

Association between chromosomal aberration of *COX8C* and tethered spinal cord syndrome: array-based comparative genomic hybridization analysis

Qiu-jiong Zhao^{1, #}, Shao-cong Bai^{1, #}, Cheng Cheng¹, Ben-zhang Tao¹, Le-kai Wang¹, Shuang Liang¹, Ling Yin³, Xing-yi Hang^{2, *}, Ai-jia Shang^{1, *}

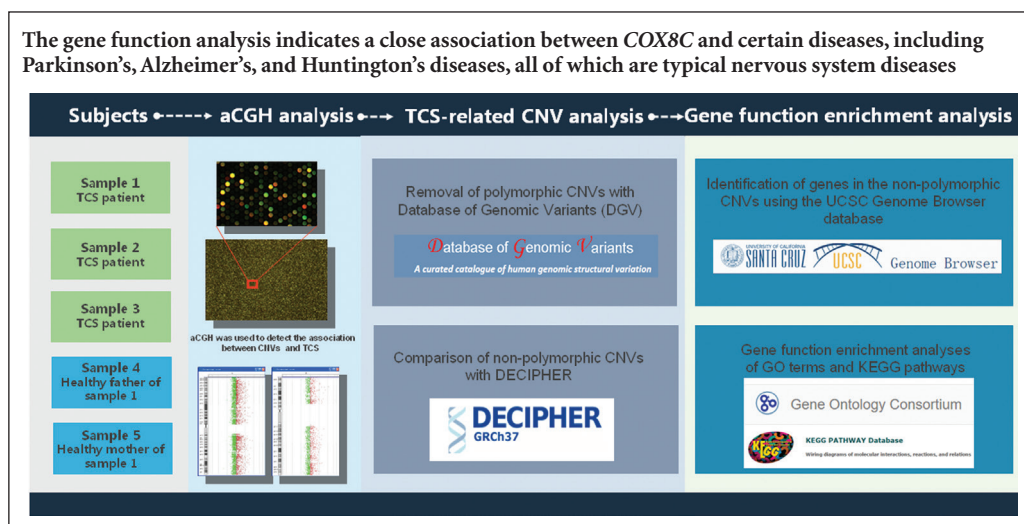
1 Department of Neurosurgery, Chinese PLA General Hospital, Beijing, China

2 iGeneTech Biotechnology Co., Ltd., Beijing, China

3 Department of Neurology, Chinese PLA General Hospital, Beijing, China

How to cite this article: Zhao QJ, Bai SC, Cheng C, Tao BZ, Wang LK, Liang S, Yin L, Hang XY, Shang AJ (2016) Association between chromosomal aberration of *COX8C* and tethered spinal cord syndrome: array-based comparative genomic hybridization analysis. *Neural Regen Res* 11(8):1333-1338.

Graphical Abstract



*Correspondence to:
Ai-jia Shang, M.D., Ph.D. or
Xing-yi Hang, Ph.D.,
shangaj@163.com or
xingyi.hang@igenetech.com.

#These authors contributed
equally to this study.

orcid:
0000-0002-4895-5442
(Ai-jia Shang)
0000-0002-3736-2203
(Xing-yi Hang)

doi: 10.4103/1673-5374.189200

Accepted: 2016-08-09

Abstract

Copy number variations have been found in patients with neural tube abnormalities. In this study, we performed genome-wide screening using high-resolution array-based comparative genomic hybridization in three children with tethered spinal cord syndrome and two healthy parents. Of eight copy number variations, four were non-polymorphic. These non-polymorphic copy number variations were associated with Angelman and Prader-Willi syndromes, and microcephaly. Gene function enrichment analysis revealed that *COX8C*, a gene associated with metabolic disorders of the nervous system, was located in the copy number variation region of Patient 1. Our results indicate that array-based comparative genomic hybridization can be used to diagnose tethered spinal cord syndrome. Our results may help determine the pathogenesis of tethered spinal cord syndrome and prevent occurrence of this disease.

