

**Original Article** 

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## **GPNMB** pre-mRNA Splicing Promotes Neuronal Survival after Hypoxie-Ischemic Encephalopathy

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#### Abstract:

HIE is a threat of neonatal life such as cerebral palsy, mental retardation, epilepsy, low intelligence, memory and visual disorder; the underlying mechanism, however, is still unclear, and no specific treatment is available. This study was designed to determine the effect of GPNMB alternative splicing on cortical neurons and associated with molecular signal in rats subjected to HIE injury. We firstly established Hypoxie-ischemic encephalopathy (HIE) module and measured blood flow of cerebral blood flow, we found that cerebral blood flow was blocked after HI, particularly in right hemisphere. Furthermore, microarray sequencing was preformed to compare and analyze the differentially expressed genes, then we validated that compared with the normal group, there were 18 upregulated genes and 3 downregulated genes in the HI group. Moreover, the Gene Ontology database and the KEGG database were employed to determine gene function. We found that generation of neurons and ErbB signaling pathway associated with differential expression of GPNMB. At the same time, we established an oxygen-glucose deprivation (OGD) model, then used Real-Time quantitative PCR (qRT-PCR) and Sequencing to compare the expression levels of GPNMB between normal and OGD groups in SY5Y cells and fetal neurons, respectively. We found that consistent with the sequencing data and qRT-PCR analysis revealed that the expression of GPNMB was up-regulated significantly both SY5Y cells and fetal neurons after OGD injury. In addition, in order to explore the role of GPNMB and mechanism of regulation, we preformed that screening, confirmation and transfection of GPNMB effective interference fragments, next, we demonstrated that compared with NC group the expression of GPNMB was decreased in the GPNMB-si group, from which we supposed that GPNMB-si could promote neurons recovery. The following step, in order to identify change of function after GPNMB interference, MTT assay, CCK-8 assay and high throughput screening were preformed, respectively. We found that GPNMB-si may promote increase of cell viability and cell number. Finally, to elucidate molecular mechanism of differential expression of GPNMB, we analyzed the alternative splicing of GPNMB expression by Splice Grapher software to model the gene that predicts a new alternative splicing event. We found that interference of 3' site in GPNMB sequence modulate alternative splicing of GPNMB. These results suggest that alternative pre-mRNA splicing may be an important regulatory mechanism for cell survival after brain injury, and might provide a novel therapeutic strategy for the treatment of HIE in future clinic trial.

#### Key words: GPNMB HIE, Microarray sequencing, Alternative splicing

#### Introduction

Hypoxie-ischemic encephalopathy (HIE) is the brain pathological changes caused by various causes of cerebral ischemia and hypoxia, the most common is neonatal HIE, but can also occur in other age. Neonatal HIE is a brain disease caused by hypoxia in perinatal neonates(Yang LJ,et al.,2014).(Gadzinowski J, et al.,2012). Every year, 7%-10% (about 1 million 400 thousand ~200 million) in the newborns in our country were born with neonatal asphyxia, which about 1/3 dead from suffocation, about 300 thousand of the asphyxia newborns live with varying degrees of disability, seriously affect the life quality

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of individuals and families and improvement of the population quality(Jiang J,et al., 1999). Cerebral ischemia can also cause many remote organ dysfunctions (Klempt ND, et al., 1992). (Perlman JM.2006). But the specific mechanism of HIE is not entirely clear, and no specific treatment is available. Therefore, effective treatment strategies should be urgently identified and developed, and it is very important to explore its' molecular mechanisms.

