Identification of HOXD4 Mutations in Spinal Extradural Arachnoid Cyst

Yoji Ogura1,2, Noriko Miyake3, Ikuyo Kou1, Aritoshi Iida1, Masahiro Nakajima1, Kazuki Takeda1,2, Shunsuke Fujibayashi4, Masaaki Shiina5, Eijiro Okada6, Yoshiaki Toyama2, Akio Iwanami2, Ken Ishii2, Kazuhiro Ogata5, Hiroshi Asahara7, Naomichi Matsumoto3, Masaya Nakamura2, Morio Matsumoto5, Shiro Ikegawa1*

1 Laboratory of Bone and Joint Diseases, RIKEN Center for Integrative Medical Sciences, Tokyo, 108–8639, Japan, 2 Department of Orthopaedic Surgery, School of Medicine, Keio University, Tokyo, 160–8582, Japan, 3 Department of Human Genetics, Yokohama City University Graduate School of Medicine, Yokohama, 236–0004, Japan, 4 Department of Orthopaedic Surgery, Kyoto University, Kyoto, 606–8507, Japan, 5 Department of Biochemistry, Yokohama City University Graduate School of Medicine, Yokohama, 236–0004, Japan, 6 Department of Orthopaedic Surgery, Saiseikai Central Hospital, Tokyo, 108–0073, Japan, 7 Department of Systems BioMedicine, Tokyo Medical and Dental University Graduate School of Medical and Dental Sciences, Tokyo, 113–8510, Japan

* sikegawa@ims.u-tokyo.ac.jp

Abstract

Spinal extradural arachnoid cyst (SEDAC) is a cyst in the spinal canal that protrudes into the epidural space from a defect in the dura mater and leads to neurological disturbances. We previously showed that familial SEDAC is caused by FOXC2 mutation; however, the causal gene of sporadic SEDAC has not been identified. To identify the causal gene of sporadic SEDAC, we performed whole exome sequencing for 12 subjects with sporadic SEDAC and identified heterozygous HOXD4 loss-of-function mutations in three subjects. HOXD4 haplo-insufficiency causes SEDAC and a transcriptional network containing HOXD4 and FOXC2 is involved in the development of the dura mater and the etiology of SEDAC.

Introduction

Spinal extradural arachnoid cyst (SEDAC) is a cyst in the spinal canal (Fig 1), which is formed by arachnoid mater protruding from a dural defect that connects the intra- and epi-dural spaces. SEDAC represents 1% of all primary spinal tumours[1] and occurs predominantly in the lower thoracic to lumbar area, posterior to the spinal cord.[2] SEDAC expands due to retention of the cerebrospinal fluid in response to changes in spinal pressure. The enlarged cyst compresses the spinal cord and leads to neurological disturbances.[3] The onset is usually after middle age since the expansion progresses gradually.

SEDAC could be secondary to inflammation, previous spinal surgery and closed spinal trauma;[4] however, in most cases, it is caused by an idiopathic, congenital dural defect. Genetic etiology of SEDAC has been suggested.[5, 6] We previously investigated 2 familial SEDAC pedigrees associated with lymphedema-distichiasis syndrome (LDS) (OMIM 153400)
and identified heterozygous loss of function mutations in FOXC2 (forkhead box C2).\[6\] In the study, we also investigated seven sporadic SEDAC patients, but found no FOXC2 mutation. Furthermore, clinical features of the sporadic SEDAC without a FOXC2 mutation were significantly different from syndromic SEDAC with a FOXC2 mutation in age of onset and number and location of the cysts,\[6\] suggesting genetic heterogeneity of SEDAC.

To identify the causal gene of sporadic SEDAC, we performed whole exome sequencing (WES) for 12 subjects with sporadic SEDAC. We identified HOXD4 loss-of-function mutations in three subjects.

**Materials and Methods**

**Subjects**

We recruited 12 sporadic SEDAC subjects (Table 1). SEDAC was diagnosed using MRI. All subjects were Japanese and had no mutations or structural abnormalities in FOXC2. All but one received surgery. Secondary SEDAC was excluded by medical examination and negative intra-operative findings. Written informed consent was obtained from all subjects. The study was approved by the ethical committees of RIKEN and other participating institutions.