Key Words: nerve regeneration; neural tube defects; tethered spinal cord syndrome; comparative genomic hybridization; *COX8C*; gene function enrichment analysis; database of genomic variants; database of DECIPHER; copy number variations; neural regeneration

Introduction

Tethered spinal cord syndrome (TCS) is a neurodevelopmental disorder that results in spinal cord malformation (Payne, 2007; Cearns et al., 2016). TCS is classified as a neural tube defect, and although the incidence of neural tube defects is approximately 1% worldwide (Feuchtbaum et al., 1999; Tunçbilek et al., 1999; van der Put et al., 2001; Khoshnood et al., 2015; Atta et al., 2016), infants born with neural tube defects account for 20–25% of all congenital malformations (Laharwal et al., 2016). The causes of neural tube

defects are multivariate, yet to date there is no convincing mechanistic evidence for their occurrence. Some possible contributing factors include gene mutations, chromosomal abnormalities, and environmental factors (Bassuk and Kibar, 2009; Joó, 2009a, b; Molloy et al., 2009; Wen et al., 2009). Recent studies have revealed novel risk factors for neural tube defects including heterozygous missense mutations in the genes, *VANGL1* and *FUZZY* (Bartsch et al., 2012; Seo et al., 2015), as well as maternal folic acid deficiency (Bartsch et al., 2012; Seo et al., 2015). Altered methylation of *MGMT*, a

DNA repair gene, is also associated with neural tube defects (Tran et al., 2012). Moreover, abnormal expression of genes coding for zinc finger proteins is reported to be risk factors (Grinberg and Millen, 2005; Costa-Lima et al., 2008).

Previous studies have shown that chromosomal imbalances due to genomic instability are closely associated with neural developmental disorders (Au et al., 2010; Zhao et al., 2013). Copy number variations (CNVs) are found in patients with neural tube abnormalities in cerebral and spinal sections (Bassuk et al., 2013; Chen et al., 2013). Array-based comparative genomic hybridization (aCGH) is a modern technique for molecular karyotype analysis that combines conventional comparative genomic hybridization and microarray analysis (Saber et al., 2014). In contrast to conventional hybridization, aCGH does not detect metaphase chromosomes. Instead, it targets genomic DNA to perform high-throughput screening of the whole genome for CNVs (Vissers et al., 2003). The aCGH approach can accurately locate CNVs on chromosomes, and clearly calculate CNV length and identify genes within variant fragments (Mosse et al., 2005). Nowadays, aCGH is commonly used for cancer and genetic disorder research (Kallioniemi, 2008; Sireteanu et al., 2012). In this study, we used aCGH to detect CNVs in three children with TCS and two healthy parents. In order to examine TCS pathogenesis at the chromosome and gene levels, we determined the relationship between these chromosomal aberrations and TCS, and consequently detected CNVs linked with occurrence and development of TCS.

Subjects and Methods

Subjects

Three children diagnosed with typical TCS based on clinical criteria (Filippidis et al., 2010) by the Department of Neurosurgery at the Chinese PLA General Hospital and the Second Artillery General Hospital, and the healthy parents of Patient 1 were enrolled in the study. Peripheral blood samples were collected from the patients and healthy controls. Before initiation of the study, written consent was obtained from the guardians of all children. The study (Project ID: S2013-117-01) was approved by the ethics committee of the Chinese PLA General Hospital, China.

Case 1 was a 2-year-old girl with a sacrococcygeal mass and right foot deformity. The sacrococcygeal mass was identified at birth. Physical examination revealed spina bifida. Strophopodia of the right foot and a second enlarging sacrococcygeal mass were first observed at 8 months of age. The patient was diagnosed with TCS with myelomeningocele.

Case 2 was a 12-year-old boy who presented with a lumbosacral mass at the age of 8 months. The patient was diagnosed with TCS with spinal cord lipoma. Surgical treatment was performed. Urinary abnormality occurred 11 years after surgery, along with urinary incontinence, nocturnal enuresis, urinary frequency, and urinary urgency. A further surgery was performed because magnetic resonance imaging showed spinal cord lipoma and recurrence of TCS.

Case 3 was a 5-year-old girl with abnormal hair growth in the lumbosacral region at birth. Physical examination re-

vealed a partial spinal canal defect. Because the hair growth increased, magnetic resonance imaging examination was performed. The results revealed a tethered spinal cord and split cord malformation (Type I). Surgery was performed to correct the malformation.

aCGH analysis

aCGH is a specific array-based genomic hybridization method that uses different fluorescent dyes to label DNA from patients and controls, to identify differences between the two groups (Sealfon and Chu, 2011; Brady and Vermeesch, 2012). By comparing the ratio of two different fluorescence signals at each target spot in the microarray, CNVs are detected in specific sequences or genes between two genomes (Gijssbers et al., 2011; Shoukier et al., 2013).

