Research Article

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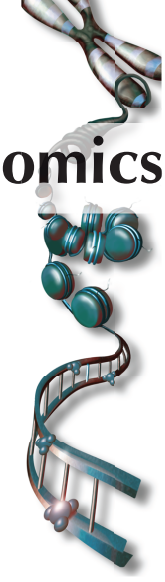
Epigenomics



Hypomethylation of *GRHL3* gene is associated with the occurrence of neural tube defects

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Aim: To investigate the relationship between *GRHL3* methylation and the etiology of neural tube defects (NTDs). **Materials & methods:** Analyze data from a genome-wide DNA methylation array. Targeted DNA methylation analysis was performed for 46 cases and 23 controls. At last, *grhl3* overexpression and gene depletion experiments were conducted in zebrafish. **Results:** Five hypomethylated CpGs were discovered in the methylation arrays performed on NTD cases. In a validation study, 15 hypomethylated CpGs were found and the overall methylation levels decreased in brain/spinal cord tissue from NTD cases. The knockdown and overexpression of *grhl3* in zebrafish damaged embryonic convergent extension processes. **Conclusion:** Hypomethylation of *GRHL3* in central nervous tissue is associated with NTDs, further supporting the importance of *GRHL3* and methylation in proper neural tube closure.

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Keywords: *GRHL3* gene • methylation • neural tube defects • PCP pathway

Neural tube defects (NTDs) are a group of severe malformations that arise following closure failure of the neural tube during early embryonic development, and affect more than 300,000 newborns every year globally [1]. Genetic factors are believed to play key roles in the occurrence of NTDs [2]. More than 300 genes have been identified as NTD-related mutations in mouse models [2,3]. While modest positive associations between gene variants such as the 677C >T of *MTHFR* gene and an increased risk for NTDs in humans, there are few other confirmed gene variation associations in humans [4]. It is highly likely that there may exist other underlying mechanisms, including epigenetic modifications, that are involved in the etiology of NTDs.

DNA methylation is an epigenetic mechanism that has been shown to play an important role in the normal closure of the neural tube. Folate is a critical component in the one-carbon metabolic pathway by providing methyl groups for a variety of biochemical reactions, including methylation of DNA [5]. Several studies have shown that supplementation with folic acid may prevent NTDs by stimulating DNA methylation [6,7]. Additional investigations have demonstrated that the *MTHFR* 677C >T polymorphism could influence global DNA methylation status through an interaction with folic acid [8,9]. Recently, our group performed a genome-wide DNA methylation array and found that hypermethylation of the *CTNNA1* and *MYH2* genes in the tight junction pathway may be associated with an increased risk of NTDs [10]. The NTD-related aberrant methylation of other genes has also been reported, including imprinted genes [11,12], transposon genes [13], DNA repair genes [14], folate receptor genes [15] and *HOX* genes [16].

The planar cell polarity (PCP) signaling pathway plays a key role in the closure of neural tube, and variants of PCP pathway genes have previously been reported to cause NTDs [17,18]. Epigenetic modification of these genes may also play a role in the formation of NTDs by perturbing their expression. One such study reported that aberration of DNA methylation, rather than mutations of *FZD3* gene (one of PCP core genes), increased the risk

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of spinal bifida [19]. Given the important and well-established roles of PCP pathway genes in the proper closure of neural tube, it is possible that aberrant methylation of other PCP genes may also be involved and worthy of further investigation. *GRHL3* gene is an important PCP pathway gene that is involved in establishing dorsal/ventral patterning and subsequent organ development in early stages of mouse embryogenesis [20]. Inactiavation of this gene was produced NTDs in the *Grhl3* knockout mice [21] and in the curly tail (Ct) mouse model [22]. Recently, Castro *et al.* reported that the conditional *Grhl3* knockout in the neural plate and node-streak border has minimal effect on closure, suggesting that abnormal functioning of the surface ectoderm is primarily responsible for early failure of spinal neurulation in Grhl3 null embryos [23]. Lemay *et al.* described eight deleterious variants in *GRHL3* in human spina bifida cases [24], demonstrating the importance of this gene in the development of human NTDs. Therefore, an investigation into potential aberrant methylation of the in *GRHL3* gene in NTD cases may enhance our understanding of the underlying mechanisms leading to the development of NTDs.

We hypothesized that aberrant DNA methylation of *GRHL3* gene is related to the formation of NTDs. To test this hypothesis, we initially examined the methylation changes in the *GRHL3* gene in the array data of ten NTD cases and eight controls. We subsequently performed a locus-specific methylation validation study in a larger sample using the MassARRAY platform. At last, we conducted overexpression and morpholino (MO) experiments using a zebrafish model to study the function of *ghrl3* gene in the development of the neural tube.

Materials & methods

Participants & samples

The subjects of this study were recruited from five counties in Shanxi Province, China (Pingding, Zezhou, Xiyang, Shouyang and Taigu) from 2011 to 2014, a province long known to have a high prevalence of NTDs [25]. Cases were electively terminated following prenatal diagnosis of an NTD; subjects with defects in other system were excluded. Controls were terminated fetuses with no gross congenital malformations. For anencephalic cases, the residual brain tissues were harvested, while for spina bifida cases, tissues were collected from the anatomical lesion site of the spinal cord. A normal brain or spinal cord tissue sample from nonmalformed control fetuses were also collected at pregnancy termination by experienced pathologists. All samples were stored at -80°C until utilized for various analyses. This study was approved by the institutional review board of Peking University and all of the participating mothers provided written informed consent.