GPNMB (Glycoprotein non-metastatic melanoma protein b) is a Protein Coding gene. The protein encoded by this gene is a type I transmembrane glycoprotein which shows homology to the pMEL17 precursor, a melanocyte-specific protein(Mi Hwang S, et al., 2017). Diseases associated with GPNMB include Glioblastoma Multiforme and Glioblastoma. Among its related pathways are Adhesion and Signaling by PTK6(Rose AA, et al., 2016), (Tomihari M, et al., 2009). GPNMB shows expression in the lowly metastatic human melanoma cell lines and xenografts but does not show expression in the highly metastatic cell lines (Qin CP,et al.,2013). GPNMB may be involved in growth delay and reduction of metastatic potential (Welinder C,et al.,2017). The physiological functions of GPNMB are mainly include inhibiting the activity of phosphatase, regulating melanogenesis in mouse melanocytes, supporting a role of OA in tissue regeneration after repetitive overuse, promoting the regeneration of damaged tissues, particularly bone tissue, revealing the role of the GPNMB fragments during developing brain, regulating of pesticide-induced immune dysfunction, promoting proliferation of developing eosinophils, mediating mesenchymal stem cell survival, proliferation, and migration(Coluzzi F,et al., 2011), (Zhang YX, et al.,2017). In addition, the pathological function of GPNMB are mainly about promoting migration of oral squamous cell carcinomas, conferring osteomimicry facilitate homing to bone to enable the development of bone metastasis, inhibiting the progression of glioma, promoting growth and invasive behavior of human lung cancer cells, counteracting amyloid deposition, promoting breast cancer glycolysis reprogramming and tumorigenesis, ameliorating mutant TDP-43-induced motor neuron cell death, targeting Glycoprotein NMB for the treatment of Osteosarcoma, modeling alveolar soft part sarcoma unveils novel mechanisms of metastasis(Li YN, et al., 2017), (Frara N, et al., 2016), (Venishetty VK, et al., 2017), (Tanaka M, et al., 2017). The previous studies shown that the tissue specificity of GPNMB is up-regulated in various cancer cells, including in glioblastoma multiforme(Rose AAN, et al., 2017). Meanwhile, many previous studies have clearly described that expressed in many melanoma cells, as well as in tissue macrophages, including liver Kuppfer cells and lung alveolar macrophages, in podocytes and in some cells of the ciliary body of the eye (at protein level), however, hardly detectable in healthy brain(Lu H,et al.,2013),(Hu X,et al.,2013),(Zhou L,et al.,2017). Throughout the whole research development history, the reports on GPNMB in damaged brain are rare. Therefore, GPNMP might be a potential factor in HIE.

Gene microarray seguening (Shlyapnikov YM,et al.,2014).as a high throughput technology of life sciences and microelectronics, has been widely investigated and is widely applied in a large number of research areas of biology and medicine in bioinformatics research in recent years. It provides important theoretical and practical values for sequence analysis, genome research and the intensity of hybridization signals of gene expression profiles. It is able to contain large-scale, high-throughput information, integrating a range of biological information (Du JY, et al.,2014). Thus, in the present study, in order to identified the potential molecular mechanisms responsible for the GPNMB-si promotes neuronal survival after HIE injury, gene microarray analysis was used.

This study was designed to determine the effect **of** GPNMB on cortical neurons and associated with GPNMB pre-mRNA splicing in rats subjected to HIE, and to explore the related molecular mechanisms using Gene microarray sequencing and Gene Ontology (http://www.geneontology.org/). The result could provide a novel strategy for the treatment of HIE in future clinic trial.

## Methods

## 1. Animal care

Neonatal rats (One-day old) were provided by Experimental Animal Center of Kunming Medical University. The animal study agreement was legally approved by the Animal Care & Welfare Committee of Kunming Medical University with the approval number: 2015-1A. All experiments identified to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health. Animals were resided in cages with a 12-h light/dark cycle and can freely obtain food and water. Aseptic environment should be maintained during surgical procedures.

## 2. Preparation of HIE model

The preparation process of HIE model was followed as previously described. In brief, firstly, preparation before the experiment: the new 7 day old SD rats were weighed and numbered before operation, then adjust the temperature and humidity of the box, so that the hypoxia box temperature is constant at 37°C, humidity 50-80%. Next, establishment of ischemia and hypoxia model: the establishment of the reference method for modeling, the first neonatal rat right carotid artery ligation and disinfection operation area, in the midline of the neck to offset to the right for about 0.5cm incision exposed the right common carotid artery, vessel coagulator and burned the right common carotid artery after skin incision suture. Postoperative newborn rats placed next to the maternal recovery 1h. After the newborn rats into containing 8% oxygen and 92% nitrogen gas mixture in a hypoxic chamber (flow of 3 L/min). Meanwhile, pay attention to the observation of neonatal rat and environmental changes and to determine the temperature of 37°C in the process of hypoxia and humidity 50-80%, 2h after out of new born SD rat into mice next to the rest. Sham operation group: after anesthesia only skin incision in the neck right common carotid artery was isolated, not by ligation of right common carotid artery suture incision, hypoxia, directly next to the mother rest.

The other condition of the whole operation process is the same as that of the ischemia and hypoxia group.

### 3. Brain injury Zea-longa neurological score

According to the standards of Zea-longa 5 grade scoring(Goldberg MP,et al., 1997). Zea-longa score test was performed to determine whether the hypoxic ischemic brain damage model was established successfully. According to the standards of Zea-longa scoring, five scales are involved in this test as following. A score of 0 indicates normal behavior without any neurological defect symptom; a score of 1 indicates a mild focal neurologic deficit with failing to extend left forepaw fully; a score of 2 shows no ability to walk straight and the body remains turning sideways, indicating moderate neurological impairment; a score of 3 indicates a severe neurological dysfunctions with falling to stand and sliding to the left when standing; a score of 4 indicates failure to walk spontaneously with consciousness loses.