Total DNA was extracted from peripheral whole blood using a commercially available DNA-isolation kit (BioChain Inc., Beijing, China), according to the manufacturer's protocol. For each aCGH experiment, purified DNA and normal sex-matched DNA (1 µg each; Promega, Madison, WI, USA) were digested with *AluI* and *RsaI* (10 U each; Promega), and differentially labelled with cyanine-5 and cyanine-3 fluorescent dyes using a Genomic DNA Enzymatic Labeling Kit (Agilent, Santa Clara, CA, USA). aCGH analysis was performed using the Agilent 8 × 60K commercial array. This platform contains 60-mer oligonucleotide probes spanning the entire human genome with an overall mean probe spacing of 50 kb. After hybridization, arrays were scanned using a dual-laser scanner (Agilent), and images extracted and analyzed using the Feature Extraction (Agilent) and Workbench genomics software, respectively. Changes in test DNA copy number at specific loci were considered only if they were < -0.38 (deletion) or > 0.38 (amplification) of the log₂ ratio values from at least five consecutive probes.

TCS-related CNV analysis

Removal of polymorphic CNVs using the Database of Genomic Variants

CNV fragments were scanned against the Database of Genomic Variants (Iafate et al., 2004; Wong et al., 2007). CNVs that completely matched those in the database were removed as they represent common polymorphic variants present in the normal population. Partially overlapping (< 40%) CNVs were considered non-polymorphic and retained for further analysis. In addition, discontinuous polymorphic fragments appearing within CNV sequences (total fragment length was shorter than half-lengths of detected CNVs) were not treated as common polymorphisms and were also retained for further analysis.

Comparison of non-polymorphic CNVs with DECIPHER

The non-polymorphic CNV fragments selected above were searched against the DECIPHER database (Firth et al., 2009). Cases were identified with CNVs similar to those reported in previously tested samples (partial overlap > 60%) or containing documented CNVs. Additionally, chromosomal abnormalities, related phenotypes, and syndromes associated with these cases were identified.

Table 1 Array-comparative genome hybridization analysis of TCS patients and controls

ID	Sample	Chromosome	Location	Type	Length (Mb)
1	1	1p21.2	99940520–100180875	R	0.24
2	1	9p13.3	33900470–34310995	R	0.41
3	1	14q32.12	93745414–93938049	R	0.19
4	2	15q11.1q11.2	20481702–22509254	D	2.03
5	3	2p11.2	89276158–90234023	D	0.96
6	3	15q11.1q11.2	20481702–22784582	R	2.30
7	1_F	Arr (1–22) × 2, XY normal male	–	N	0
8	1_M	7q11.22q11.23	72044007–72286210	D	0.24
9	1_M	19p12	20785680–21001208	R	0.22

Samples 1–3 are the three TCS patients. 1_F is the healthy father of Patient 1. 1_M is the healthy mother of Patient 1. Type R: Repeat; Type D: deletion; Type N: normal; TCS: tethered spinal cord syndrome.

Table 2 DECIPHER search results for non-polymorphic copy number variations (CNVs)

Sample	Chromosome locations of CNVs	DECIPHER location	DECIPHER ID
1	chr9:33900470–34310995	chr9:33893072–34050307	251082
		chr9:33919201–34096914	260013
		chr9:34032428–34130165	271046
2	chr14: 93745414–93938049 chr15:20481702–22509254	–	–
		chr15:20487211–22734426	1989
		chr15:20408283–22558756	250510
		chr15:20481702–23179948	262593/262622
		chr15:20603042–23132102	263404
		chr15:20481702–30322138	271693
3	chr15:20481702–22784582	chr15:20487211–22734426	1989
		chr15:20408283–22558756	250510
		chr15:20481702–23179948	262593/262622
		chr15:20603042–23132102	263404
		chr15:20481702–30322138	271693

Table 3 Syndromes and clinical phenotypes linked to non-polymorphic copy number variations

