Clinical research

Loss-of-function variation in the DPP6 gene is associated with autosomal dominant microcephaly and mental retardation

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The molecular basis of autosomal dominant microcephaly, a disorder associated with small head circumferences that results in variable mental retardation, is largely unknown. In the present study, we conducted a variation analysis of the DPP6 gene in patients with autosomal dominant microcephaly and variable mental retardation. The copy number variation analysis of DPP6 was performed on DNA samples from 22 patients with microcephaly using high-resolution, array-based genomic hybridization, and sequence analysis was performed to screen mutations in another 50 microcephalic patients. Two de novo deletions and one missense mutation in familial microcephalic patients were identified. The transfection of plasmids encoding green fluorescent protein-pLLU2G-shDPP6 fusion proteins in mouse brains revealed that the decreased expression of the DPP6 gene slightly reduced the weight of the mouse brains and resulted in mouse learning disabilities compared with their wild-type littermates. Our data indicate that the loss-of-function variations in DPP6 are associated with autosomal dominant microcephaly and mental retardation. DPP6 appears to play a major role in the regulation of proliferation and migration of neurons in neurogenesis, most likely by participating in neuronal electrical excitability, synaptic integration, and plasticity.

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1. Introduction

Microcephaly is a rare condition characterized by a small occipitofrontal head circumference (OFC) that is 2 or more standard deviations below the mean (−2 SD) according to age and gender [1]. It has an approximate incidence rate of 0.1–2% in the general population [2]. Microcephaly can present in syndromic and non-syndromic forms, often accompanied by variable degrees of mental retardation. The severity of mental retardation has been related to the degree of microcephaly and the abnormalities in the brain anatomy [3]. Isolated microcephaly has been defined by giving the following clinical features: uncomplicated by other abnormalities; present at birth; associated with normal pregnancies, deliveries, and postnatal periods; associated with or without early psychomotor retardation; and non-progressive. The brain has a normal architecture except for being small [4]. The genetic factors that can cause isolated microcephaly are mostly Mendelian autosomal dominant, recessive, or X-linked genes, and some cases are caused by other rare chromosomal aberrations [5]. Individuals with autosomal dominant microcephaly exhibit some differences from those with autosomal recessive inheritance: Firstly, the stature of the patients with autosomal dominant microcephaly can be in the normal range; Secondly, patients with autosomal dominant microcephaly are generally categorized as being moderately to mildly or borderline retarded, and many of them are capable of leading relatively normal lives [6]. Moreover, investigators have identified families that have autosomal dominant microcephaly and short stature with normal intelligence [7,8]. The molecular basis of autosomal dominant microcephaly is largely unknown. Dipeptidyl-peptidase-like protein 6 (DPP6), a critical component
protein in voltage-gated potassium (Kv) transmembrane channels in neurons, is located on chromosome 7q36.2 and is thought to play distinct roles in brain development. The DPP6 protein is an integral membrane glycoprotein with a short cytoplasmic N-terminal domain, one transmembrane domain, and a long extracellular C-terminal domain. The function of DPP6 is associated with Kv4 channels, facilitating the surface expression and modifying the kinetic and steady-state properties of the channel by regulating subthreshold-activating A-type K+ currents [9,10]. In mice, the loss of DPP6 has been proven to result in abnormal electrophysiologic neuron characteristics [11,12]. To date, abnormal variations in DPP6 have been associated with amyotrophic lateral sclerosis (ALS) [13] and idiopathic ventricular fibrillation [14]. Collectively, DPP6 plays fundamental roles in neuronal and excitable cell function, and DPP6 dysfunction has been implicated in neurological disorders and diseases.

In the present study, we performed whole genome copy number variation analysis of 22 patients with either sporadic or familial microcephaly and mental retardation, and two de novo genomic deletions harbouring the DPP6 gene were found in 2 patients. These findings prompted us to search for DPP6 mutations in other microcephaly patients and construct a DPP6-knockdown mouse model using siRNA technology to further evaluate the microcephalic phenotype.