The human study involved two phases. At the discovery phase, pathological brain/spinal cord samples from ten NTD cases (six anencephaly, four spinal bifida) and brain/spinal cord tissue from eight nonmalformed controls recruited between 2011 and 2012 were used in a genome-wide methylation analysis [10]. At the validation phase, 46 NTD cases (20 anencephaly, 26 spinal bifida) and 23 controls which were 2:1 pair matched by gender and gestational weeks and recruited during 2013–2014 were included. Pathological or normal brain/spinal cord tissue samples were used to examine locus-specific methylation differences within *GRHL3* gene.

DNA methylation analysis using the Illumina 450 K BeadChip array

Fetal tissue DNA was extracted by using QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany). The concentration of DNA was measured by using a NanoDrop2000 Ultramicro spectrophotometer (Thermo Fisher Scientific, MA, USA). All of the DNA samples were stored at -80°C until used for specific assays.

Infinium Human Methylation 450 (HM450K) Bead Chip assay (Illumina, CA, USA) was performed to analyze genome-wide DNA methylation level in neural tissues from both cases and controls, as previously described in more detail elsewhere [10]. Briefly, bisulfite conversion of the DNA (500 ng) was performed using the EZ DNA methylation kit (Zymo Research, CA, USA). The bisulfite conversion reaction was performed in duplicate for each sample to minimize potential bias caused by variable conversion efficiency, and pooled bisulfite treated DNA was used for subsequent array analysis. Arrays were processed according to the manufacturer's protocol.

Cluster analysis and component analysis with the methylation array data showed that the NTD case group and the nonmalformed group could be distinguished from each other [10]. The data for the *GRHL3* gene was extracted from the genome-wide array.

Methylation of CpGs using the Sequenom EpiTYPER

Target verification of differentially methylated CpG islands in human gDNA was performed using the Sequenom EpiTYPER (Sequenom, CA, USA). Bisulfite conversion of 1 mg DNA was conducted using an EZ DNA Methy-

lation Kit (Zymo Research) according to the manufacturer's instructions. In this step, the unmethylated cytosine is converted into uracil while the methylated cytosine remains unchanged.

The bisulfite treated DNA was amplified by PCR experiment. The PCR primers of *GRHL3* gene were designed by the online tool Epidesigner (www.epidesigner.com). Five CpG sites whose methylation intensity was significantly different between NTD cases and controls, were located far away from each other and one amplicon could not cover all five sites. As such, we designed two amplicons to target the two aberrant CpG sites of which differences between cases and controls were higher than 0.2 in HM450K array. Additionally, considering the important role of the CpG island, which is considered to be a region with a high frequency of CpG sites, we designed another two amplicons to target the CpG island of *GRHL3* gene in the validation study. The primer sequence was presented in Supplementary Table 1. The reaction volume of PCR was 5 µl. The PCR reaction program was as follows: 94°C for 4 min; 45 cycles of (95°C for 20 s; 56°C for 30 s; 72°C for 60 s); 72°C for 3 min. The PCR product was incubated using shrimp alkaline phosphatase for 20 min at 37°C and then purified at a temperature of 85°C for 5 min. Enzyme digestion was performed using T cleavage enzyme (T Cleavage Mix) at 37°C for 3 h and the product was desalinized using 384 dimple plates. Subsequently, the methylation of the DNA was analyzed by the MassARRAY system. The results were analyzed using EpiTYPER software. To control for the quality of the methylation assay, we used both positive controls (completely methylation) and negative controls (nonmethylation) in our analyses.

grhl3 overexpression & depletion in zebrafish

Tübingen (Tu) strain zebrafish were raised according to standard protocols. The *GRHL3* gene is highly conservative between humans and zebrafish. In this study, we used human mRNA to conduct overexpression experiments. To produce *GRHL3* mRNA, the ORF cDNA of *GRHL3* was cloned into the pCMV6-Entry vector (Origene Technologies, Beijing, China). The cDNA was transfected into *Escherichia coli* and the plasmid containing the *GRHL3* cDNA was extracted and enzyme digested. At last, the capped and polyadenylated *GRHL3* mRNA was produced by *in vitro* transcription using mMESSAGE mMACHINE™ T7 Transcription Kit (Ambion, TX, USA). The synthesized mRNA was diluted in phenol red to different concentrations for microinjection. The MO reagent with an ATG-blocking (initiation codon) *grhl3* sequence (5′-TGAGAGCCTCAATCTCCTTGGTCAT-3′) was obtained from GeneTools LLC, Co. (OR, USA) [26].

Different doses of MO reagent (6–14 ng) and mRNA (100–300 pg) were microinjected into each zebrafish embryo at its one-cell stage. Noninjected embryos were used as controls. All embryos were observed using fluorescent stereo microscopy at 24 and 48 h postfertilization (hpf). Images of each phenotype were captured by using the charge coupled device (CCD) image system under bright field. All the embryos were divided into four categories according to the severity of the malformations. All involved embryos were independently observed and counted by two blinded researchers to reduce subjective error.

