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Research Report

Impaired methylation modifications of *FZD3* alter chromatin accessibility and are involved in congenital hydrocephalus pathogenesis

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ABSTRACT

Congenital hydrocephalus is heterogeneous in its etiology, and in addition to a genetic component, has been shown to be caused by environmental factors. Until now, however, no methylation alterations of target genes have been connected with congenital hydrocephalus in humans. Frizzled 3(*FZD3*) is a planar cell polarity (PCP) gene required for PCP signaling. Partial restoration of frizzled 3 activities in *FZD3* mutant mice results in hydrocephalus. To analyze the possible roles of epigenetic modifications of the *FZD3* gene in congenital hydrocephalus pathogenesis, DNA methylation in the promoter region of *FZD3* was assayed using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Gene expression and chromatin accessibility were also determined to assess the role of methylation alterations. Our study found methylation levels of the *FZD3* gene were increased in congenital hydrocephalus, especially in males (10.57 ± 3.90 vs. 7.08 ± 0.94, $p=0.001$). Hypermethylation of *FZD3* increased congenital hydrocephalus risk, with an odds ratio of 10.125 ($p=0.003$). Aberrant methylation modification of *FZD3* altered both chromatin structure in this region and *FZD3* expression levels. Totally, aberrant methylation modification of the *FZD3* gene increases the risk of congenital hydrocephalus by altering chromatin structure and disturbing gene expression.

1. Introduction

Hydrocephalus is a medical condition caused by excessive accumulation of cerebrospinal fluid within brain ventricles, resulting in ventricular dilatation and damage to the surrounding brain parenchyma. Almost 50% of hydrocephalus cases are congenital and usually associated with adverse neurological outcomes (Schrander-Stumpel and Fryns, 1998).

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Congenital hydrocephalus (CH) may occur alone (non-syndromic) or as part of a syndrome with other anomalies (syndromic) (Partington, 2001; Teebi and Naguib, 1988). The reported incidence of CH ranges between 0.4 and 3.16 per 1000 live births (Bajpai, 1997; Fernell et al., 1986; Persson et al., 2005).

CH is a heterogeneous disorder with no well-established risk factors (Van Landingham et al., 2009). Although genetics is believed to play an important role in the etiology of non-syndromic CH (Ibanez-Tallon et al., 2004; Stoll et al., 1992; Vogel et al., 2012), only about 40% of hydrocephalus cases have a possible genetic etiology (Haverkamp et al., 1999; Kenwrick et al., 1996; Maruta et al., 1996; Okamoto et al., 1996; Zhang et al., 2006). In syndromic cases, it is hard to define defective genes because of the association with other anomalies (Vogel et al., 2012). To date, mutations in only one human gene (*L1CAM*) have been definitively linked to development of CH, yet these cases only account for 5% to 15% of likely hereditary cases (Izumoto et al., 1996; Jouet and Kenwrick, 1995; Kenwrick et al., 1996). A wide variety of environmental factors have been shown to cause hydrocephalus in animal models, including alcohol consumption, x-ray radiation, infections, nutritional abnormalities and chemical exposure during gestation (Adeloye and Warkany, 1976; Aolad et al., 1998; Bearer, 2001; Ramanathan et al., 1996). Moreover, a number of therapeutic drugs, multiparous pregnancies and prenatal care in the first trimester can cause an increased risk of CH (Gonzalez et al., 1998; Kazy et al., 2005; Orioli and Castilla, 2000; Van Landingham et al., 2009).

Recently, research has found that folate metabolism plays a part in CH etiology (Cains et al., 2009a), and folate supplementation during pregnancy decreases the risk of CH (Cains et al., 2009b), although the underlying mechanism is unclear. In cells, folate acid is a methyl-donor, and interrupts methylation modification of DNA and protein, and is also involved in abnormal neurogenesis pathologies, such as neural tube defects (Li Wang et al., 2010; Tran et al., Zhang). Until now, no research has reported methylation abnormalities to be involved in CH pathogenesis.