DECIPHER ID	Phenotype	Syndrome
251082	Microcephaly, autism, intellectual disability, prominent fingertip pads, and joint laxity	–
262593	–	Angelman syndrome Prader-Wolli syndrome
262622	–	Angelman syndrome Prader-Wolli syndrome
263404	–	Angelman syndrome Prader-Wolli syndrome

Table 4 Genes contained in non-polymorphic copy number variations

Gene ID	Symbol	Description	Chromosome location	Length (Mb)
55833	UBAP2	Ubiquitin associated protein 2	9:33921691–34048947	0.127256
54926	UBE2R2	Ubiquitin-conjugating enzyme E2R 2	9:33817565–33920402	0.102837
25853	DCAF12	DDB1 and CUL4 associated factor 12	9:34086385–34127397	0.041012
55727	BTBD7	BTB (POZ) domain containing 7	14:93703896–93799438	0.095542
341947	COX8C	Cytochrome c oxidase subunit VIII C	14:93813537–93814702	0.001165
57578	UNC79	Unc-79 homolog (Caenorhabditiselegans)	14:93799565–94174222	0.374657
646096	CHEK2P2	Checkpoint kinase 2 pseudogene 2	15:20487997–20496839	0.008842
283755	HERC2P3	Hect domain and RLD 2 pseudogene 3	15:20587869–20711433	0.123564
606	NBEAP1	Neurobeachinpseudogene 1	15:20862967–20893737	0.03077
440225	NF1P2	Neurofibromin 1 pseudogene 2	15:22133181–22152165	0.018984
283694	OR4N4	Olfactory receptor, family 4, subfamily N, member 4	15:22382382–22383507	0.001125
729786	GOLGA8C	Golgin A8 family, member C	15:20767700–20781019	0.013319
727832	GOLGA6L6	Golgin A6 family-like 6	15:2073709–20747114	0.01002