Statistical analysis

In the discovery stage, potential sources of technical bias were excluded prior to further analysis. Probes were excluded from further analysis if >95% of the samples had a detection value >0.01 [16]. Illumina Genome Studio software (Illumina, CA, USA) was used to extract signal intensities for each probe, perform the initial control quality checks and estimate β-scores and detection p-values. β-score was defined as the proportion of total signal from the methylation-specific probe or color channel. Detection p-value was defined as the 1-p-value computed from the background model characterizing the chance that the target sequence signal was distinguishable from the negative controls. Independent t-tests were used to identify differentially methylated CpG sites. The p-value was adjusted for multiple testing using the Benjamini–Hochberg false discovery rate (FDR) methods to control for the false discovery rate. Differentially methylated CpG sites were identified by the following criteria: the false discovery rate $p < 0.05$; the absolute β-value difference >0.05 [16]. Some more detailed data analysis was shown in the Supplementary Methods.

In the replication stage, the differences in proportions of population characteristics between groups were examined with Pearson's χ^2 test. The distribution of methylation values of cases and controls were tested by Shapiro–Wilk test. Paired t-test was used to identify CpGs that were differentially methylated between the NTD cases and controls. Binary logistic regression was performed to investigate the relationship between methylation levels of *GRHL3* with the risk for NTDs. In order to make it better to explain the regression coefficient, we take the negative value of the methylation data into the model, and whether or not taking folic acid was further adjusted in the regression model. Maternal folate supplementation (yes vs no) was considered a potential confounding factor because folate

Table 1. Methylation of *GRHL3* gene using the Human Methylation 450 Bead Chip assay.

Target ID	Chr	Mapinfo	Case	Control	Diff	ad-p	Region
cg24860886	1	24645397	0.205	0.546	-0.341	0.005†	TSS1500
cg27298252	1	24645380	0.876	0.930	-0.054	0.048†	TSS1500
cg06376426	1	24648203	0.675	0.720	-0.045	0.285	TSS1500
cg21273275	1	24648328	0.367	0.283	0.084	0.310	TSS1500
cg09322349	1	24645184	0.532	0.601	-0.069	0.438	TSS1500
cg22176324	1	24648851	0.072	0.069	0.003	0.722	TSS1500
cg05036846	1	24648984	0.073	0.072	0.001	0.911	TSS1500
cg14616251	1	24648696	0.076	0.076	0.000	0.995	TSS1500
cg13987674	1	24649494	0.854	0.913	-0.059	0.106	TSS200
cg09662754	1	24645805	0.081	0.099	-0.018	0.188	TSS200
cg12757705	1	24645802	0.051	0.057	-0.006	0.464	TSS200
cg11709405	1	24649398	0.394	0.448	-0.055	0.489	TSS200
cg15050111	1	24645850	0.075	0.073	0.002	0.817	TSS200
cg20242797	1	24645853	0.114	0.118	-0.005	0.852	TSS200
cg18196063	1	24652248	0.811	0.891	-0.080	0.012†	5′UTR
cg26365938	1	24645953	0.077	0.089	-0.012	0.074	5′UTR
cg21289280	1	24645885	0.049	0.058	-0.010	0.166	5′UTR
cg12405265	1	24655961	0.843	0.833	0.010	0.678	5′UTR
cg04552500	1	24645917	0.099	0.095	0.004	0.711	5′UTR
cg23820945	1	24646022	0.145	0.153	-0.008	0.906	5′UTR
cg14167629	1	24646046	0.117	0.105	0.011	0.659	First exon
cg20325200	1	24667622	0.590	0.812	-0.222	0.000†	Body
cg16734875	1	24678010	0.883	0.943	-0.060	0.011†	Body
cg19697558	1	24646085	0.070	0.068	0.002	0.916	Body
cg16155382	1	24646191	0.129	0.101	0.028	0.373	Body
cg01119512	1	24646205	0.230	0.201	0.029	0.408	Body
cg02984188	1	24646392	0.364	0.482	-0.118	0.240	Body
cg11155431	1	24664451	0.886	0.890	-0.004	0.830	Body

Target ID is identified according to HM450k. The nucleotide position is based on NCBI build 37/hg19. Region is defined relative to the nearest open reading frame: within 1500 (TSS1500) or 200 bp (TSS200) of a transcription start site, in the 5′ UTR, the first exon of a transcript (exon) and in the body of gene (body). Differentially methylated CpG sites were identified by two criterions: the false discovery rate <0.05, which was analyzed by independent t-tests with multiple comparison tests; the absolute β-value difference >0.05[16].
†The CpG site was aberrant methylated in case group.
ad-p: Adjusted p-value; Chr: Chromosome; diff: Difference; Mapinfo: Nucleotide position; UTR: Untranslated region.

is a one-carbon unit donor in the methylation pathway. In the zebrafish study, Pearson's χ^2 test and the Fisher test were used to analyze the distribution of malformed zebrafish. A two-tailed p-value of <0.05 was considered statistically significant. SPSS 20.0 software (IBM Co., NY, USA) was used for analyzing the result in this study.

Results

DNA methylation analysis of *GRHL3* gene detected by microarray: discovery

Methylation data for CpGs in *GRHL3* gene in the discovery stage were extracted from the dataset obtained from HM450K array-based genome-wide methylation analysis by using DNA isolated from central nervous tissue from ten NTD cases and eight unrelated nonmalformed controls. No significant differences were found in gestational age and gender distribution between cases and controls [10].