## 4. Tissue harvest

After 20 days of operation, the cerebral blood flow of rat was measured in normal and HI groups by laser Doppler blood flow apparatus (Perimed, Beijing). The specific steps were as follows: firstly, determine the monitoring position that selected position in the same side of the fontanelle after the center line a few millimeters of position. Next, anesthesia, then cut the brain of the mouse skin, and clean the surface fascia of the skull. The following step, fixed the probe to the skull. Finally, the reperfusion blood flow of normal and HI groups was monitored continuously.

## 5. Microarray sequencing

Microarray sequencing was performed according to previously described procedure with certain modifications(Zhang Y,et al., 2010), (Liao M,et al., 2012). Briefly, RNA was used to synthesize double stranded cDNA, and subsequently transcribed into biotintagged cRNA using the MessageAmpTM Premier RNA Amplification Kit (Ambion, Austin, TX). The cRNA was purified and fragmented to strands of 35-200 bases in length according to the protocols from Affymetrix. The fragmented cRNA was then hybridized to Affymetrix Gene Chip® Rat Genome 230 2.0 Array containing 28, 700 transcripts, which was performed at 45°C and 60 rpm with rotation for 16 h using an Affymetrix Gene Chip Hybridization Oven 640. After hybridization, the Gene Chip arrays were eluted and then stained (streptavidinphycoerythrin) on an Affymetrix Fluidics Station 450 followed by scanning on a Gene Chip Scanner 3000. The scanned images were firstly assessed by visual inspection, and subsequently analyzed using the default setting of Gene Chip Operating Software (GCOS 1.4). An invariant set normalization procedure was performed to normalize the different arrays using DNA-chip analyzer (dChip). In a comparison analysis, a two-class unpaired method was used in the Significant Analysis of Microarray software (SAM) to identify significantly differentially expressed genes between the operated group and the sham-operated group. The gene, which meets a selection threshold of false discovery rate (FDR) < 5% with fold change more than 1.5 or less than 0.667, was determined to be significantly differentially expressed.

## 6. Data analysis.

Signal processes were detected using the Gene Ontology database (http://www.geneontology.ogy.org/); the analysis includes biological process (BP) that are composed by orderly composition of molecular function and a process of multiple steps; cell components (CC), namely the position of the cell in which the gene product is located in the cell or gene products group (e.g., the rough endoplasmic reticulum, nucleus or ribosomes and proteasomes); and molecular function (MF) that describes the activity in molecular biology. Each section has 4 small parts: 1, the analysis of gene number (count), namely the number of differentially expressed genes measured in this function group; 2, P-value trees, namely thecascade relationship of biological pathway; 3, enrichment factor (fold enrichment), that is, the proportion of the changes in genes than proportions in the GO database in this function group (such as, the more proportion, the more reliability, which was regarded as the more significant changes in molecular function); 4, enrichment points, with enrichment factor empathy. Pathway analysis was performed using the KEGG database (http:// www.genome.jp/kegg/pathway.html) or DAVID Bioinformatics Resources (https://david.ncifcrf. gov/).

## 7. SY5Y cells culture

The stock vial of SHSY5Y cell line was kept in liquid nitrogen at vapor phase. The vial of cells was thawed at 37°C for 2 min by gentle agitation before the vial contents were transferred to a 75cm2 culture flask containing 12–15 mL of culture medium and cultured for 1–2 wk. When the cells became confluent, they were dissociated using trypsin-EDTA and subcultured at a dilution of 1:10. The culture medium was a 1:1 mixture of MEM and Ham's F12 medium, with 10% FBS, containing l-glutamine at 2 mm, sodium pyruvate at 1 mm, non-essential amino acids at 0.1 mm, sodium bicarbonate at 1.5 g/L, and penicillin and streptomycin at 50 units/mL. The culture medium was changed twice a week.

#### 8. Primary cultures of fetal cortical neurons

Aborted fetuses were used to culture primary cortical neurons. Firstly, aborted fetuses were decapitated at the base of the foramen magnum after sterilization, the cranium were bisection along the sagittal suture. Next, the cerebral cortexes were harvested, minced, and isolated by 0.25% trypsinase for 10 min at 37°C; then, the tissue was eluted with the 10% BSA. The following step that cells were collected by centrifugation at 1,000 rpm for 10 min, resuspended by 10%BSA, and plated in 24-well plates at the density of 105cells/ml. After incubation at 37°C with 5 % CO2 for 4h. After 4 hours, the whole medium was changed into the special medium for neurons (basal + 2 % b27, no serum). After this, half of the medium was refreshed every three days.