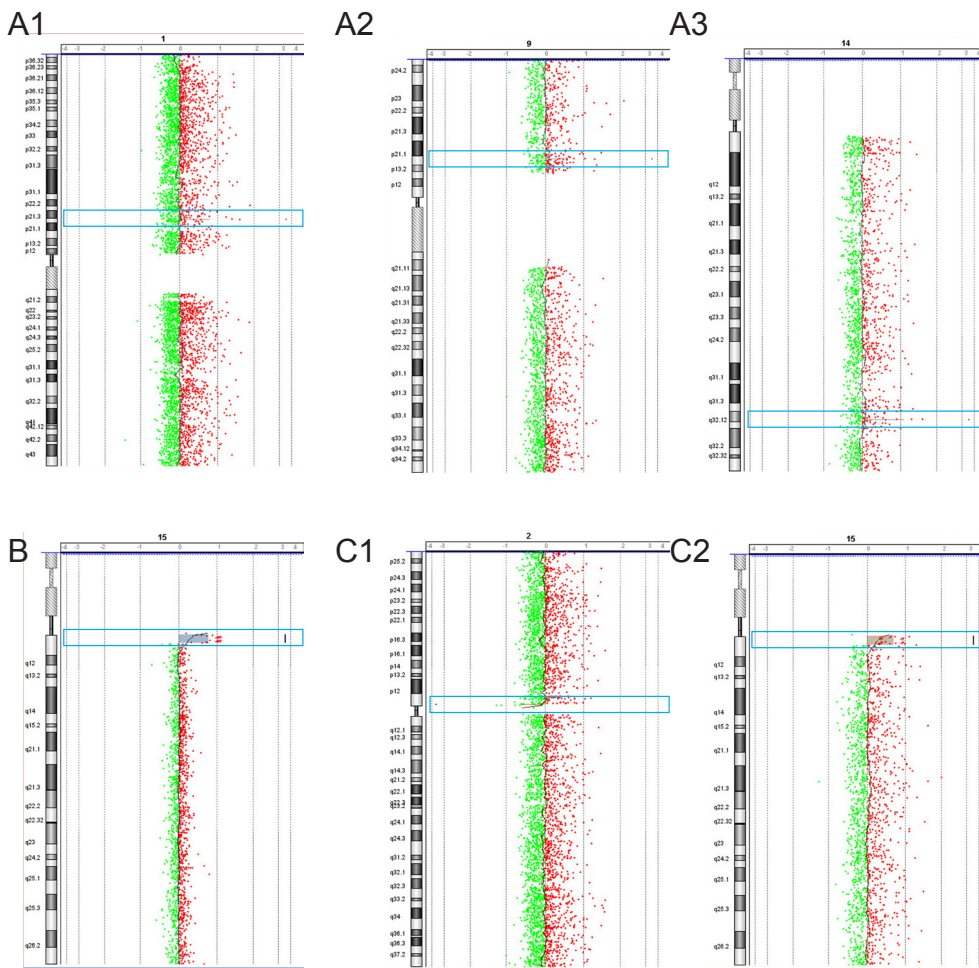


Figure 1 Chromosome maps of the three patients with tethered spinal cord syndrome.
 (A1–A3) The three CNVs identified in Patient 1 were: 1p21.2, 9p13.3, and 14q32.12. (B) The CNV identified in Patient 2 was: 15q11.1q11.2. (C1, C2) The two CNVs identified in Patient 3 were: 2p11.2 and 15q11.1q11.2. Blue squares in the figure indicate the location of chromosome micro-variations. CNVs: Copy number variations.

Table 5 Enrichment results for gene ontology (GO) analysis

GO ID	Function type	Description	P-value	Gene name
GO:0004842	Molecular function	Ubiquitin-protein ligase activity	0.002187	<i>UBE2R2, HERC2P3</i>
GO:0005743	Cellular component	Mitochondrial inner membrane	0.002229	<i>COX8C, HERC2P3</i>
GO:0007276	Biological process	Gamete generation	0.003439	<i>HERC2P3, CXADR</i>
GO:0016021	Cellular component	Integral to membrane	0.027279	<i>COX8C, UNC79, CXADR, OR4N4, GOLGA6L6</i>

Gene function enrichment analysis

Entire genes incorporated in non-polymorphic CNVs were identified using the University of California, Santa Cruz (UCSC) Genome Browser database (<http://genome.ucsc.edu/>). Gene function enrichment analyses were performed for the genes identified, including Gene Ontology (GO) (<http://geneontology.org/>) and Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway (<http://www.genome.jp/kegg/>) analyses.

Enrichment P-values for each GO term or KEGG pathway were calculated using the hyper-geometric distribution method. P-values were then corrected for multiple hypotheses testing using the false discovery rate method. A P-value of 0.05 was set as the threshold value for significant gene enrichment for each GO term or KEGG pathway.

Results

Gene micro-repeat fragment location in TCS patients

Results of the aCGH analysis for all three patients and two par-

ents are shown in **Table 1**. Three micro-repeat fragments were detected in DNA isolated from Patient 1. A micro-deletion fragment was detected in Patient 2, while a micro-deletion and micro-repeat were detected in Patient 3. The father of Patient 1 had a normal karyotype, whereas the mother’s chromosome map showed micro-deletion and micro-repeat fragments. The micro-deletion fragment in Patient 2 and micro-repeat fragment in Patient 3 were located in the same region: 15q11.1q11.2 (**Figure 1**).

Database searching of CNVs

The eight identified CNVs were searched against the Database of Genomic Variants. The results showed that four CNVs were normal chromosomal polymorphisms, specifically, the 1p21.2 micro-repeat in Patient 1, 2p11.2 micro-deletion in Patient 3, and 7q11.22q11.23 micro-deletion and 19p12 micro-repeat in the mother of Patient 1.

Investigation of the other four non-polymorphic CNVs

Table 6 Gene enrichment analysis

KEGG ID	KEGG pathways	P-value	Gene name
5416	Viral myocarditis	0.000828	CXADR
4260	Cardiac muscle contraction	0.001001	COX8C
5012	Parkinson's disease	0.002835	COX8C
190	Oxidative phosphorylation	0.002921	COX8C
4120	Ubiquitin mediated proteolysis	0.003054	UBE2R2
5010	Alzheimer's disease	0.004646	COX8C
5016	Huntington's disease	0.005561	COX8C
4740	Olfactory transduction	0.023898	OR4N4

KEGG: Kyoto Encyclopedia of Genes and Genomes.

in DECIPHER revealed eight specific CNVs in these regions (Table 2). Non-polymorphic CNVs in Patients 2 and 3 (ID 4 and 6 in Table 1) shared the same chromosomal initiation site, indicating that multiple CNVs occur in the same location. Further analyses revealed that these CNVs are associated with two syndromes (Angelman and Prader-Willi) and one phenotype (microcephaly) (Table 3).