As shown in Table 1, a total of 28 CpGs on the *GRHL3* gene were detected by the HM450K array. Of these, 17 CpGs were found to be hypomethylated in NTD fetuses, and five of these 17 CpGs (cg24860886, cg27298252, cg18196063, cg20325200 and cg16734875) were significantly hypomethylated in NTD cases (β-difference of -0.341, -0.054, -0.080, -0.222 and -0.060, respectively). The location of the five significantly aberrant CpGs is shown in Figure 1. Of these five CpGs, two CpG probes (cg24860886 and cg27298252) were located in the TSS1500 region; one CpG probe (cg18196063) was located at 5′UTR; the remaining two CpG probes (cg20325200 and cg16734875) were located within the body of the *GRHL3* gene.

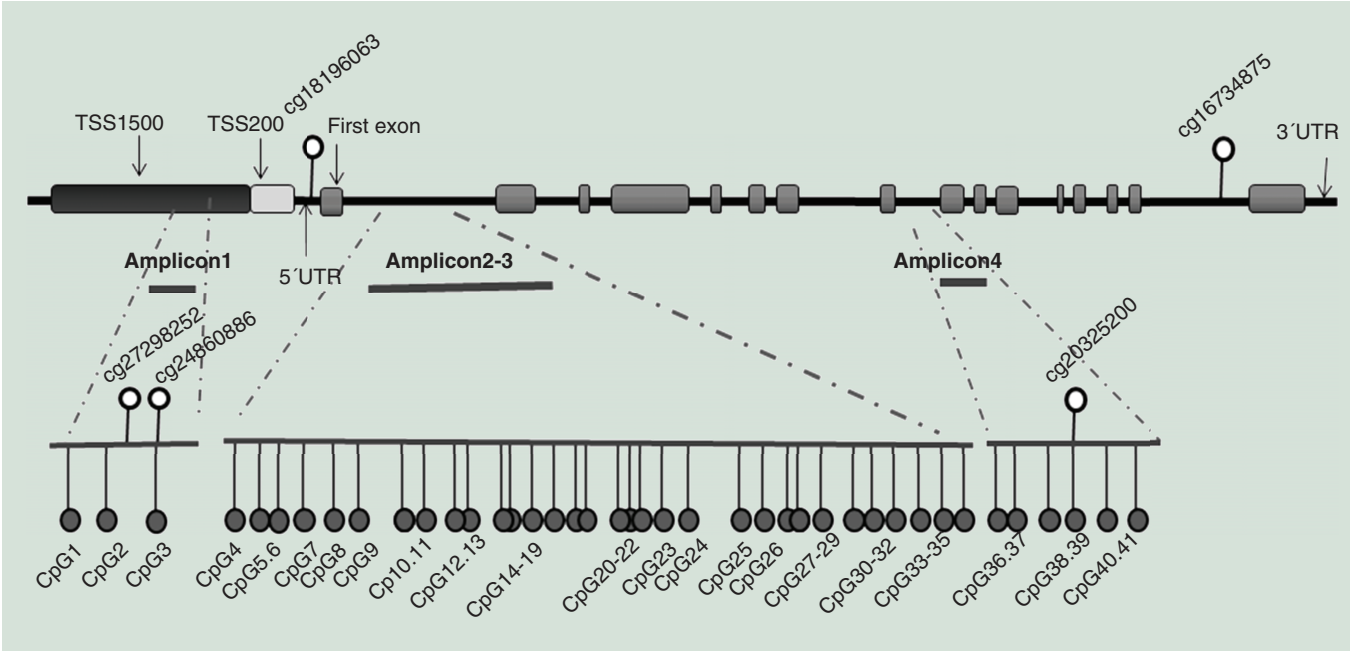

Figure 1. Locations of the CpG sites detected by HM450k and Sequenom EpiTYPER. In the validation study with the Sequenom EpiTYPER assay, four DNA amplicons were involved; amplicon 1 covered three CpG sites (CpG1 to CpG3) which were located in the TSS1500 region of the *GRHL3* gene. Amplicon 2 and amplicon 3 covered the CpG island region of *GRHL3* predicted using UCSC (chr1:24648202–24648985). These two amplicons included 32 analytical CpG sites (CpG4 to CpG35). Amplicon 4 covered six CpG sites (CpG36 to CpG41) within the gene body. Nucleotide positions accord to the NCBI build 37/hg19. The differentially methylated CpG units identified by 450 K Array (cg27298252, cg24860886, cg18196063, cg20325200 and cg16734875) are also indicated.

Table 2. Characteristics of subjects included in the analysis of Sequenom EpiTYPER.

Characteristic	Total	Case (n = 46)	Control (n = 23)	p-value
Gestational week	24.23 ± 5.50	24.07 ± 5.38	24.65 ± 5.83	0.679
Gender:				1.000
– Male	27 (39.1)	18 (39.1)	9 (39.1)	
– Female	42 (60.9)	28 (60.9)	14 (60.9)	
Occupation:				
– Farmer	52 (75.4)	32 (76.2)	20 (74.1)	0.842
– Others	17 (24.6)	10 (23.8)	7 (25.9)	
Educational level:				
– Primary school or lower	7 (10.1)	4 (9.5)	3 (11.1)	0.810
– Junior school	44 (63.8)	28 (66.7)	16 (59.3)	
– High school or higher	18 (26.1)	10 (23.8)	8 (29.6)	
Folate supplementation:				
– No	37 (53.6)	23 (54.8)	14 (51.9)	0.813
– Yes	32 (46.4)	19 (45.2)	13 (48.1)	

Pearson's χ^2 test for gender, occupation, educational level and folate supplementation. Independent samples test for gestational week.