# 9 Screening and validation of effective interference fragments

Firstly, in the NCBI access GPNMB gene sequence, in Guangzhou Ruibo company design three RNA interference fragment and a random garbage control fragments. Next, recovery of SY5Y cells inoculation in 6-well plates, at 37°C, 5% CO2 incubation box cultured for 24 hours, cell fusion rate of 40% after the screening of the effective RNA interference. According to the operation manual, the cultured SY5Y cells were randomly divided into normal group, reagent group, NC group, GPNMB-F1 group, GPNMB-F2 group, GPNMB-F3 group and CY3 group, and each group set of 3 holes cells. The following steps that 12000rpm centrifugal interference RNA frozen powder 1min. 1X will be diluted into 10X buffer with ddH2O, and according to the specification will eventually reflect the system in accordance with the following configuration: each hole to join the 1Xbuffer+5ul siRNA +5ul reagent 60ul, room temperature static 15-30min. Finally, to reflect the system before 1ml culture base out six well plates, leaving 1ml in the hole. After 24h, 48h was added to the culture medium, qPCR was used to verify the interference effect, and the most effective interfering fragments were selected for the following experiment.

## 10. Transfection of effective interference fragments

After fetal neurons of human cultured 5 days, and randomly divided into normal group, OGD group, OGD+reagent group, OGD+NC group, OGD+GPNMB-si group and CY3 group, and each group set of 3 holes cells cell morphology was observed on the live cell workstation and photographed. Then according to the operation instruction, the interference RNA transfection system was configured, each hole add to 60ul 1Xbuffer+5ul siRNA +5ul reagent, place in room temperature for 15-30min. Suck the culture medium for 1ml and left 1ml in the hole before adding the reflection system. After 24h, 1ml medium was added to the culture medium, the cell morphology was observed and photographed in the living cell workstation after 48h. In order to calculate the number of cells, 5 randomly selected fields of view were selected for cell counting200X).

## 11.OGD model

Oxygen-Glucose Deprivation (OGD) model was established in the study. The OGD was performed as

previously described (Milner R,et al., 2008). In short, well-differentiated SY5Y cells were washed with PBS for three times and resuspended in pre-warmed (37°C) DMEM medium that contains all the standard components except glucose. Then cells were allowed to grow in a hypoxia chamber (Thermo scientific, Waltham, USA) with a compact oxygen controller to maintain the oxygen concentration was less than 0.8 %, 95 % N2, 5 % CO2, 37 °C for 5 h. After hypoxia, the cells were then transferred back to normal DMEM medium containing 4.5 g/l glucose under an atmosphere of 95% air and 5% CO2, and incubated for 24 h for reoxygenation. the welldifferentiated SY5Y cells culture medium was replaced with a sugar-free medium, and incubated in the incubator for 2 hours: After 2 hours, the culture medium was used as a special medium for neurons, and cultured in 5 % CO2, 37 °C incubator for 24 hours. Meanwhile, cultured primary fetal cortical neurons also performed the above steps.

# **12.** MTT assay and morphological observation for the determination of cell viability

MTT assay was performed following the protocols of Agrawal et al (Agrawal M, et al., 2013). with some modification. In brief, cells  $(1 \times 104)$  were seeded in 96well microculture plates that had been pre-coated poly-Llysine and cultured overnight incomplete DMEM medium in a CO2 incubator (95% air/5% CO2). The treated cells were visualized using a Leica DMI-3000B phasecontrast fluorescence microscope (Leica, Heidelberg, Germany) equipped with a digital camera. Then, cells were incubated in a serum-free medium containing MTT solution in the darkness for 4 h at 37°C. The MTT solution was discarded, and 100 µl DMSO was added to each well to dissolve the formazan crystals. The value of optical density was measured at wave length of 562 nm using a Bio-Rad microplate reader (Bio-Rad, Hercules, USA). Cell survival foreach treatment was calculated as a percentage of the control.

# **13.** CCK-8 assay and morphological observation for the determination of cell viability

CCK-8 assay was performed in the study. In short, firstly, after the pancreatic enzyme digestion of the cells in each experimental group in logarithmic phase, the complete medium was resuspended into cell suspension and counted. Next, determining the cell density (mostly 2000 cells / well) according to the cell growth rate, repeat 100  $\mu$ l perzwell and 3-5 holes in each group. According to the experimental design, the number of plates (such as 5 days, board). The following step, after a unified paved, until the cells completely precipitated down, then observe the experimental group of cell density under the microscope, if the density is not uniform, then fixed a group, fine-tuned the amount of other groups of cells again plate (such as: More, reduce the amount of cells

again plate) and put in the cell incubator culture; then from the second day after plating, add 10  $\mu$ L of CCK-8 reagent to the wells 2 to 4 hours before the start of the treatment, without changing the fluid. Finally, after 4 h, the 96-well plate was placed on the shaker for 2-5 min, and the OD value was measured at 450 nm and analyzed data.