Gene function enrichment analysis

Within the four non-polymorphic CNVs regions, 13 genes were identified by the UCSC Genome Browser (Table 4). Function enrichment analysis of GO terms and KEGG pathways were performed for these genes. The results included a number of biological functions (*e.g.*, gamete generation), molecular functions (*e.g.*, ubiquitin-protein ligase activity), two cellular components (mitochondrial inner membrane and integral membrane component), as well as eight KEGG pathways, including viral myocarditis, cardiac muscle contraction, Parkinson's disease, oxidative phosphorylation, ubiquitin-mediated proteolysis, Alzheimer's disease, Huntington's disease, and olfactory transduction. From these results, we found that the *COX8C* gene is closely related to neural system diseases such as Parkinson's disease, Alzheimer's disease, and Huntington's disease (Tables 5, 6).

Discussion

Advantages of using aCGH for detection of rare chromosomal micro-variations

Chromosomal sub-microscopic variations are strongly associated with human disease (Feuk et al., 2006). In clinical settings, the definite diagnosis of several diseases cannot be achieved using existing techniques. Consequently, some rare syndromes are labelled idiopathic or unexplained. Most of these syndromes are due to genomic imbalances created by chromosomal micro-variations such as micro-deletions and micro-repeats (D'Angelo et al., 2014). The aCGH approach efficiently detects chromosomal micro-aberrations and aids elucidation of idiopathic or unexplained diseases.

Significance and limitations of aCGH analysis

The main objective of this study was to identify non-random CNVs and evaluate their association with TCS. The main questions regarding the CNVs we identified are: (1) whether the CNVs are inherited; (2) whether they are found in the

normal population; (3) whether their lengths are sufficient to contain genes with functional annotations; (4) whether they are linked to diseases in DECIPHER; and (5) whether any are unreported, unidentified, or novel. Although the Database of Genomic Variants and DECIPHER, which are globally representative databases, were used to determine the type of CNVs identified, ethnic differences are inevitable when using international databases.

Diseases similar to TCS that are associated with COX8C

CNVs similar to the ones we detected are found in the DECIPHER database. These CNVs are associated with Angelman and Prader-Willi syndromes, and microcephaly. All of these disorders involve significant neural abnormalities (Mabb et al., 2011; Mahmood et al., 2011; Cassidy et al., 2012). Furthermore, gene function analysis indicated a close association between *COX8C* and certain diseases including Parkinson's, Alzheimer's, and Huntington's diseases, all of which are typical nervous system diseases (Bassil and Mollaei, 2012; Pogledić and Relja, 2012; Gazewood et al., 2013). By comparing the CNVs from Patient 1 with those identified in her parents, we excluded the possibility of TCS being hereditary. Thus, we propose that the condition may be acquired during neural development.

Conclusion

In this study, we used high-resolution aCGH to identify pathogenic CNVs in samples from patients with typical TCS. Our findings suggest an association between certain CNVs and nervous system disease. Our data may be used in the future as a reference for the integration of available data, or for further studies with larger sample sizes. Our study demonstrates specific transformation research, and shows that a molecular method can be used to clinically diagnose TCS. Our findings may help to shed new light on the pathogenesis of TCS.

Acknowledgments: We are very grateful to the staffs of iGene-Tech Biotechnology Co., Ltd. in China for some of the experiment operations.

Author contributions: QJZ and SCB performed the experiment. CC, BZT and LKW collected patients, and conducted clinical communication and treatment. SL and LY provided technical and capital supports. QJZ and XYH analyzed and explained data. AJS and XYH served as principle investigators. All authors approved the final version of the paper.

Conflicts of interest: None declared.

Plagiarism check: This paper was screened twice using Cross-Check to verify originality before publication.

Peer review: This paper was double-blinded and stringently reviewed by international expert reviewers.

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Copyedited by James R, Frenchman B, Yu J, Qiu Y, Li CH, Song LP, Zhao M