**Exome sequencing**

We sheared genomic DNA (3 μg) by Covaris S2 system (Covaris) and processed with SureSelect All Exon 5 kit (Agilent Technologies). We sequenced the captured DNAs with HiSeq 2000 (Illumina) with 101 base pair-end reads with seven indices. We performed the image analysis
and base calling by HiSeq Control Software/Real Time Analysis and CASAVA1.8.2 (Illumina) and mapped the sequences to human reference genome hg19 by Novoalign 3.00.02 (P1–P7) and Novoalign 3.00.04 (P8–P12). We removed PCR duplication by the Picard tools. The variants were called by Genome Analysis Toolkit (GATK) v1.6–5 or v2.7–4 using the thresholds recommended in GATK best practices v.3 with hand filtering, and annotated by ANNOVAR (2012 February 23).

Analysis of exome sequence data

We hypothesized that SEDAC was inherited in an autosomal-dominant fashion since all subjects were sporadic. We filtered out these variants with the script created by BITS (Tokyo, Japan) according to following conditions: 1) variants registered in ESP6500, 2) variants found in our in-house controls (n = 575), 3) synonymous changes, 4) non-flagged rare variants registered in dbSNP build 137 (MAF < 0.01), and 5) variants within segmental duplications.

Sanger sequence

We sequenced PCR products from genomic DNAs and plasmid clones of the expression vectors using 3730xl DNA Analyzer (Applied Biosystems) according to the manufacture’s instruction.

in silico analyses of mutation and gene

To evaluate the variants identified by the sequence analyses, we considered the following variants as deleterious: 1) nonsense variants (stop codon and frame shift), 2) deletion of > 3 amino acid, and 3) missense variants with high in silico prediction scores (PolyPhen-2 > 0.95 and SIFT < 0.05). We performed in silico structural analysis as previously described.[7, 8] Briefly, since no experimental structure was available for the human HOXD4 homeodomain, we used a crystal structure of the Antennapedia homeodomain in complex with DNA (PDB code 9ANT) by searching an analogous structure of human HOXD4 using SWISS-MODEL server. To evaluate the impact of the deletion on the homeodomain structure, we calculated free

| Subject ID | Age at diagnosis (years) | Sex | Cyst Number | Location | Surgery | Associated feature |
|------------|--------------------------|-----|-------------|----------|---------|-------------------|
| P1         | 64                       | M   | 1           | T12-L2   | –       | –                 |
| P2         | 36                       | M   | 1           | T11-L3   | +       | –                 |
| P3         | 45                       | M   | 1           | T12-L2   | +       | –                 |
| P4         | 60                       | F   | 1           | T12-L2   | +       | –                 |
| P5         | 50                       | F   | 1           | T12-L2   | +       | –                 |
| P6         | 38                       | M   | 1           | T12-L1   | +       | –                 |
| P7         | 45                       | F   | 1           | T11-L3   | +       | –                 |
| P8         | 49                       | F   | 1           | T11-L2   | +       | –                 |
| P9         | 59                       | M   | 1           | T11-S    | +       | –                 |
| P10        | 38                       | M   | 1           | L2-4     | +       | –                 |
| P11        | 38                       | F   | 1           | L2-4     | +       | –                 |
| P12        | 61                       | F   | 2           | L2-3/ L5-S| +       | –                 |

Subjects harboring HOXD4 mutations are in bold.

Distichiasis, lymphedema and skeletal anomalies.

Table 1. Clinical data of spinal extradural arachnoid cyst subjects.

doi:10.1371/journal.pone.0142126.t001
energy change upon the deletion using the FoldX software (version 3.0β5). We used STRING 9.1. (http://string-db.org/) for in silico protein–protein interaction analysis. We examined expression patterns of Hoxd4 and Foxc2 in mouse embryo using EMBRYS database (http://embrys.com/).