### 14. High throughput screening

Cells were captured using phase contrast microscopy (NIKON ECLIPSE TS100) and Digital Sight (NIKON DS-Fi2). Images were imported to ImageJ (NIH, Baltimore, Maryland, USA) to measure neurite length, which was defined as the total length of the neurite cylinder axis. The ratio of neurites to number of cells in the picture was also measured. Images were captured following the incubation cells in differentiation and culture medium for 24 h; following the incubation of differentiated cells with 0.1-100 µM paclitaxel for 24h in SY5Y cells; and following the incubation of differentiated cells exhibiting paclitaxel-induced neurite retraction (1  $\mu$ M) in differentiation medium with TJ24, TJ68, and NGF for 72 h without paclitaxel). High-throughput screening (HTS), that in, full-hole scanning parameters, 96 views per hole in three consecutive holes, next, the average number of cells in the three pores is calculated.

#### 15. Statistical analysis

All data in the experimental process are presented as mean  $\pm$  standard deviation (SD). Statistical analysis Figure.1



Fig. 1 Measurement of cerebral blood flow. (a) Showed the cerebral blood flow of normal brain. (b) Showed the cerebral blood flow after HI. (c) Showed the blood flow of cerebral blood flow in normal brain, and the blood flow of cerebral blood flow after HI-2h-1, HI-2h-2 and HI-2h-3 groups. (d) Showed the blood flow of cerebral blood flow in left and right hemispheres after 2h of HI. Data are presented as mean  $\pm$  standard deviation (SD). \* P<0.05.

was performed by using SPSS 16.0 software. Statistical comparisons were performed using one-way ANOVA with Student-Newman-Keuls (SNK) post hoc test. P<0.05 value was considered to be statistically significant. \* P<0.05.

## **Result:**

# 1. Morphological changes and amount of cerebral blood flow after HI.

The typical morphology of cerebral blood flow under laser Doppler blood flow apparatus was showed in Fig. 1. After HI, cerebral blood flow was blocked (Fig. 1b). The blood flow of cerebral blood flow in normal group is higher compares with HI-2h-1, HI-2h-2 and HI-2h-3 groups (\*P<0.05) (Fig. 1c). The blood flow of cerebral blood flow in left hemisphere is higher compares with right hemisphere (\*P<0.05) (Fig. 1d). These results revealed that HI might block blood flow of cerebral blood flow.

#### 2. Microarray sequencing of GPNMB

In order to elucidate the molecular mechanisms effects of GPNMB on HI, we used Agilent Feature Extraction software to compare and analyze the differentially expressed genes. Genes in signal intensity (normalized intensity) ratio >2 or 0.5 were defined as differentially expressed genes. We found that, compared with the normal group, there were 18 upregulated genes (GPNMB, Hbegf, Gimap9, Igf2bp3, Fam115c, Nod1, Tp53, Zfp746, Zyx, Zfp212, Ezh2, Egfr, Hif1a, Pdia4, Lrrk2, Tpk1, Casp2 and Cul1) and 3 downregulated genes (chst15, Ahsa2 and Plxncl) in the HI group (Fig. 2).

#### Protein-protein interaction (PPI) network analysis

To analyze the interaction relation hip of proteins, the target proteins from NSC group and OEC group were separately input into the string software, then the protein interaction diagram was obtained. the results showed, In the up-regulated factor the PPI network with 3 proteins and 1 edges was constructed (Fig. 3A). In this network, EGFR chain ITGB3. In addition, the top 2 pairs with the greatest combined score were EGFR-ITGB3,0.641, ITGB3-EGFR(0.641). Values in brackets are the combined score value. In the down-regulated factor the PPI network with 7 proteins and 6 edges was constructed (Fig. 3B). In this network, CCL4 chain CSF2,CX3CL1 and CXCL10,CX3CL1 chain CSF2 and CXCL10. In addition, the top 5 pairs with the greatest combined score were CCL4-CSF2,0.678, CXCL10-CCL4 (0.857), CCL4-CXCL10(0.857), CXCL10-CSF2(0.737), CSF2-CXCL10(0.737). Values in brackets

## Figure.2



*Fig. 2 Heatmap of differentially expressed genes. There were 18 upregulated genes and 3 downregulated genes in the HI group.* 

are the combined score value. In the all factor the PPI network with 10 proteins and 8 edges was constructed (Fig. 3A). In this network, EGFR chain ITGB3 and ITGB3,CXCL10 chain CX3CL1,CCL4 and CSF2. In addition, the top 5 pairs with the greatest combined score were CCL4-CXCL10(0.857), CXCL10-CCL4(0.857), CSF2-CXCL10(0.737), CXCL10-CSF2(0.737), EGFR-CSF2(0.720).

## Kyoto Encyclopedia of Proteins and Genomes (KEGG) analyses for differentially proteins

To analyze the enriched pathways of NSC vs OEC, KEGG analysis was performed. The results showed that the significant enriched pathways of the all regulated NSC vs OEC, are Cytokine-cytokine receptor interaction TNF signaling pathway,Chemokine signaling pathway,Cytosolic DNA-sensing pathway,Salmonella infection,Toll-like receptor signaling pathway (Fig 4).

