STUDY OF NEURAL TUBE DEFECTS AND DNA METHYLATION MODIFICATIONS THROUGH THE PPI NETWORK

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ABSTRACT

Background: *Failure of neural tube closure results in neural tube defects (NTDs), which may have severe neurological consequences or be fatal.*

Methods: *Differential analysis of NTDs and controls was performed separately for spinal and brain samples in GSE33111. Enrichment analysis for common genes of two groups of differentially expressed genes (DEGs). Differential methylation analysis was performed on NTDs and controls of spinal and brain samples from GSE69502, respectively. Enrichment analysis was performed on the intersection genes. Comparing the expression and methylation differences of genes obtained NTDs related genes subjected to methylation modification. PPI network analysis of common genes identified significant methylation marks.*

Results: *We found 2288 DEGs in the brain samples and 2267 DEGs in the spinal samples, for a total of 531 common genes. These genes were mainly enriched in type I interferon signaling pathway, and necroptosis. In addition, we found 2004 CPGs in brain samples and 3104 CPGs in spinal samples, for a total of 249 intersection genes. Methylation marks were significantly enriched in sensory organ morphogenesis, calcium ion transport into cytosol. Through the PPI network constructed on common genes, we identified the genes subjected to methylation modification in the network, which were considered as methylation marks (ANXA2, WIPF1 and KDM4B). Their methylation levels were reduced in NTDs compared to controls.*

Conclusion: *The interferon signaling pathway and calcium ion may be the molecular dysregulation mechanisms of NTDs. ANXA2, WIPF1 and KDM4B were identified as potential markers and target genes for NTDs.*

Keywords: *Neural tube defects, methylation, target genes, calcium ion.*

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Introduction

Neural tube defects (NTDs) are severe central nervous system birth defects caused during embryogenesis by failure of the morphogenetic process of neural tube closure[1]. The two most common NTDs are spina bifida and anencephaly[2]. The former result from failure of neural tube closure in the spinal region, while the latter result from failure of neural tube closure in the cranial region. The CNS is formed during vertebrate neurulation, which occurs in human embryos between days 17 and 28 post fertilization[3]. With an incidence of 1 in 1000 to 10, NTDs are among the most common severe birth defects[4]. Globally, it is estimated that approximately 300000 infants suffer from NTDs each year[5]. Approximately 10% of neonatal mortality is due to malformations of the nervous system in the embryonic period[6].

Neural tube closure defects are among the most common human birth defects, but their etiology remains unclear. Population and family studies suggest that the etiology of NTDs is complex and involves both environmental and genetic factors. Recognized risks of NTDs include maternal diabetes, obesity, reduced socioeconomic status, increased

temperature and exposure to certain teratogens during conception[7]. The heritability of NTDs is about 60% and involves many susceptibility genes[8]. With the implementation of the national folic acid (FA) fortification program, the incidence of neural tube defects (NTDs) in Canada has decreased by approximately 40%[9]. In a rural area of northern China, the incidence of NTDs was 6/1000 before folic acid supplementation in women of reproductive age, and the incidence decreased to 2/1000[10]. Thus, maternal blood folate status is an independent risk factor for NTDs[11].

Gene expression and regulation are complex processes in the pathogenesis of neural tube defects, and current research on these defects is focused on key genes that cause neural tube defects. More than 300 genes have now been found to cause NTDs in mice, and in humans, 82 genes have been implicated as genetic risk factors[12]. In addition to the potential effects of multiple risk alleles, the possibility exists that epigenetic alterations also contribute to the occurrence of NTDs by mediating interactions between fetal genetics and environmental factors[13]. One important mechanism by which gene expression can be altered within the globe is through the action of epigenetic regulators[14]. DNA methylation is one of the best understood epigenetic mechanisms[15]. Aberrant DNA methylation of key pathway genes may increase the risk of abnormal embryonic development and NTDs[16]. The current study aimed to identify differentially expressed genes and identify key genes associated with NTDs. Further exploration of the biological functions and molecular regulatory mechanisms involved in these genes is warranted. These results provide insights into regulatory mechanisms associated with embryonic cell growth during neural tube development.

Materials and methods

Data collection

GSE33111 included mRNA expression profiles by array of spinal cord and brain residual samples from 3 NTDs with those of 4 normal controls in the 2nd trimesters of pregnancy.

The data was preprocessed by Bioconductor lumi package. GSE69502 included DNA methylation profiling by array of spina bifida (SB, n=23) with those of 9 controlsand anencephaly (AN, n=9) from NTDs with those of 11 controlsin the 2nd trimesters of pregnancy. Raw data were preprocessed by with champ method.

