGA database using cBioPortal. Co-expressed genes relevant to iNOS/NOS2 were screened by LinkedOmics. Gene ontology (GO) and KEGG pathway enrichment for iNOS/NOS2 and co-expressed genes was performed using LinkedOmics. Overall survival (OS) and disease-free survival (DFS) outcomes between iNOS/NOS2 mRNA high and low expression groups were compared using a log-rank test. Twenty-two glioma patients who underwent operation were included in the present work. A real-time PCR assay was used to detect iNOS/NOS2 mRNA expression in tumor tissue and normal brain tissue.

Results There was no statistical difference in iNOS/NOS2 mRNA expression levels between tumor and normal tissues of glioma. A real-time PCR assay indicated that iNOS/NOS2 mRNA expression in tumor tissue and normal brain tissues were not statistically different ($p > 0.05$). A mutation rate of 0.8% for the iNOS/NOS2 gene was found using 1044 glioma patients from two datasets. The mutation types include deep deletion (0.4%), truncating

(0.2%) and missense (0.2%). The top positive and negative co-expressed gene with iNOS/NOS2 were COL25A1 ($r_{\text{pearson}} = 0.4734$, $p < 0.05$) and ALCAM ($r_{\text{pearson}} = 0.4734$, $p < 0.05$), respectively. For KEGG pathway analysis, iNOS/NOS2 was mainly enriched in calcium signaling pathway, Wnt signaling pathway, GnRH signaling pathway, HIF-1 signaling pathway and pathways in cancer. The overall survival (HR=2.0, $p < 0.05$) and disease-free survival (HR=1.6, $p < 0.05$) values were significantly different between iNOS/NOS2 high and low expression groups.

Conclusion OS and DFS were significantly decreased in high iNOS/NOS2 mRNA expression groups. iNOS/NOS2 can be used as a poor prognostic biomarker for glioma.

Keywords: Bioinformatics analysis; inducible nitric oxide synthases; glioma; prognosis.

Introduction

Nitric oxide (NO) is a kind of bioactive molecule in the human body. Recent studies have shown that NO is closely related to tumorigenesis and development of a variety of malignant tumors [1-3]. NO in tumor tissues is mainly synthesized by inducible nitric oxide synthase (iNOS). iNOS, known as inducible type NOS, is non-calcium-dependent [4, 5]. It exists in the cytoplasm of leukocytes, glial cells and many other cells. When the cells are stimulated, such as by inflammation, degeneration, and tumorigenesis, iNOS is activated and expressed. The NO produced by iNOS widely participates in various pathological processes [6].

Most studies have shown that expression of iNOS in gliomas is significantly higher than that of normal tissues, and iNOS expression is positively correlated with the degree of malignancy. However, Giannopoulou et al. [7] found that expression of iNOS in astrocytomas increased

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with increased pathology grading of I, II and III. However, iNOS expression levels were significantly decreased in grade IV astrocytomas. Therefore, iNOS expression patterns in human glioma and their correlation with patients' prognoses were not yet completely clear.

Methods

Bioinformatics analysis

Nitric oxide synthases (iNOS/NOS2) expression of tumor and corresponding normal tissues of glioma patients were analyzed using the TCGA databases and the online analysis tool GEPIA [8]. The mutation statuses of iNOS/NOS2 genes were also explored in the TCGA database using cBioPortal. Co-expressed genes relevant to iNOS/NOS2 were screened by LinkedOmics [9]. Gene ontology (GO) and KEGG pathway enrichment for iNOS/NOS2 and co-expressed genes was performed using LinkedOmics in the aspects of biological function(BP), cellular component(CC) and molecular function(MF).

Real-time PCR assay

Twenty-two glioma patients who underwent operation were included in the present work. This work was approved by the ethical committee of Tianjin Huanhu Hospital. A real-time PCR assay was used to detect iNOS/NOS2 mRNA expression in tumor tissue and normal brain tissue. Total RNA from tissues and cells was isolated using RNA Trizol (Invitrogen, Carlsbad, CA, USA). Using agarose gel electrophoresis and BioPhotometer Plus (Eppendorf AG, Hamburg, Germany), RNA integrity and amount were assayed. Then, 2 µg of total RNA was reverse transcribed into cDNA using reverse transcriptase (Invitrogen) according to the manufacturer's protocols. The primers of iNOS/NOS2 were Forward primers: 5'-GGATGACTTTCGAGGACATGC-3' and Reverse primers: 5'-GGGCCCTCTGGTCATACTTTT-3'. PCR was performed using a ABI 7500 Real-Time PCR system (Applied Biosystems, Austin, TX, USA). Relative expressions of mRNAs were calculated using 2^{-ΔΔCt} method.