## Discussion

The transplantation of neural stem cells and olfactory ensheathing cells promotes the recovery of spinal cord injury, but the degree of repair movement and sensory function are different. We try to explore its mechanism by analyzing its intrinsic factors and mechanism changes. Current research has found that injury produce an inhibitory environment that's not good for nerve Fig. 3 3. **Data analysis** 

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## **3.1 Biological process analysis of the genes**

In the differentially expressed genes in the fetal neurons of human, according to the enrichment points, we used the GO database to select the top 10 biological process (Fig.3 BP): system development, organ development, generation of neurons, tissue development, localization of cell, cell migration, epithelium development, negative regulation of protein metabolic process, behavior and cellular response to oxidative stress.

# **3.2 Related signaling pathway analysis of differently expressed genes**

To further study the function of the differentially expressed genes in neurons, we used the KEGG database for pathway analysis. The differentially expressed genes were sorted in descending order according to the enrichment score. The top 10 signaling pathways (Fig.3 Pathway) were as follows: proteoglycans in cancer, pathways in cancer, ErbB signaling pathway, non-small cell lung cancer, bladder cancer, melanoma, glioma, endometrial cancer, pancreatic cancer and epithelial cell signaling in Helicobacter pylori infection.

#### 4. Molecular verification after OGD injury

The typical morphology of cultured fetal neurons and SY5Y cells growing under light microscope was showed in Fig. 4. After OGD injury, SY5Y cells were decreased and fetal neurons were small with relatively round cell bodies and short neurites (Fig. 4A). Higher expression of GPNMB in OGD group compares with normal group in SY5Y cells (\*P<0.05) (Fig. 4B); and higher expression of GPNMB in OGD group compares with normal group in fetal neurons of human(\*P<0.05) (Fig. 4C). It revealed that OGD might up-regulate the level of GPNMB in cultured fetal neurons and SY5Y cells, respectively. The expressional fold change of GPNMB in SY5Y cells and fetal neurons were validated by qRT-PCR and Seqnencing, respectively. We identified that consistent



Fig. 3 GO analysis and KEGG analysis: Biological process (BP) and related signaling pathway (Pathway) analysis of the differentially expressed genes.

with the sequencing data and qRT-PCR analysis revealed that the expression of GPNMB was upregulated after OGD injury (Fig.4D).

## 5. Successful transfection of effective interference fragments.

In order to detect the role of GPNMB, we designed the RNA interference (RNAi) of GPNMB, which can suppress the expression of genes specially. qRT-PCR showed that compared with NC group, the expression of GPNMB was decreased in the GPNMB-F3 group, which showed the GPNMB-F3 was the most effective interference fragments, and this difference are statistically significant (p<0.05) (Fig.5B). After screening the effective interference F3, there were necessary to further confirm the interference F3 by CY3 staining. As shown in Fig.5A, we use CY3 to stain the cells in SY5Y, which proved the successful transfection in SY5Y cells.

#### 6. The change of function after GPNMB interference

In order to identify change of function after GPNMB interference, MTT assay, CCK-8 assay and high throughput screening were preformed, respectively. Both MTT assay and CCK-8 assay whose stand for cell viability, we found that both relative values of MTT and CCK8 are lower in OGD + NC group compares with OGD + reagent and GPNMB-si groups (p<0.05) (Fig.6A, B). We also found that higher cell number in normal group compares with OGD group (p<0.05), and showed that higher cell number in GPNMB-si group compares with NC and reagent groups (p < 0.05) (Fig.6C). We drew cell growth curve by high throughput screening, we identified that higher variation trend of growth curve in GPNMB-si group compares with normal group, however, lower variation trend of growth curve in NC and reagent groups compares with normal group (Fig.6D).

## 7. Molecular mechanism analysis of differential expression of GPNMB: alternative splicing

To elucidate that changes in GPNMB alternative splicing, we analyzed molecular mechanism of the alternative

#### Figure.4



Fig. 4 Validation of GPNMB expression in cultured fetal neurons and SY5Y cells after OGD injury. (A) Showed the morphology of fetal neurons (up) and SY5Y cells (down) after OGD injury. (B) Showed the GPNMB expression of SY5Y cells in OGD injury. (C) Showed the GPNMB expression of fetal neurons of human after OGD injury. (D) Comparison between qRT-PCR and Sequencing results, which showed the expressional fold change of GPNMB in SY5Y cells and fetal neurons of human by qRT-PCR and sequencing, respectively. Data are presented as mean  $\pm$  standard deviation (SD). \* P<0.05.