Difference analysis

Differential expression analysis was performed using the limma R package. Genes with an P value <0.05 in GSE33111 were assigned as differentially expressed genes between NTDs and normal controls. Differentially methylated positions (DMPs) between NTDs and normal controls were obtained through limma R software package. Set screening threshold with |deltaBeta| >0.1 and P<0.05.

Enrichment analysis for selected genes

Gene Ontology (GO) enrichment analysis in this study included biological progression, cellular components, and molecular functions. The GO and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis for differentially expressed genes were performed using Enrichr database. The P value <0.05 were considered significantly enriched.

Protein-protein interaction (PPI) network

The PPI network was constructed with common genes was identified using the STRING database. The combined score >0.7 was considered significant. The PPI network was visualized by Gephi software.

Results

Neural tube defect related genes

To identify aberrantly expressed genes in patients with neural tube defects, we performed a comparative analysis of gene expression between NTDs patients and controls. In the brain samples, we found 2288 differentially expressed genes (DEGs), and in the residual samples, we found 2267 differentially expressed genes (DEGs) (Figure 1A). Among the two sets of DEGs, there were 531 common genes (Figure 1B). Aberrant expression of these genes may be associated with NTDs.

Biological functions of NTDs associated genes

Enrichment analysis of common genes identified 241 biological processes (BP), with the most significantly enriched terms including type I interferon signaling pathway, leukocyte cell adhesion, and cytokine mediated signaling pathway (Figure 2A).

There were also 20 cellular components (CC), and the most significantly enriched terms included azurophilic granule lumen, azurophilic granule, and G-protein coupled receptor dimeric complex (Figure 2B). And 56 molecular functions (MF), with the most

significantly enriched terms including phosphatase binding, protein serine/threonine kinase activity, and phospholipase inhibitor activity (Figure 2C). In addition, the KEGG pathway enrichment results showed 55 significant enrichments, mainly including the RIG-I-like receptor signaling pathway, necroptosis, and cell adhesion molecules (CAMs) (Figure 2D).

Identification of methylation marks

Using DNA methylation profiles of NTDs patients and controls, we identified differentially methylated probes (CPGs) between the them. In brain samples we found 2004 CPGs, in spinal samples we found 3104 CPGs (Figure 3A, B).

Among the top 70 genes with higher methylation levels, hypoprobe was much more than hyperprobe, especially in the brain samples (Figure 3C, D). We found 249 genes with methylation modifications in both tissues (Figure 3E).

Importantly, 10 aberrantly expressed genes associated with NTDs were subjected to methylation modification (Figure 3F).

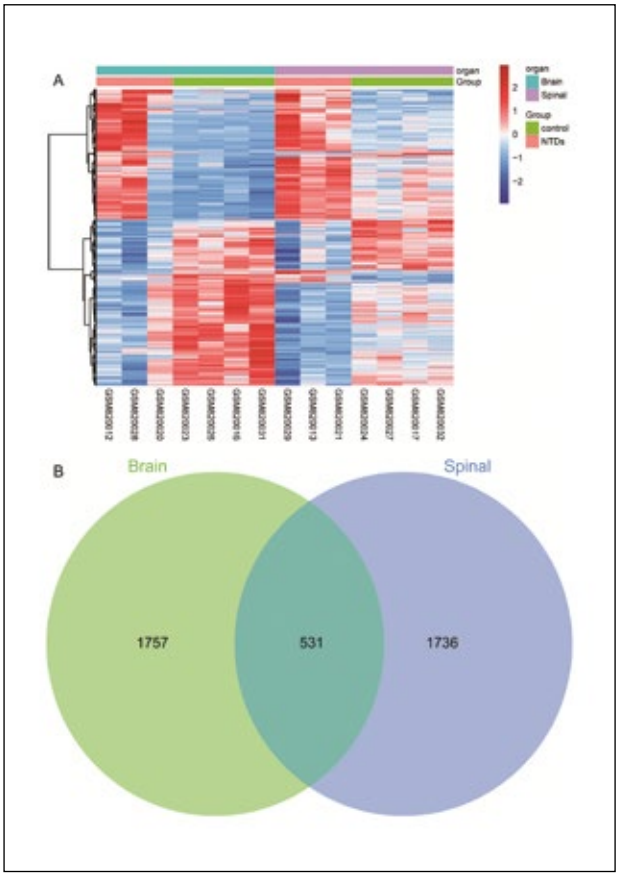

Figure 1: Genes associated with neural tube defects.
A. Heatmap of differentially expressed genes between NTDs and controls in brain and residual samples. B. Intersection of two groups of differentially expressed genes.