Targeted DNA methylation analysis of *GRHL3* gene in a larger sample: validation

To verify the relationship between NTDs and *GRHL3* hypomethylation identified in the discovery phase, 46 NTD cases and 23 gestational age and gender-matched controls were included in the validation study using the Sequenom EpiTYPER technology. The demographic characteristics of the subjects are depicted in Table 2. As shown in Figure 1, we completely amplified the four DNA amplicons. Amplicon 1 covered three CpG sites (CpG1 to CpG3), in which the CpG3 was the same as the cg24860886 CpG site identified to be differentially hypomethylated in the discovery phase. Amplicon 2 and 3 covered the CpG island of *GRHL3*, including 32

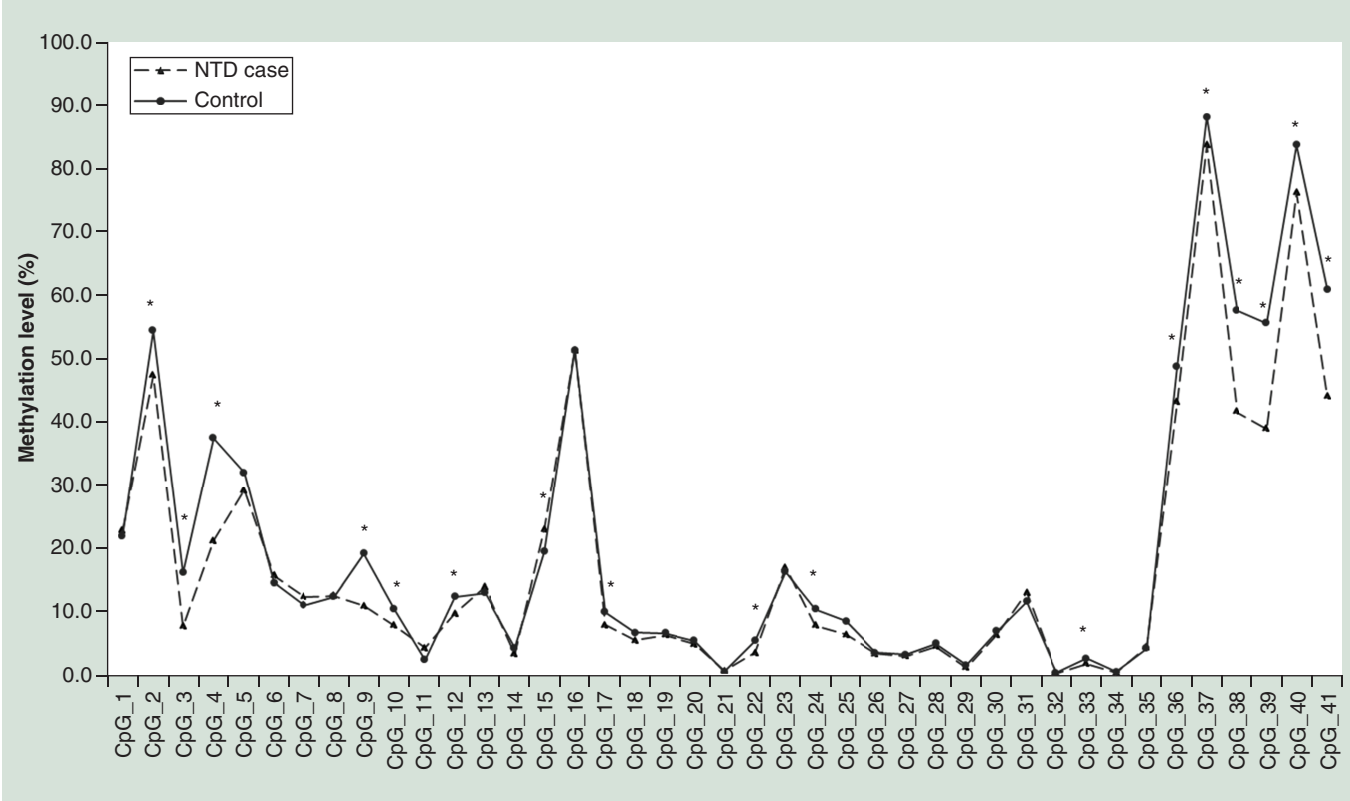

Figure 2. Methylation pattern of *GRHL3* gene by using Sequenom EpiTYPER. Methylation level for each CpG unit between NTD cases and control group within the amplicons were analyzed. Paired t-test was performed.
*The methylation of CpG site had significant difference between case and control group ($p < 0.05$).
NTD: Neural tube defect.

analytical CpG sites (CpG4 to CpG35). Amplicon 4 covered six CpG sites (CpG36 to CpG41) within the gene body, in which the CpG39 was the same as the hypomethylated CpG site of cg20325200 identified in the discovery phase.