Luciferase assay of the recombinant HOXD4 mutations
We cloned the cDNA of the wild-type HOXD4 into the XbaI and EcoRV sites of the pFLAG-CMV-4 expression vector (Sigma Aldrich). We introduced the two HOXD4 mutations by site-directed mutagenesis using PrimeSTAR Mutagenesis Basal Kit (Takara). For Western blotting, HeLa cells were transfected in a 12-well plate using 500 ng of the HOXD4-pFLAG construct. Transfected HeLa cells were washed with PBS, were harvested by scraping after 48 hours of incubation, and were resolved by SDS-PAGE. The N-terminal FLAG epitope was detected by immunoblot with a mouse anti-FLAG monoclonal antibody (Sigma Aldrich). We cloned the murine Hoxd4-responsive luciferase vector into the NheI and BglII sites of the pGL3-Basic vector (Promega). The homeodomains of mouse and human HOXD4 have completely identical amino acid sequences.[9] For the luciferase assay, HeLa cells were transfected in a 24-well plate using 150 ng of the HOXD4-pFLAG construct, 300 ng of the luciferase reporter vector and 1 ng of the pRLSV40 control vector (Promega). Transfected cells were grown for 48 hours under 5% CO2 at 37°C. The cells were washed with PBS and dissolved in 150 μl of a passive lysis buffer. We performed the dual-luciferase assays using the Promega Dual Luciferase Assay kit (Promega) according to the manufacturer’s protocol.

Results
Whole exome sequencing
The mean depth of coverage for reads was 109.5×, and 92.8% of the targeted bases had sufficient coverage (more than 20 reads on average) (S1 Table). By the WES, we identified heterozygous variants in HOXD4, c.633_634insA (p.D212Rfs*3) in P5 and c.680_691del (p.S227_S230del) in P6 and P9. The results were confirmed by the Sanger sequence (Fig 2A). These variants were not found in any public databases including WES database of 1,208 Japanese people (Human Genetic Variation Browser). HOXD4 is a two-exon gene (Fig 2B) on chromosome 2p. c.633_634insA is considered to lead to a truncation of the HOXD4 protein rather than to cause nonsense-mediated mRNA decay, because the mutation is located in the last exon (Fig 2B). c.680_691del produces a deletion in the C-terminal of the homeodomain (Fig 2B). We then examined the presence of structural abnormalities, such as deletion and duplication, in HOXD4 using TaqMan real-time quantitative PCR method as previously described;[6] however, no abnormality was found in the subjects (data not shown).

in silico analysis of the HOXD4 variants
We evaluated the effect of the two HOXD4 variants on their ability to bind to DNA using in silico structural analysis as previously described.[6, 7] c.633_634insA (p.D212Rfs*3) that affects the hydrophobic core of the homeodomain was predicted to disturb the folding of the tertiary structure and thereby impairs DNA binding activity of the homeodomain. No implications could be drawn about the impact of c.680_691del (p.S227_S230del) from the analysis.

in vitro analysis of the transcriptional activities of the HOXD4 variants
We then analyzed their transcriptional activity of the variants in vitro. We confirmed the protein production using a Western blot analysis (Fig 2C) and measured the transactivation
activity of the HOXD4 constructs using luciferase assay. The mutant constructs with p.
D212Rfs*3 and p.S227_S230del had significantly reduced luciferase activities compared to the
wild type (Fig 2D), indicating that the two mutations were loss of function mutations. This
result was replicated in HEK293 cells (data not shown).

in silico analysis of the relation of HOXD4 and FOXC2
HOXD4 and FOXC2 are both homeobox transcription factors involved in various develop-
mental processes. To examine the relation of the two transcription factors, we evaluated their
interaction using STRING 9.1. We found that HOXD4 and FOXC2 proteins had indirect, but close interactions with each other (S1 Fig). We then investigated the expression patterns of Hoxd4 and Foxc2 during early development of the mouse and found that they had similar temporal and spatial expression pattern in the somite of mouse embryo (S2 Fig).