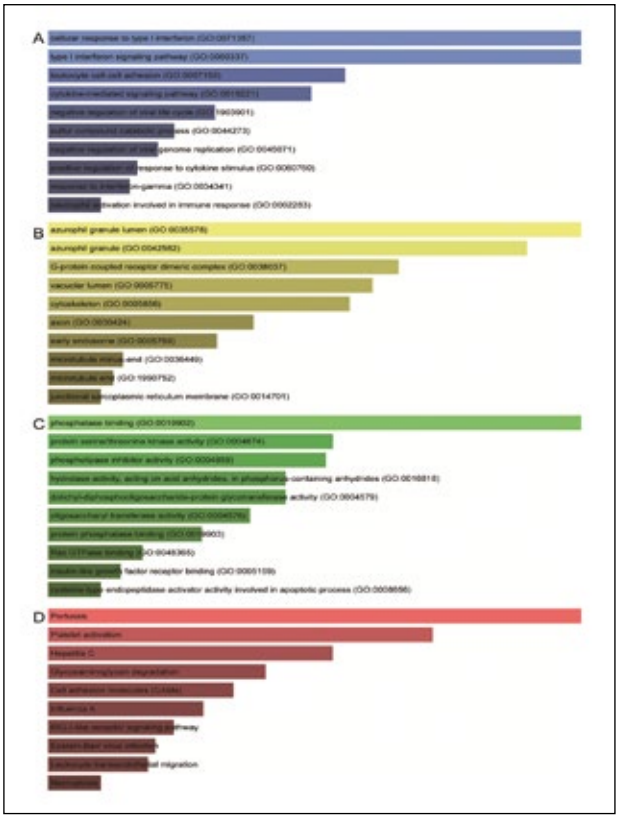

Figure 2: Significant enrichment results for common genes.
A. Top 10 terms of significantly enriched biological processes. B. Top 10 terms of significantly enriched cellular components. C. Top 10 terms of significantly enriched molecular functions. D. Top 10 terms of significantly enriched KEGG pathway for common genes.

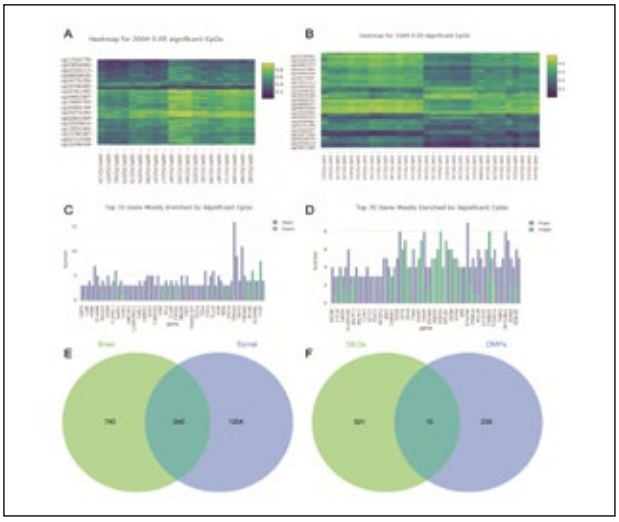

Figure 3: Identification of methylation marks associated with neural tube defects.
A. Heatmap of the CpG probes with significant differences in brain samples. B. Heatmap of the CpG probes with significant differences in residual samples. C. The distribution of CpG probes. C. Hyper probe and Hypo probe of CpG for the top 70 significant differentially methylated genes in brain samples. D. Hyper probe and Hypo probe of CpG for the top 70 significant differentially methylated genes in residual samples. E. Intersection of two groups of differentially methylated marks. F. Common genes modified by methylation.

Biological functions of methylation marks

Enrichment analysis of 249 methylation marks identified "sensory organ morphogenesis", "eye development", "metal homeostasis" as significantly enriched biological processes (Figure 4A).

In KEGG pathway results, "calcium ion transport into cytosol", "adrenergic signaling in cardiomyocytes", "parathyroid hormone synthesis, secretion and action" were significantly enriched (Figure 4B).