As shown in Figure 2, among the 41 detected CpG sites, 29 CpGs showed hypomethylation in NTD cases, 15 of which were significantly different between the cases and controls. In the validation phase, both the HM450K array-matched CpG sites CpG3 (cg24860886) and CpG39 (cg20325200) showed significant hypomethylation in cases with a β-difference of 7.2 and 16.7%, respectively. CpG15 showed significant hypermethylation in cases with a smaller β-difference of 3.5% ($p = 0.027$). After the Benjanmini–Hochberg correction, CpG38, CpG39 (cg20325200) and CpG40 still demonstrated significant hypomethylation in the NTD cases. The details are represented in Supplementary Tables 2–4.

The average methylation level of *GRHL3* gene in the case group was 16.5% (95% CI: 14.0, 19.0%), significantly lower than the control group (20.1%; 95% confidence interval [CI]: 16.9, 23.3%). The logistic regression showed that hypomethylation of GRHL3 gene was a risk factor for NTDs (ad-OR of 1.657; 95% CI: 1.279–2.146), indicating that as the average methylation level is reduced by one unit, the risk of NTDs increases by 65.7%. We further analyzed the association between different subtypes of NTDs, namely spina bifida and anencephaly, with the average methylation level of *GRHL3* gene. As shown in Table 3, the average methylation levels of both subgroups were significantly lower than those of the control group and hypomethylation could increase the risk of spina bifida and anencephaly.

GRHL3 mRNA overexpression & *grhl3* downregulation in zebrafish

Functional studies of the *GRHL3* gene (Gene ID: 57822) were performed using the zebrafish model. *Grhl3* gene (Gene ID: 794613) is well-conserved between humans and zebrafish, which indicates a high similarity of encoded

Table 3. Mean methylation level of *GRHL3* gene in neural tube defect cases and controls.

Group	n	Mean methylation (%)	OR† (95% CI)	Adjusted-OR (95% CI)
NTDs	46	16.5 (14.0,19.0)	1.552 (1.232–1.954)	1.657 (1.279–2.146)
Controls	23	20.1 (16.9,23.3)	1.000	1.000
Spinal bifida	26	15.9 (13.4,18.4)	1.514 (1.121–2.045)	1.562 (1.140–2.140)
Controls_1	13	19.3 (15.9,22.7)	1.000	1.000
Anencephaly	20	17.4 (15.0,19.8)	1.800 (1.165–2.780)	2.114 (1.178–3.792)
Controls_2	10	21.1 (18.5,23.7)	1.000	1.000

Using logistic regression to investigate the relationship between methylation of GRHL3 with NTDs and subtypes of NTDs.
†In order to make it better to explain the regression coefficient, we take the negative value of the methylation data into the model.
Adjusted-OR: Adjusted by folic acid supplementation; CI: Confidence interval; control_1: The control matched with spinal bifida case; control_2: The control matched with anencephaly case; NTD: Neural tube defect; OR: Odds ratio.

amino acids between these two species [22]. We performed gene overexpression and gene knockdown experiments by microinjecting synthetic *GRHL3* mRNA and a spliced *grhl3* MO reagent, respectively.

After injection of different concentrations of *GRHL3* mRNA into one-cell stage embryos, the convergence extension process of the embryos was damaged at 24 and 48 hpf, including shortening and bending of the body axis. Based on the severity of the observed phenotype, we divided the embryos into four grades (grade 1: wild-type like; grade 2: mild; grade 3: moderate; grade 4: severely abnormal) (Figure 3A). The distributions of these four grades at the different concentration groups at both 24 and 48 hpf were examined. Compared with the control group, the rate of abnormal embryos was significantly higher in the groups receiving 100, 200 and 300 pg mRNA. The malformation rate was increased with increasing concentrations of mRNA at both 24 and 48 hpf (p for trend <0.05) (Figure 3B). In addition, those embryos injected with different dosages ranging from 6 to 14 ng of *grhl3*-MO showed higher malformation rates than the control group, and the malformed rate increased with the increasing MO dosages at both 24 and 48 hpf (Figure 3C).

Discussion

Using a two-phase design and functional studies in zebrafish models, we found that hypomethylation of the *GRHL3* gene may be involved in the etiology of some NTDs. In the discovery phase, we found five hypomethylated CpGs of the *GRHL3* gene in NTD cases. Subsequently, we detected 15 hypomethylated CpGs in NTD cases in the validation phase with a larger case–control sample. The zebrafish experiment demonstrated that the depletion of functional *grhl3* gene and the overexpression of *GRHL3* mRNA could severely compromise the convergent extension process, leading to NTD-related phenotypes.

GRHL3 is essential to the formation of dorsal/ventral and subsequent organ development in early embryonic stage [20]. Most animal studies to date have focused on gene mutations including deletion or loss of function in the *grhl3* gene. A previously published study has shown that MO-mediated knockdown in zebrafish of the *grhl3* led to severe hypoplasia of the lower jaw cartilages [26]. All the *Grhl3*$^{-/-}$ mutant mice showed spinal bifida and curled tail, and 2% of them had a defect analogous to anencephaly. The *Grhl3*$^{-/-}$ mice also showed resistance to folate and inositol [21]. Lemay *et al.* recently reported eight deleterious variants in *GRHL3* in human spina bifida cases [24]. In our study, we found that most CpGs of *GRHL3* were hypomethylated in the NTD cases and the mean methylation level in NTDs was lower than in nonmalformed controls. Further subgroup analysis suggest that the hypomethylation of *GRHL3* gene was present in both spinal bifida and anencephaly subtypes, the two major subtypes of human NTDs. Our study provides novel evidence on the role of epigenetic modifications of the *GRHL3* gene in human NTDs.