Survival analysis

According to the median iNOS/NOS2 expression level in tumor tissue of the glioma cases, patients were divided

into high ($\geq$ median expression) and low ($<$ median expression) iNOS/NOS2 expression groups. The OS and DFS between the high and low iNOS/NOS2 expression groups were compared by log-rank test and Cox regression analysis and shown with a Kaplan-Meier survival plot.

Statistical analysis

Stata15.0 software (<https://www.stata.com/>) was used for data analysis. Measurement data was expressed as $\bar{x} \pm s$, and a t test was used to make comparisons between groups. Counting data was expressed by number, and a chi-square test was used to make comparisons between groups. iNOS/NOS2 expression, mutation and survival analysis was performed using the corresponding online software of service. Two tailed $P < 0.05$ was considered statistically significant.

Results

iNOS/NOS2 mRNA relative expression

iNOS/NOS2 mRNA levels in glioma are shown in **Figure 1**. There was no statistical difference in iNOS/NOS2 mRNA expression level between tumor and normal tissues of glioma. iNOS/NOS2 mRNA expression levels in other carcinomas are shown in **Figure 2**. iNOS/NOS2 mRNA seem to be upregulated in colorectal cancer and prostate carcinoma.

Real-time PCR assay

The real-time PCR assay indicated that iNOS/NOS2 mRNA expression in tumor tissues of glioma patients was downregulated compared to expression in normal brain tissue, but without statistical difference ($p > 0.05$), **Figure 3**.

iNOS/NOS2 mutation analysis

iNOS/NOS2 gene mutation analysis was performed using the TCGA databases and the online analysis tool cBioPortal. A total mutation rate of 0.8% for iNOS/NOS2 genes was found using 1044 glioma patients from two datasets (Figure 3A). The mutation types include deep deletion (0.4%), truncating (0.2%) and missense (0.2%).

Figure 1: iNOS/NOS2 mRNA relative expression scatter plot of tumor tissues and corresponding normal tissues of glioma patients.

Figure 2: Figure 1. iNOS/NOS2 mRNA relative expression levels in multiple carcinomas.

Co-expression analysis

The top 50 positive and negative co-expressed genes for iNOS/NOS2 are shown in **Figure 5**. The top positive and negative co-expressed genes for iNOS/NOS2 were COL25A1 ($r_{\text{pearson}}=0.4734$, $p<0.05$) and ALCAM ($r_{\text{pearson}}=0.4734$, $p<0.05$), respectively, **Figure 5**.

GO enrichment analysis

Enrichment results for biological process (BP), cellular component (CC) and molecular function (MF) analyses of iNOS/NOS2 and co-expressed genes are shown in **Tables 1-3**. With regard to KEGG pathways, iNOS/NOS2 was mainly enriched in calcium signaling pathway, Wnt signaling pathway, GnRH signaling pathway, HIF-1 signaling pathway and pathways in cancer, **Table 4**.

iNOS/NOS2 expression and prognosis

According to the median iNOS/NOS2 expression in tumor tissues of glioma subjects, patients were divided into iNOS/NOS2 high and low expression groups. The overall survival (HR=2.0, $p<0.05$) and disease-free survival (HR=1.6, $p<0.05$) were significantly different between the iNOS/NOS2 high and low expression groups. The overall survival and disease-free survival in the iNOS/NOS2 high

expression group were significantly lower than those of the low expression group, **Figure 6**.

Discussion

Epidemiological data indicates that gliomas are the most frequently clinically diagnosed primary tumor of the central nervous system [10]. Gliomas are generally divided into 4 types (I to IV) according to the pathology characteristics and biological behavior of the tumor cells. The general prognosis is poor, especially for grade IV gliomas, with a median survival period of 1 to 1.5 years after diagnosis, even if comprehensive treatment is provided [11-13].

As in other solid malignant tumors, the inflammatory environment plays an important role in the tumorigenesis of gliomas [14, 15]. Nitric oxide (NO) is a radical molecule that acts as a ubiquitous messenger and can be induced by an inducible isoform (iNOS or NOS2) and sustained at high levels in tumor cells. Several studies [16-18] have found that LPS, IFN - γ , TNF - α and other cytokines can upregulate expression of iNOS. Mattace et al. [19] found that PDTC and TLCK, two inhibitors of NF- κ B, can significantly reduce the ability of LPS, IFN - γ , TNF - α to promote expression of iNOS. Therefore, it has been indicated that LPS, IFN - γ , TNF - α regulate expression of iNOS through the NF- κ B pathway. Kim et al. [20] found

Figure 3: Scatter plot of iNOS/NOS2 mRNA expression levels in tumor tissues and normal brain tissues of glioma patients.