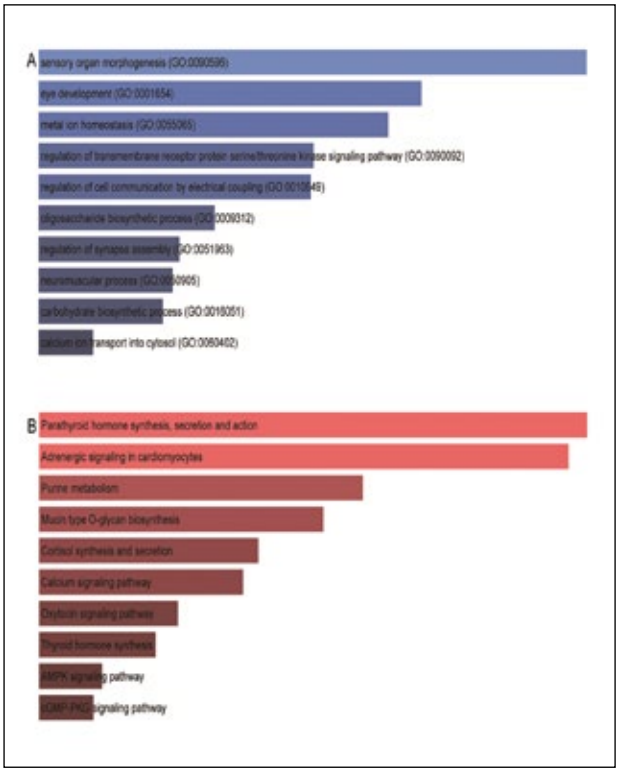

Figure 4: Biological functions and signaling pathways in which methylation marks participate.
A. Biological processes enriched by methylation marks. B. KEGG pathways enriched by methylation marks.

Key methylation marks

Further, a PPI network was constructed for the common genes, 181 significant differentially expressed genes were screened, and these genes were ranked by degree of connectivity (Figure 5A).

Among the network genes, we found 3 (ANXA2, WIPF1 and KDM4B) methylation marks (Figure 5B). At the expression level, ANXA2 and WIPF1 were upregulated and KDM4B was downregulated in NTDs patients compared to controls (Figure 5C).

At the methylation level, ANXA2, WIPF1 and KDM4B were downregulated in NTDs patients compared to controls (Figure 5D).

They were considered key methylation marks of neural tube defects.

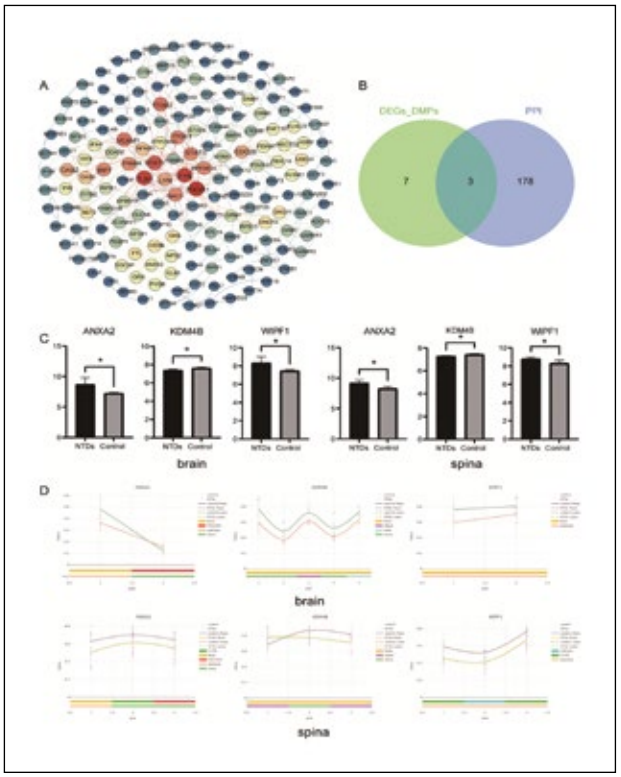

Figure 5: Identification of key methylation marks.
A. PPI network constructed by common genes.Nodes are colored from blue to red, indicating increased connectivity of genes. B. Methylation marks in PPI networks. C. Differential expression of key methylation marks between neural tube defects patients and controls. D. Differential methylation levels of key methylation marks between neural tube defects patients and controls.

Discussion

In mammals, during neural tube closure, the initially flattened neural plate bends and elevates bilaterally, producing neural folds that then fuse at the midline. Failure at any step in this process results in neural tube defects (NTDs)[17]. Aberrant DNA methylation has been implicated as a cause of NTDs and suggested as a mechanism by which folic acid prevents NTDs. Herein, we sought to identify NTDs associated methylation marks and the underlying molecular mechanisms by comparing differentially expressed and differentially methylated genes between NTDs and controls.