DNA methylation in mammals is a postreplication modification that is predominantly found in cytosine residues of the dinucleotide sequence CpG. Methylation is now recognized to be a major contributor to the stability of gene expression, and it is essential for vertebrate development [27]. The loss of methylation may lead to apoptosis in embryos [28]. Hypomethylation may upregulate the expression of mRNA of genes which is suggestive for *GRHL3* overexpression. Furthermore, in the present study, *GRHL3* overexpression experiment was performed in zebrafish to examine possible biological mechanisms of *GRHL3* hypomethylation underlying the development of NTDs. The zebrafish experiments showed that the overexpression of *GRHL3* mRNA could lead to a disturbance of convergent extension which created different degrees of NTD-related phenotypes, including bending and curling of the body

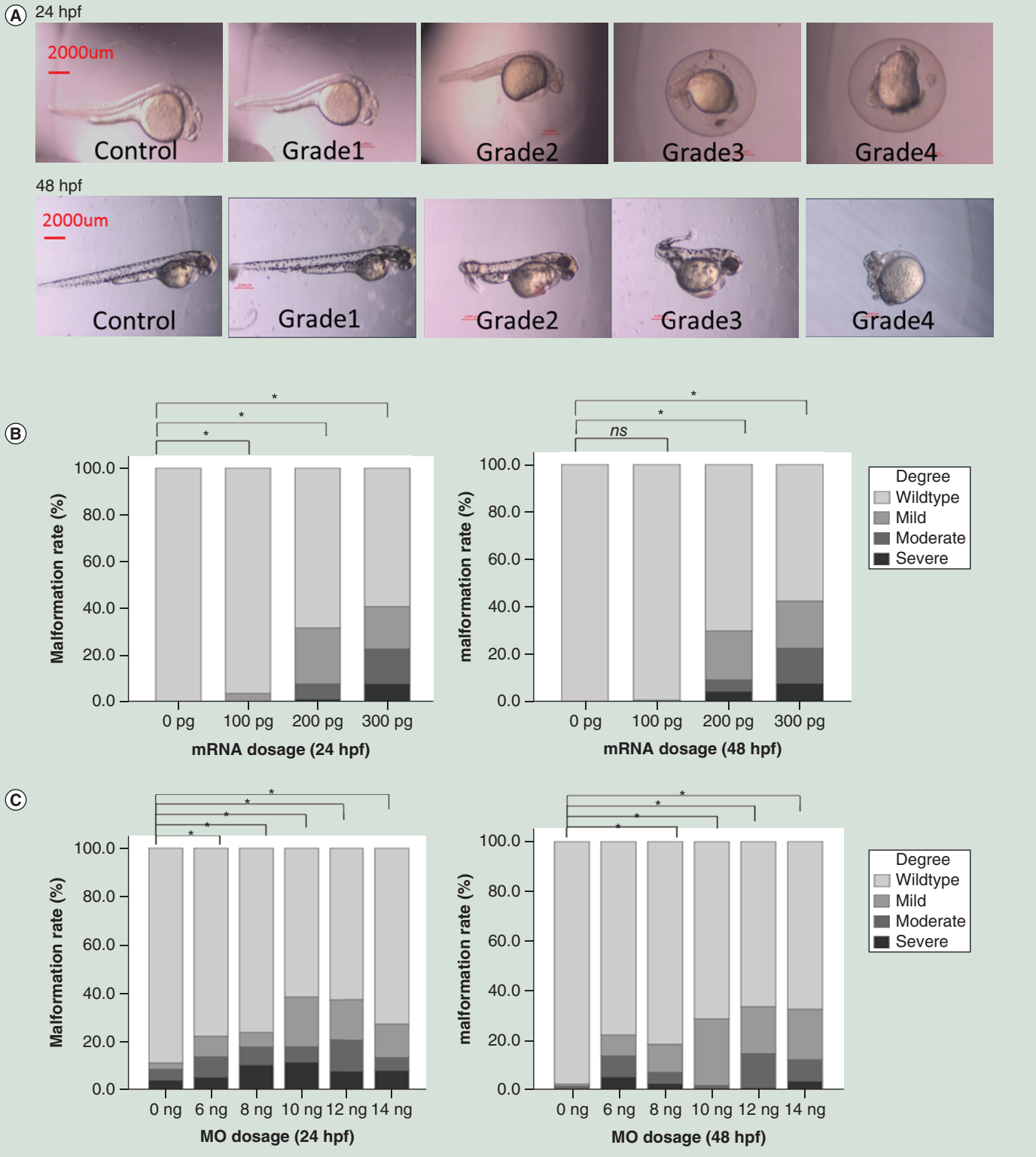

Figure 3. Phenotype analysis of *GRHL3*-overexpression and *grhl3*-MO in zebrafish embryos. (A) Phenotype analysis of zebrafish embryos treated with *GRHL3* mRNA: based on the phenotype severity, we divided the phenotypes into four grades: grade 1: no difference comparing with wild-type, no shortening of the body axis; grade 2: mild deformity, curling body axis with the length reduced about 1/4 relative to the wild-type; grade 3: moderate deformity, severe curvature and body axis shortened nearly 1/2; grade 4: severe deformity, no body axis. **(B)** Phenotype analysis of zebrafish embryos after microinjection with *GRHL3* mRNA ranging from 100 to 300 pg. The abnormal rates of overexpressed groups were significantly higher than control group. With an increase of the mRNA, the malformation rate showed a rising tendency. **(C)** Phenotype analysis of MO experiment. Distributions of these four grades of the different dose groups at 24 hpf and 48 hpf were analyzed separately.
$^{*}p < 0.05$ by χ^2 test.
MO: Morpholino.