Figure 4: iNOS/NOS2 mutation analysis using TCGA databases and the online analysis tool cBioPortal.

Figure 5: Co-expressed genes relevant to iNOS/NOS2 analysis (A: Heat-map of genes positively correlated with iNOS/NOS2; B: Top gene positively correlated with iNOS/NOS2; C: Heat-map of genes negatively correlated with iNOS/NOS2; D: Top gene negatively correlated with iNOS/NOS2).

Figure 6: Survival curve of iNOS/NOS2 expression and prognosis in patients with glioma (A: Overall survival of iNOS/NOS2 low and high expression groups; B: Disease-free survival of iNOS/NOS2 low and high expression groups)

Table 1: Biological process enrichment for iNOS/NOS2 and co-expressed genes.

Description	Size	Expect	Ratio	P-value
peptidyl-aspartic acid hydroxylation	4	0.0032	312.56	0.0032
positive regulation of circadian sleep/wake cycle, non-REM sleep	5	0.004	250.05	0.00399
peptidyl-aspartic acid modification	5	0.004	250.05	0.00399
axonogenesis involved in innervation	6	0.0048	208.38	0.00479
positive regulation of circadian sleep/wake cycle, sleep	6	0.0048	208.38	0.00479
positive regulation of dopamine secretion	6	0.0048	208.38	0.00479
peptidyl-lysine monomethylation	7	0.0056	178.61	0.00559
sebaceous gland development	7	0.0056	178.61	0.00559
negative regulation of feeding behavior	9	0.0072	138.92	0.00718
negative regulation of synaptic transmission, glutamatergic	9	0.0072	138.92	0.00718

Table 2: Cellular component enrichment for iNOS/NOS2 and co-expressed genes.

Description	Size	Expect	Ratio	P-value
intercellular canaliculus	4	0.00792	126.25	0.0079
3M complex	5	0.0099	101	0.00986
kainate selective glutamate receptor complex	5	0.0099	101	0.00986
Cul7-RING ubiquitin ligase complex	5	0.0099	101	0.00986
chromaffin granule membrane	6	0.01188	84.167	0.01182
invadopodium membrane	6	0.01188	84.167	0.01182
chromaffin granule	8	0.01584	63.125	0.01574
NMDA selective glutamate receptor complex	11	0.02178	45.909	0.02158
platelet dense granule lumen	13	0.02574	38.846	0.02545
apical dendrite	14	0.02772	36.071	0.02738

Table 3: Molecular function enrichment for iNOS/NOS2 and co-expressed genes.

Description	Size	Expect	Ratio	P-value
dopamine beta-monooxygenase activity	2	0.00532	188.04	0.00531
Rab GDP-dissociation inhibitor activity	2	0.00532	188.04	0.00531
8-oxo-dGDP phosphatase activity	2	0.00532	188.04	0.00531
ADP-sugar diphosphatase activity	2	0.00532	188.04	0.00531
nitric-oxide synthase activity	3	0.00798	125.36	0.00796
peptide YY receptor activity	3	0.00798	125.36	0.00796
peptide-aspartate beta-dioxygenase activity	3	0.00798	125.36	0.00796
oxidoreductase activity	4	0.01064	94.019	0.0106
tetrahydrobiopterin binding	4	0.01064	94.019	0.0106
ADP-ribose diphosphatase activity	4	0.01064	94.019	0.0106

Table 4: KEGG pathway enrichment for iNOS/NOS2 and co-expressed genes.