2. Materials and methods

2.1. High-resolution array-based comparative genomic hybridization

Peripheral blood samples from all participants were collected after informed consent and study approval by the Guangzhou Women and Children’s Medical Center Institutional Review Board. Genomic DNA was extracted from blood using the Qiagen DNA blood Mini Kit (Qiagen, Germany). To identify candidate loci for microcephaly we first carried out a genome-wide copy number scan in 22 patients associated with microcephaly and mental retardation, and two de novo genomic deletions harbouring the DPP6 gene were found in 2 patients. These findings prompted us to search for DPP6 mutations in other microcephaly patients and construct a DPP6-knockdown mouse model using siRNA technology to further evaluate the microcephalic phenotype.

2.2. DPP6 gene mutational analysis

Genomic DNA was extracted from peripheral blood leukocytes from the parents of the above two individuals (Case BY0712 and Case BY2018), 50 other random microcephalic patients and 50 normal control subjects. All 26 exons of the DPP6 gene were amplified by means of the polymerase chain reaction (PCR) with the use of DPP6-exon primer, respectively. Sequences of the primers used to amplify the exons of DPP6 are available on request. Direct cycle sequencing of the PCR products was performed with the use of the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) and analyzed with the use of the ABI Prism 310 Genetic Analyzer (Applied Biosystems).

2.3. Construction of plasmids

Full-length human shRNA oligo (shDPP6) were synthesized and annealed into double-strand. Vector pLLU2G (515 ng/µl) was digested by restriction enzymes HpaI (D1064A, TAKARA) and XhoI (D1094A, TAKARA). shDPP6 and Vector pLLU2G were ligated to generate pLLU2G-shDPP6(D2011A, TAKARA). The resulting plasmid encodes a fusion protein eGFP-pLLU2G-shDPP6 (eGFP fused to the upstream of hUbC promoter). DPP6-knockdown constructs pLLU2G-shDPP6-1, pLLU2G-shDPP6-2, pLLU2G-shDPP6-3 and pLLU2G-shDPP6-4 were generated from the wild-type construct by PCR reaction. After all the clones were sequence-verified (Invitrogen), the plasmid DNAs were prepared for microjection (Cyagen).

2.4. Immunohistochemistry analysis on the DPP6-knockdown mice brain tissue

After the hemi brain frozen sections were prepared, block endogenous peroxide blocking 3% H2O2 RT 10 min, washed with 0.01 M PBS (pH 7.2–7.4) for 2 min × 3 gentle shaking and block section with 5% BSA at room temperature for 15 min with gentle shaking, then incubate sections in primary antibody (DPP6, 1:50, abcam) at 4 °C overnight, and then washed with 0.01 M PBS (pH 7.2–7.4) for 2 min × 3 gentle shaking. Incubate sections in PV-6001

Fig. 1. The diagrammatic presentation of array-CGH detection in microcephalic patients of Case BY0712 and Case BY2018. The array result displays the scatterplot of genomic DNA copy number. The log 2 ratio of normal two copies is corresponding to base line “0” on the scatterplot, the log 2 ratio less than 0 indicates deletion (both cases). The deletion with 336 kb size in Case BY0712 maps to chromosome position 153,649,777–153,985,995 and deletion with 362 kb size in Case BY2018 maps to position: 153,829,386 on chromosome 7q36.2 region (red arrow), within which harbouring DPP6 gene.
for 30 min in RT, wash with 0.01 M PBS (pH 7.2–7.4) for 2 min ×3 gentle shaking. Prepare 3,3′-diaminobenzidine tetrahydrochloride (DAB) (Wuhan Boster Biological Technology Laboratory) following manufacturer’s protocol. Incubate sections in DAB mixture to when a brownish colour developed on each section, then washed with distilled water. After serial sections were stained with Haematoxylon–Eosin (HE), sections were dehydrated in successive baths of 75%, 85% and 95% alcohol (20 s each), respectively, further in bath of absolute alcohol for 5 min andylene for 10 min, a distilled water bath for 5 min, and then prepared to visualize under the light microscopy.