axis. These findings suggest that the hypomethylation of *GRHL3* gene may cause abnormal closure of neural tube by secondary to overexpression *GRHL3* mRNA in humans.

Transcription start site (TSS) controls the levels of gene expression [29]. Extensive previous work on DNA methylation focused on the CpG island at TSS, and showed that aberrant methylation in TSS plays an important role in regulation of gene expression [30]. Additionally, CpG islands within the gene body, defined as the entire gene from the TSS to the end of the transcript, were also important for the gene's function [31], and several studies reported the abnormal methylated CpG islands within gene body associated with NTDs [16,32]. In the *GRHL3* gene, the CpG island is located in the gene body. To investigate the methylation level of *GRHL3* gene, we not only detected the CpG sites that showed significant hypomethylation within TSS1500 region and gene body identified by microarray, but also detected the CpG island within *GRHL3* gene body. We found that the CpG sites in both TSS region and gene body in *GRHL3* gene showed significantly decreased methylation levels in NTD cases, implying that hypomethylation of *GRHL3* gene may be a risk factor for NTDs.

CpG methylation is thought to be dynamic and tissue specific [29], so it is critically important to detect the methylation levels of DNA from pathological central nervous tissue, rather than lymphocyte, as is commonly done in the study of human NTDs. Prior to our study, only one genome-wide microarray analysis was performed to detect the aberrant methylation in NTD cases. However, DNA from blood lymphocytes was used for methylation profiling [16,32], which may have different methylation profiles compared with the more biologically relevant central nervous tissue. Our team performed genome-wide methylation microarray analysis using DNA isolated form the cells harvested close to the NTD lesion in affected fetuses, and from brain/spinal cord tissue from nonmalformed controls [10]. In the present study, the methylation experiments were all performed using specific lesion site neural tissue, which provided direct evidence of methylation changes of the target tissue/organ. The limitation of our study was that we did not investigate the expression of *GRHL3* gene in NTD cases and controls because the RNA extracted from the neural tissues failed to pass the quality control to enable us to successfully perform RNAseq studies. However, in our study, we used the zebrafish model to perform the functional analyses of the *GRHL3* gene, which provided us with unique insight on the association of *GRHL3* mRNA expression with the development of NTDs. In addition, the view that the methylation status assayed in this study reflects to the real status when NTD happens is challenging, for methylation is an ever-changing process. Given that the nearly impossible goal of obtaining the neural tissue when neural tube is closing, which happens during 21st to 28th day postconception, the continuum of neural tube development at about 5 months of gestation makes the current study the best approximation possible.

Conclusion & future perspective

In conclusion, we are able for the first time to provide evidence that the *GRHL3* gene hypomethylation in central nervous tissue is associated with the failure of neural tube closure. Our findings also provide novel insight into the mechanisms of PCP pathway genes underlying the development of NTDs. Further studies are warranted to examine how abnormal methylation of *GRHL3* gene interrupts neural tube development, and investigate whether the aberrant methylation of other PCP pathway genes have causative relationships with the etiology of NTDs.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.futuremedicine.com/doi/suppl/10.2217/epi-2018-0016

Financial & competing interests disclosure

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Ethical conduct of research

This study was approved by the institutional review board of Peking University and the mothers provided written informed consent.

Summary points

- Although the importance of planar cell polarity (PCP) pathway genes to the occurrence of neural tube defects (NTDs) has been previously reported, very few studies have investigated whether aberrant methylation of PCP pathway genes could lead to NTDs. Our study showed that hypomethylation of *GRHL3* gene, which is a PCP pathway gene, was positively associated with an increased risk for NTDs.
- Most of the previous research on the association between DNA methylation and NTD risks used samples from blood lymphocytes. Considering that DNA methylation is dynamic and tissue specific, for the present study, the methylation experiments were all performed using specific lesion site neural tissue, which provided direct evidence on methylation changes of the target organ.
- We screened five hypomethylated CpGs of GRHL3 gene in NTD cases by using genome-wide DNA methylation analysis, and we detected 15 hypomethylated CpGs in NTD cases in the validation phase by using targeted methylation analysis.
- We found that the CpG sites in both transcription start site and gene body in *GRHL3* gene showed significantly decreased methylation levels in NTD cases.
- The subgroup analysis suggests that the hypomethylation of *GRHL3* gene was present in both spina bifida and anencephaly subtypes, the two major subtypes of human NTDs.
- We demonstrated that the overexpression of *GRHL3* mRNA could lead to disturbance of convergent extension and appeared different degrees of NTD-related phenotypes in zebrafish, including bending and curling of the body axis.

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