Description	Size	Expect	Ratio	P-value
Calcium signaling pathway	181	0.42576	9.395	0.00069
Wnt signaling pathway	143	0.33637	8.9187	0.00417
GnRH signaling pathway	92	0.21641	9.2418	0.0192
HIF-1 signaling pathway	97	0.22817	8.7655	0.02121
Phosphatidylinositol signaling system	97	0.22817	8.7655	0.02121
Melanogenesis	101	0.23758	8.4183	0.02288
Phospholipase D signaling pathway	140	0.32931	6.0732	0.0418
Adrenergic signaling in cardiomyocytes	144	0.33872	5.9045	0.04399
Arginine biosynthesis	20	0.04705	21.256	0.04607
Oxytocin signaling pathway	149	0.35049	5.7064	0.04679
Cushing syndrome	152	0.35754	5.5938	0.0485
Fatty acid elongation	23	0.0541	18.484	0.05281
Biosynthesis of unsaturated fatty acids	23	0.0541	18.484	0.05281
Tuberculosis	176	0.414	4.831	0.063
Proteoglycans in cancer	196	0.46104	4.338	0.07609
Tyrosine metabolism	36	0.08468	11.809	0.08149
Arginine and proline metabolism	44	0.1035	9.6619	0.09873
Fatty acid metabolism	44	0.1035	9.6619	0.09873
Pathways in cancer	519	1.2208	2.4574	0.11775
Glycerolipid metabolism	53	0.12467	8.0212	0.11776

that IL-1 β is an early protein that promotes expression of iNOS in rat C6 glioma. It can regulate expression of iNOS through the p38 MAPK and NF- κ B pathways. Xu et al. [21] also confirmed that p38 MAPK, rather than p42 / p44mapk, is involved in the expression of iNOS in C6 glioma.

In the present work, we investigated the expression of inducible nitric oxide synthases (iNOS/NOS2) in human glioma and its correlation with patients' prognoses through bioinformatics analysis. We found that iNOS/NOS2 mRNA expression levels were not different between tumor and normal tissues of glioma patients. However, the tumor tissues had a trend of upregulated expression compared to normal tissues. Further analysis of iNOS/NOS2 in other carcinomas showed that the difference was not statistically significant for most solid cancers. Therefore, we suspect that the biological function of iNOS/NOS2 in carcinoma maybe not depend on concentration level, a hypothesis which is partly supported by previous studies. We also noticed a total mutation rate of 0.8% for iNOS/NOS2 based on analysis of the TCGA datasets. The mutation types include deep deletion (0.4%), truncating

(0.2%) and missense (0.2%). This indicated that iNOS/NOS2 mutations are not common in glioma. Prognosis analysis demonstrated that the overall survival (HR=2.0, $p<0.05$) and disease-free survival (HR=1.6, $p<0.05$) values were significantly different between the iNOS/NOS2 high and low expression groups. The OS and DFS was significantly decreased in the high iNOS/NOS2 mRNA expression group. iNOS/NOS2 can be used as a poor prognostic biomarker for glioma.

The potential molecular mechanism by which iNOS/NOS2 affects glioma patients' prognoses may include promotion of neovascularization and inhibition of tumor cell apoptosis. NO plays contradictory roles in tumor progression, and its function in promoting or inhibiting cancer depends on the tumor type. NO levels in normal tissues are relatively stable and play a certain physiological role. In malignant tumor tissues, iNOS is activated by various cytokines, thus releasing more NO. The role of NO in tumorigenesis is complex. It has been reported that the production of a certain amount of NO in tumors can increase tumor blood flow and promote

angiogenesis, thus promoting tumor growth [22]. However, high concentrations of NO have toxic effects on tumor cells, promote cell apoptosis, block tumor angiogenesis and play an anti-tumor role. Generally, NO in tumor tissue mainly plays a role in promoting tumor growth. The molecular mechanism of interaction between NO and VEGF may be as follows: ① NO, as an important signal transduction factor in the binding process of VEGF and its receptor produced by tumor cells, plays an important role in inducing tumor angiogenesis [23]; ② NO promotes VEGF-induced increase of vascular permeability, increases the nutritional supply of tumor tissue, and ultimately promotes tumor growth [24].

iNOS is also closely related to apoptosis of tumor cells. In mammalian cells, NO is involved in apoptosis as an intracellular biological messenger or cytotoxic effector [25]. NO initiates apoptosis or cell survival signals according to its concentration and intracellular redox state. Most scholars [26-28] believe that NO produced by malignant tumor cells not only plays a role in increasing tumor blood supply and promoting tumor angiogenesis, but also inhibits tumor cell apoptosis.

In conclusion

iNOS is widely involved in the angiogenesis, proliferation, apoptosis and chemotherapy tolerance of glioma, which are closely related to the carcinogenesis and development of glioma. Therefore, iNOS may be a new potential target for comprehensive treatment of glioma. However, most of these results were based on data mining from several databases, and further cell and clinical experiments are needed to validate the findings.

Data Availability Statement: The datasets generated during or analysed during the current study are available from the corresponding author on reasonable request.

Conflict of interest: Authors state no conflict of interest

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