#### Figure.5



Fig. 5 Screening, confirmation and transfection of GPNMB effective interference fragments. (A) Showed GPNMB effective interference fragment transfected successfully in SY5Y cells. (B) Showed screening of GPNMB interference fragments F1, F2, F3 and choosing the most effective interference.

splicing in GPNMB expression by Splice Grapher software to model the gene that predicts a new alternative splicing event (Fig.7). We found that interference of 3' site in GPNMB sequence modulate alternative splicing of GPNMB.

#### Disscussion

The main finding of this study is that GPNMB premRNA splicing promotes neuronal survival after HIE injury. The present study may provide a new therapeutic strategy for HIE and may act as a guide for fundamental research and clinical research in the future.

## Figure.6



Fig. 6 The detection of GPNMB interference function. (A) and (B) showed relative values of MTT and CCK-8 after interfering GPNMB in OGD + NC, OGD + reagent and GPNMBsi groups, respectively. (C) Showed the change of cell number in normal and OGD groups, and NC, reagent and GPNMB-si groups after OGD injury, respectively. (D) Showed variation trend of cell growth curve in normal, NC, reagent and GPNMB-si groups with days post transfection. Data are presented as mean  $\pm$  standard deviation (SD). \* P<0.05.

#### Successful establishment of the HIE model

In this study, we successfully established a HIE model of rat in vivo. Zea-longa neurological score was used to confirm the successful establishment of the HIE model. The results demonstrated that the blood flow of cerebral blood flow was blocked after HI. As previously reported, HIE can lead to organ dysfunction(Sun L, et al.,2010),(Hu Y,et al.,2017),(Min J, et al.,2009),(Min J, et al.,1997), particularly effect on neurological function (Jain SV,et al.,20117),(Shah GS,et al.,2005).characterized by neurons were small with relatively round cell bodies and short neurites after HIE(Arevalo JC, et al.,2006). In present study, the results demonstrated that the blood flow of cerebral blood flow was blocked after HI, particularly in the left hemispheres. These findings confirmed that the model of HIE was successfully established.

#### Functional effects after GPNMB interference

In present study, we demonstrated that down-expression of GPNMB promotes neuronal proliferation after HIE. Therefore, after HIE, effective interference fragments of GPNMB might be regarded as a promoting factor for neural repair. It was known that GPNMB plays important roles in various types of cancer and amyotrophic lateral sclerosis (ALS)(Fiorentini C, et al., 2014). The previous studies confirmed that GPNMB-si is an effective factor that could inhibiting the progression of glioma, it therefore is useful for the recovery of neuron in the present experiment(Huang JJ, et al., 2012), (Maric G, et al.,2013). Meanwhile, some researchers identified that GPNMB was over-expressed and secreted by breast cancer cells, and the use of blocking antibodies suggested an autocrine loop mediating cell resistance to apoptosis, which revealed that GPNMB is involved in breast cancer. Therefore, they suggested that GPNMB contribute to breast cancer cell survival and can serve as prospective targets in attempts to inhibit tumor growth(Smuczek B, et al.,2017),(Gabriel TL, et al.,2014),(Wang J, et al.,2014), which indicated that down-expression of GPNMB-si may serve as an effective strategy for the treatment of tumor. In addition, the precious study also demonstrated that patients with high serum levels of GPNMB levels survived for longer than those with low levels, and

in CRC patients, serum levels of GPNMB cannot be used as markers of disease progression or tumor stage, while they may serve as prognostic indicators(Belone Ade F,et al.,2015),(Furuya M,et al.,2015),(Tian F, et al.,2013),(Kanematsu M, et al.,2015). This finding may be explained by the psychoactive treatments administrated to the CRC patients. By membrane protein library/ BLOTCHIP-MS technology to analyze all cell membrane proteins as binding partners of the GPNMB extracellular fragment, the researcher has identified that the alpha subunits of Na(+)/K(+)-ATPase (NKA) as a possible binding partner(Okita Y,et al., 2017). What's more, it was showed that endogenous GPNMB extracellular fragment bound to and colocalized with NKA alpha subunits. In addition, exogenous GPNMB extracellular fragment, for example, human recombinant GPNMB, also bound to and colocalized with NKA alpha subunits. Moreover, it was found that the GPNMB extracellular fragment had neuroprotective effects and activated the phosphoinositide 3-kinase (PI3K)/Akt and mitogen-activated protein kinase (MAPK)-extracellular signal-regulated kinase (ERK) kinase (MEK)/ERK pathways via NKA. To sum up, all the findings indicated that NKA may act as a novel "receptor" for the GPNMB extracellular fragment, offering additional molecular targets for the treatment of GPNMB-related diseases, including various types of cancer and ALS(Ono Y,et al.,2016), (Ono Y, et al.,2016). In our study, the low level of GPNMB needs to be amplified by inference technology to decrease the level of GPNMB for eventual functional recovery after HIE. Therefore, down-expression of GPNMB interference may

serve as an effective strategy for the treatment of HIE in future clinic practice.