3. Results

3.1. High-resolution array-based comparative genomic hybridization

The study conformed to the guidelines of the institutional review board, and all tests were conducted as part of the diagnostic procedures approved by the institutional review board. Informed consent was obtained from all subjects and their guardians. To identify candidate loci for microcephaly we first carried out a genome-wide copy number scan in 22 patients associated with microcephalic phenotypes using Affymetrix GeneChip Cytoscan HD Array containing 2,696,550 markers for copy number analysis. The array results revealed two small deletions with sizes of 336 kb [mapping position: 153,649,777–153,985,995 (GRCh37/hg19)] and 362 kb (mapping position: 153,829,386–154,191,684) mapping to chromosome 7q36.2 in two patients (Case BY0712 and Case BY2018, respectively). Both deletions were de novo and harboured the DPP6 gene only (Fig. 1). We further performed quantitative real-time polymerase chain reaction (RT-PCR) to confirm the deletions (see Supplemental Fig. 1).

3.2. Mutational analysis

To examine the relationship between microcephaly and the DPP6 gene, mutation screening of the DPP6 gene was performed on the parents of the above two patients (Case BY0712 and Case BY2018) and other 50 random microcephalic patients by DNA sequencing. All 26 exons of the DPP6 gene were amplified by PCR, and the products were sequenced. A heterozygous missense mutation, c.1153A>C, was identified in exon 11 in patient BY2950 (Fig. 2A) and resulted in the amino acid change p.Met385Leu. The family study of patient BY2950 was further expanded, and the DPP6 mutation screen was performed on all family members. The same mutation was identified in all four other affected individuals through three generations in the family of patient BY2950 (Fig. 2B), and no mutation was found in the unaffected family members. We performed the DPP6 mutation screen on 100 unrelated healthy controls, but no identical mutations were detected. To exclude the possibility of adjacent gene effects caused by HPE3 and SHH dysfunction, which are thought to be responsible for holoprosencephaly, we also performed DNA sequencing analysis of the HPE3 and SHH genes in all of the above DPP6-variant patients, and no mutations were identified. In addition, all the patients with DPP6-variant had a brain magnetic resonance (MRI) scanning showed no signs of holoprosencephaly. The clinical manifestations of the DPP6-variant patients in this study are summarized in Table 1.

3.3. Construction and evaluation of a mouse model

To further investigate DPP6 as a causative gene in microcephaly, we constructed DPP6-knockdown mice using siRNA technology to induce acute knockdown expression of the DPP6 gene in cooperation with Cyagen Biosciences Inc. Since there are several alternative transcripts for the DPP6 gene, here we chose to knockdown the transcript 1 of DPP6 (NM_130797), because that transcript had been widely studied for neuronal K+ channels. Specifically, we used the mammalian expression vector pLLU2G,
Table 1
Summary phenotypes of patients with loss-of-function variation in the DPP6 gene.