Neural tube injury is a multifactorial process that involves many molecular mechanisms. Type I interferons mediate disorders such as fetal death and severe growth restriction in pregnant mice through IFNAR signaling within the fetal placenta[18]. Elevated type I interferon activity is a factor which associated with the development of preeclampsia in patients with systemic lupus erythematosus[19]. The interferon signaling pathway has been implicated in

brain cases and brain developmental abnormalities following disease[20]. Interferon γ stimulates Shh signaling, suggesting a interaction with important neurodevelopmental pathway[21]. Interferon γ acts through the down-regulation of neurogenin 2, a pro neural factor of neural differentiation[22]. Loss of leukocyte receptor tyrosine kinase (LTK) in the cranial neural crest impairs migration and leads to increased levels of apoptosis[23]. In most cell types, RIG-I-like receptors (RLRs), which are essential for antiviral responses, elicit the production of type I and III IFNs and proinflammatory cytokines[24-26]. RLRs can also induce the activation of NF - κ B and MAPKs[27, 28]. Necroptosis is a regulator for necrotic cell death, and can be activated under apoptosis deficient conditions[29]. When tumor necrosis factor α stimulates apoptosis deficient cells, necroptosis can occur[30]. In addition to mediating cell adhesion, neural cell adhesion molecules can initiate signaling of regulation of neural progenitor survival, axonal growth[31]. Studies have shown ascidian cell adhesion molecule is necessary for closure of the anterior neuropore[32]. Aberrant methylation of the genome during embryogenesis is associated with developmental abnormalities at birth, including NTDs[33]. The results of our analysis show that genes subject to methylation modification in NTDs are involved in a variety of disease-related molecular functions. This includes processes of gastric tube formation and neural tube formation, neuronal polarity, hair bundle orientation of sensory cells in the inner ear[34-36]. Parathyroid hormone (PTH) interacts with G-protein-coupled receptors on bone cells to release calcium from long bones into the blood, and the parathyroid gland is required for the regulation of calcium homeostasis in the body[37].

In the examination of amniotic fluid, the level of calcium is significantly associated with NTDs[38]. Neural plate cells exhibit Ca^{2+} transients, and this signal regulates neural plate cell proliferation and migration[39]. Studies have also shown that T-type Ca^{2+} channels expressed in the anterior neural fold are required for neural tube fusion and closure in primitive chordates[40].

In addition, we identified several key methylation marks that could be potential targets for neural tube defects. Annexin A2 (ANXA2) is a widely distributed family of calcium dependent phospholipid binding proteins[41]. Loss of ANXA2, which is expressed in mouse primary sensory neurons, causes neuropathic pain[42]. ANXA2 is involved in vesicular trafficking, a fundamental cellular process that is also important for the formation of new tissues in multiple cell types[43]. In addition, ANXA2 has also been implicated in vascular homeostasis (44). LPS/IFNγ stimulation activates differential expression of WAS/WASL-interacting protein family member 1 (WIPF1) in microglia[45]. WIPF1 expression is important for thyroid cancer cell migration and invasion, suggesting that wipf1 is a novel target for thyroid cancer therapy[46]. Moreover, upregulated expression of WIPF1 is associated with the regulation of the cytoskeleton stimulated by glufosinate ammonium[47]. Neuroproteomic analysis has also shown that WIPF1 is involved in the reorganization of the actin cytoskeleton[48]. KDM4B is one of the histone demethylase KDM 4/ jmjd 2 family members[49]. Neuron specific KDM4B knockout mice exhibit neurodevelopmental disorders including spinal cord malformations and hippocampal damage[50]. KDM4B modifies the expression of a variety of genes involved in embryonic development[51, 52]. Our results raise the possibility that key methylation marks may be associated with NTDs.

Our study also has certain limitations. First, the small sample size of our analysis may have biased the interpretation of the results. In addition, the key methylation marks we identified were not found to have direct evidence for association with NTDs. Second, a significant portion of the analyzed results lack validation by molecular experiments, and a large number of subsequent experiments are needed to analyze the significance and clinical significance of these results.

Conclusion

In summary, by combining methylation data with transcriptome data, we described the molecular dysregulation mechanisms and potential methylation marks associated with NTDs. This study provided a new rationale for hypomethylation of ANXA2, WIPF1 and KDM4B in NTDs. Further studies are needed to evaluate the expression and methylation outcomes of ANXA2, WIPF1 and KDM4B in brain and residual NTDs and to analysis their potential role as biomarkers for NTDs.

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