## Gene microarray sequencing

We used Go analysis and pathway analysis to analyze the microarray results of the neurons. Gene chip technology and data analysis has been successfully applied in many fields, such as clinical diagnosis, medication guide, drug screening(Srinivasan A, et al., 2013), as well as basic medicine (Jiang XS, et al., 2014). Gene chip technology is able to analyze a high content of information, in a highthroughput manner, and is rapid and accurate(Yoshida M,et al.,2014). In addition, microarray and bioinformatics are complementary to Gene chip technology that were developed to be able to rapidly obtain large amounts of genetic information; they can provide the necessary database for bioinformatics research, while data analysis of gene chips also greatly depends on bioinformatics. Therefore, the combination of both provides a fast channel to study molecular biology (Retraction. et al.,2015). Through gene microarray sequencing, we found that compared with normal group, 15 genes were upregulated and 5 genes were downregulated in HI group. These indicated that the effect of differential expression genes therapy on the cerebral ischemia was a multigene regulated complex process. In addition, the functions of the identified differentially expressed genes were involved in various biological processes, cellular component, molecular function and various related signal pathways.

It has been shown that under brain injury conditions,



Fig. 7 The alternative splicing pattern of the gene. The picture above reflects the splicing of a gene from different angles. From top to bottom four boxes were described the following points: the original splicing model of the gene, different splicing forms with different color markers; the distribution of the reads on the gene; the number of splicing interface (Splicing Junction) and supported mapped reads; the newly predicted splicing event of the gene, with different types of alternative splicing events use for different color markers.

#### Figure.7

siRNA exert an important regulatory on silencing of GPNMB(Zhang P, et al.,2012). Moreover, researchers have found that the gene over-expression of early GPNMB played a deteriorated role in the development of the breast cancer (Awolaran O, et al.,2016), and a monoclonal antibody against GPNMB could be antiapoptotic(Zhang P, et al.,2013). Thus, these studies combined with our results, indicated that the effects of GPNMB-si on the treatment of HIE were mediated by the interacting effects of multiple genes involved in the regulation of multiple systems.

## Alternative splicing of GPNMB

Given the critical role of GPNMBS in cell viability of the nervous system, it is not surprising that alternative splicing of GPNMB pre-mRNA is tightly modulated under normal physiological conditions. In this study, we identified that the importance of splicing regulation in pathological HIE in vivo. GPNMB splicing is altered after HIE of aborted fetus from GPNMB-si, lead to increased expression of GPNMBS relative to GPNMBL. Moreover, specifically knocking down the GPNMBL isoform (with concomitant changes in GPNMB isoform ratios) prior to HI in neonatal rats resulted in alleviation of brain injury. Previous studies have shown that overexpression of GPNMBL in similar HIE models also aggravates brain injury (Nakano Y, et al., 2014), (Hou L, et al.,2015). Collectively, these data indicate that the ratio of GPNMB isoforms plays a critical role in brain injury, and suggest that the coordinate control of both isoforms via splicing regulation may have additive or synergistic effects. The pre-mRNA splicing machinery is extremely complex involving numerous cis-acting splicing sequences, trans-acting splicing factors, interactions with transcription regulation and mRNA export (Chen M,et al.,2009). To elucidate that changes in GPNMB alternative splicing, we analyzed molecular mechanism of the alternative splicing in GPNMB expression by Splice Grapher software to model the gene that predicts a new alternative splicing event. We found that interference of 3' site in GPNMB sequence modulate alternative splicing of GPNMB.

Though pre-mRNA splicing may result in wide-spread changes in RNA splicing, further study may identify a target that specifically regulates cell survival pathways. Exactly, several studies have shown that "master switch" splicing regulators may coordinately regulate specific unified functions such as synapse-interacting proteins encoded by Nova RNA targets(Ule J,et al.,2005). Because of the antagonistic activities of the two GPNMB isoforms, the splicing machinery serves as an attractive therapeutic target, in which a single manipulation may coordinately alter the expression of both a pro- and antiapoptotic proteins. Several other apoptotic genes appear to undergo alternatively splicing resulting in mRNA isoforms with antagonistic roles, such as caspase-2 and -9, adaptor proteins Apaf-1, Mcl-1 and IG20 (Wang L,et al.,1994),(Bae J,et al.,1998),(Bae J, et al.,2000),(Efimova EV,et al.,2004),(Xiao Q, et al.,2012).

In conclusion, by means of neurological injury models in vitro and in vivo, we have found that regulation of GPNMB alternative splicing modulates neural cell survival. Our results state clearly that alternative splicing might be an important regulatory mechanism for apoptosis after HIE injury, and that splicing regulators may be potential targets for alleviating CNS injury.

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