<table>
<thead>
<tr>
<th>Patient ID</th>
<th>Sex</th>
<th>Age (year)</th>
<th>OFC</th>
<th>Clinical manifestations</th>
</tr>
</thead>
<tbody>
<tr>
<td>BY0712</td>
<td>M</td>
<td>12</td>
<td>47 cm (&lt;−3SD)</td>
<td>Small stature (110 cm, &lt;−3SD), low body weight (29 kg, &lt;−3SD), microcephaly and moderate mental retardation. He could not concentrate his work. He could only speak single words with ambiguous pronunciation. Skeletal radiographs showed delayed bone age.</td>
</tr>
<tr>
<td>BY2018</td>
<td>F</td>
<td>15</td>
<td>48 cm (&lt;−3SD)</td>
<td>Small stature (130 cm, &lt;−3SD), low body weight (30.5 kg, &lt;−3SD), microcephaly and moderate mental retardation. She can only sing a single word with an ambiguous pronunciation. She also had scoliosis and congenital ambylophia resulting from choriotelial degeneration.</td>
</tr>
<tr>
<td>BY2950</td>
<td>F</td>
<td>5</td>
<td>44 cm (&lt;−3SD)</td>
<td>Small stature (93 cm, &lt;−3SD), low body weight (13 kg, &lt;−3SD), microcephaly and severe mental retardation. She could not able to recognize colours and numbers. She had psychomotor delay which equal to 2-year level and her activities were hyperactive.</td>
</tr>
<tr>
<td>Casell-2</td>
<td>F</td>
<td>29</td>
<td>50 cm (&lt;−3SD)</td>
<td>Small stature (156 cm, &lt;−2SD), low body weight (44 kg, &lt;−3SD), mild mental retardation. There was no sign of dominant phenotypic abnormality except for microcephaly and mild mental retardation (IQ measured with the Wechsler Adult Intelligence Scale was 65).</td>
</tr>
<tr>
<td>Casell-3</td>
<td>F</td>
<td>26</td>
<td>50 cm (&lt;−3SD)</td>
<td>Normal stature (162 cm), low body weight (45 kg, &lt;−3SD), severe mental retardation. She suffered from severe cognitive deficiency and memorial problems. She couldn’t comprehend a complete sentence, such as instructions on how to take her medicine. She was not able to remember the events that happened 3 or 4 years ago, including her childhood anecdotes and the year of her wedding (2006). In the daily life, she was afraid to get far away from the house because she was not able to find her way back home.</td>
</tr>
<tr>
<td>Casel-1</td>
<td>M</td>
<td>56</td>
<td>51 cm (&lt;−2SD)</td>
<td>Small stature (163 cm, &lt;−2SD), low body weight (49 kg, &lt;−3SD), borderline mental retardation. There was no sign of dominant phenotypic abnormality except for microcephaly and mild mental retardation (IQ measured with the Wechsler Adult Intelligence Scale was 66). He had a very poor performance in math.</td>
</tr>
</tbody>
</table>

Sex: M = male; F = female; OFC = occipitofrontal head circumference; SD = standard deviation.

which contained the hUbC promoter inserted upstream of eGFP (see Supplementary Fig. 2). The detailed processing data associated with the generation of the transgenic mice are available on request. Of the DPP6-knockdown mice generated, twelve ICR mouse strains heterozygous for DPP6-knockdown were positively identified. All protocols involving mice were approved by the local Experimental Animals Ethics Committee. The learning and memory capacity of the mice was evaluated using the standard Morris water maze test at 16 weeks of age before further analysis was conducted. The Morris test revealed obviously marginalized motion trajectories indicating brain spatial memory defects and learning disabilities in the DPP6-knockdown mouse compared with their wild-type littermates (see Supplementary Fig. 3). The mice were sacrificed at their 17+ weeks old, their brains were dissected out and weighed. ANOVA was used to compare the brain weights between the DPP6-knockdown and wild-type mice, and SPSS Version 17.0 was used to analyze the data. The average brain weight for heterozygous DPP6-knockdown mice was 0.4341 g, compared with 0.5306 g for the knockdown mice was 0.4341 g, compared with 0.5306 g for the wild-type littermates (*P* < 0.01, see Supplemental Table 1). The immunohistochemistry analysis further revealed that DPP6 protein expression was significantly decreased in the DPP6-knockdown mouse brains compared with the brains of their wild-type littermates (*P* < 0.01, Fig. 3).

4. Discussion

Abnormalities of chromosome 7q36 were previously reported to be associated with microcephaly in several patients [15–17]. However, the precise genotype-phenotype relationship was not determined in those cases, as the authors simply classified their patients’ microcephaly in the phenotypic spectrum of holoprosencephaly (MIM 600725), which results from HPE3 and SHH gene dysfunction. In the present study, this unclear relationship between holoprosencephaly and isolated microcephaly has been clarified by performing sequencing analysis of both the HPE3 and SHH genes. Moreover, brain MRI scanning in our present patients showed no signs of holoprosencephaly, and the learning disabilities and brain weight decreases in the DPP6-knockdown mice resemble the phenotype of microcephaly and mental retardation observed in our patients. Therefore, our data indicate that loss-of-function variations in the DPP6 gene appear to cause autosomal dominant microcephaly.