FZD3 is a member of the frizzled gene family, and encodes 7-transmembrane domain proteins that are receptors for the Wingless family of proteins (Kirikoshi et al., 2000). *Fzd3*−/− mice, with targeted deletion of the mouse *Fzd3* gene, show severe defects in several major axon tracts within CNS forebrain regions (Wang et al., 2006a, 2006b). *FZD3* gene dysfunction due to aberrant methylation may be involved in CH etiology. To understand the role of methylation modifications of the *FZD3* gene in CH, we measured and compared the methylation level of the *FZD3* gene in CH cases and controls. Chromatin accessibility is altered by methylation modifications, and therefore was also assessed, to understand the role of methylation modifications in target genes.

2. Results

2.1. *Hypermethylation modification of FZD3 in congenital hydrocephalus*

Methylation levels of *FZD3* in nervous tissue were analyzed in both CH cases and controls. In total, five primer pairs were designed to amplify a genomic DNA region extending from −1443 to −436 bp before the transcription start site (TSS) of the *FZD3* gene, in 38 CH and 34 controls. Using bisulfite conversion, 14 CpG units digested from 17 CpG sites were analyzed using the MassARRAY Compact system. Mean methylation levels were calculated from the average level of all 14 CpG units, and compared between CH and controls using Student's *t*-test. Mean methylation levels of the *FZD3* gene in CH were significantly higher than in controls (10.73±5.69% versus 7.17±1.04%, $p<0.001$, Fig. 2). Notably, the differences in methylation levels between syndromic or non-syndromic CH and controls, were both significant (10.64±2.73% or 10.78±6.80% versus 7.17±1.04%, $p=0.014$ or 0.002, respectively). This prompted us to compare methylation levels between the two CH subgroups (syndromic versus non-syndromic CH), however no significant difference was detected ($p=0.92$).

Methylation levels of every CpG unit in the region were also evaluated. In controls, methylation levels varied at the different CpG sites (Fig. 2), being relatively high in regions distant from the TSS site (before −900 bp), and decreasing (to <10%) closer to the TSS site (8th–14th CpG unit). However, regardless of the methylation modification level, methylation differences between CH and controls were significant in all (except the 12th and 14th) CpG units located in close proximity to the TSS site.

Methylation levels were also compared according to gender. Neither mean levels nor methylation levels of individual CpG units of *FZD3*, differed statistically in males or females (Table 3). However, it is interesting to note that aberrant methylation of *FZD3* was mainly present in males, regardless of the diagnosis of either syndromic or non-syndromic CH. Methylation alterations in females were not significant, although there was a trend towards increased methylation levels in females with CH.

Altogether, compared with control samples, methylation modification of the *FZD3* gene was impaired in both syndromic and non-syndromic CH, and especially in male CH patients.

2.2. *Increment of risk for developing congenital hydrocephalus*

In an attempt to develop a model for assessing the risk of developing CH based on *FZD3* methylation levels, we categorized CH according to the methylation level quartiles found in controls. Based on these methylation levels, 71.1% of CH patients were grouped into the highest quartile (methylation level ≥8.16%). Only three samples (7.9%) were grouped into the lowest quartile (methylation level ≤6.43%). High methylation levels of the *FZD3* gene increased the risk of CH by approximately 10-fold (OR, 10.125; 95% CI, 2.200–46.589) compared with low methylation levels (Table 4). Because aberrant methylation mainly existed in male cases, we adjusted the OR for gender and found the risk of CH associated with high methylation levels was still significant (AOR, 10.681; 95% CI, 2.272–50.220).

2.3. *Hypermethylation alters the chromatin structure of FZD3*

Previous studies have suggested that alterations in the chromatin structure of genes may be connected to gene silencing caused by hypermethylation modifications. To examine this possibility,