The function of the DPP6 gene has not yet been fully elucidated. Nadal et al. reported that the DPP6 protein was an important component of neuronal K⁺ channels [18]. Voltage-gated potassium (Kv) channels play important roles in regulating the excitability and plasticity of neurons and other excitable cells by regulating subthreshold-activating A-type K⁺ currents [19,20]. In neuronal dendrites, DPP6 establishes the A-current gradient, and the loss of DPP6 can lead to a decrease in the A-type current, resulting in hyperexcitability of dendrites by enhancing dendritic AP back-propagation, calcium electrogenesis, and the induction of long-term synaptic potentiation [12]. Therefore, neuronal and muscle hyperexcitability caused by DPP6 dysfunction appears to be the pathophysiological cause of the epileptic seizures and hyperactive behaviours observed in our paediatric patients. As a corollary, the DPP6 protein and its associated components have the potential to be new therapeutic targets in epilepsy and other neurological disorders in such patients. A previous study proved how channel activity influences neuronal migration in the mammalian nervous system [21], and changes in voltage-dependent potassium channels have been shown to be associated with the differentiation and maturation of neurons in Xenopus embryos [22]. In addition, DPP6 has been reported to have an important potential role in embryonic development [23], with the disruption of DPP6 causing the RwyRw [the mouse rump white (Rw) mutation] lethality and pigmentation defects in heterozygotes [24]. Therefore, DPP6 dysfunction can disturb the voltage-dependent ion channel properties and lead to a decrease in the proliferation rate of neurons. The end result causes a smaller-than-normal brain and microcephaly. Moreover, neuronal synapses have been shown to play important roles in learning and memory [25]. Mental retardation in our patients and learning disabilities in DPP6-knockdown mice may therefore be explained by abnormalities in DPP6 that disturb the regulation of neuronal synapse integration and plasticity. However, the mechanism underlying the above events still needs to be further elucidated.

Autosomal dominant microcephaly represents a group of phenotypically and genetically heterogeneous conditions, and decreased penetrance and marked variability in expression are
also common features in humans. The appearance of affected individuals in the family of our patient BY2950 was in accordance with an autosomal dominant inheritance pattern. The possible mechanism of the familial transmission of pathogenic mutation c.1153A > C may be the conservation of the nucleotide, and then resulted in familial segregation with the phenotypic traits. A small head circumference, moderately to mildly retarded intelligence and the lack of a distinctive facial appearance in the affected individuals were consistent with the characteristics of previously studied autosomal dominant microcephalic patients [7,26]. In particular, the manifestation of congenital amblyopia resulting from choroidal degeneration in one of our patients (patient BY2018) is remarkably similar to the manifestation of congenital amblyopia in the patients investigated by Simonell et al. [27]. In addition, the variation in the severity of microcephaly and mental retardation observed in our patients may be explained by the incomplete penetrance of the DPP6 gene. This variable expression of features has also been observed in cases of amyotrophic lateral sclerosis associated with DPP6 gene in European populations [13], but cases of amyotrophic lateral sclerosis in other populations, such as Italians and Chinese, have not been associated with alterations in the DPP6 gene [28,29].

Therefore, we conclude that the loss-of-function variations we identified in the DPP6 gene may cause the autosomal dominant microcephaly in humans. Our data indicate that DPP6 plays a major role in the regulation of proliferation and migration of neurons in neurogenesis, most likely by participating in neuronal electrical excitability, synaptic integration, and plasticity. However, to fully elucidate the mechanisms underlying such events, further intensive studies with larger patient groups must be conducted.

Competing financial interests

The authors declare no competing financial interests.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmg.2013.06.008.

References