Discussion

We have found two heterozygous mutations in HOXD4 in SEDAC. Both are thought to be loss-of-function mutations. To our knowledge, only one HOXD4 mutation has been reported; a germline mutation, c.242A>T (p.E81V) (Fig 2B) was found in two acute lymphoid leukemia patients, one with skeletal abnormalities (bilateral cervical ribs and L5 sacralization) and another without skeletal abnormalities.[10] The mutation also showed a partial loss of transcriptional activity. The association of SEDAC with acute lymphoid leukemia has not been reported. The phenotype discrepancy between the present and reported mutations may be due to the difference of the position and/or the effect of the mutations. Alternatively, based on the fact that c.242A>T of HOXD4 in the two leukemia patients is linked to a specific haplotype composed of three variants in other HOXD genes (c.746-79_746-68del and c.1025T>G in HOXD10 and c.557G>A in HOXD12), van Scherpenzeel Thim et al. speculated that the HOXD4 mutation is insufficient to confer leukemia susceptibility on its own, necessitating combined action of other HOXD variants.[10] In our patients, those HOXD variations and other HOX variants common to the two SEDAC subjects were not found.

Pathomechanism of the SEDAC by HOXD4 mutations has not been clarified. HOXD4 belongs to the homeobox (HOX) gene family of transcription factors. HOX genes play important roles in morphogenesis and are critical in the establishment of body axes during embryogenesis.[10–12] HOXD4 is involved in determining positional values in the developing spine.[10] The dural defect of SEDAC is considered to be due to abnormal dura mater development. FOXC2, the disease gene for syndromic SEDAC[5, 6] also encodes a homeobox transcription factor and expressed in the developing mesodermal mesenchyme which forms the dura mater. [13–15] Dura mater development would be impaired by FOXC2 loss of function mutations. On the other hand, HOXD4 plays an important role in morphogenesis by determining positional values in the developing spine.[16] In fact, Northern blot analysis revealed that HOXD4 transcripts were expressed in spinal cords of 5–9 week-old human fetuses.[17] HOXD4 may also be involved in dura mater development. Interestingly, in silico interaction analysis showed that HOXD4 and FOXC2 proteins had close interactions with each other (S1 Fig). Furthermore, Hoxd4 and Foxc2 showed similar spacio-temporal expression patterns in the somite of mouse embryo (S2 Fig). These findings suggest that the regulatory network of the transcription factors containing HOXD4 may be important in dura mater development and involved in the etiology and pathogenesis of SEDAC. The network may include other causal genes of SEDAC.

Supporting Information

S1 Fig. Protein-protein interaction analysis between HOXD4 and FOXC2 protein. Interaction analysis between HOXD4 and FOXC2 proteins using STRING database. Stronger associations are represented by thicker lines. Nodes are colored (if they are directly linked to the input) or white (nodes of a higher iteration) as defined by STRING database. HOXD4 and FOXC2 proteins had indirect, but strong interaction.

(SDOCX)

S2 Fig. Embryonic gene expression of Hoxd4 and Foxc2. Hoxd4 (top) and Foxc2 (bottom) expression in mouse during E9.5–11.5. Pink, dark-yellow, orange, light-yellow, blue, and red
represent somite, tail bud, maxillary process, mandibular arch, hyoid arch, and eye, respectively. The stronger color density represents more expression. Both genes had strong and similar expression in the somite.

S1 Table. Summary of the exome sequencing performance.

Acknowledgments

We are grateful to the individuals who participated in this study. We thank T. Isono, T. Kusadokoro, Y. Takanashi, and S. Tominaga for technical assistance. We also thank N. Atsumi for checking English. This study is supported by research grants from Japan Agency For Medical Research and Development (AMED) (contract No. 14525125).

Author Contributions

Conceived and designed the experiments: YO MM M. Nakamura N. Matsumoto SI. Performed the experiments: YO HA N. Miyake N. Matsumoto SI. Analyzed the data: YO N. Miyake MS KO N. Matsumoto SI. Contributed reagents/materials/analysis tools: YO N. Miyake IK A. Iida M. Nakajima KT SF MS EO YT A. Iwanami KI M. Nakamura HA SI. Wrote the paper: YO N. Miyake MS SI.

References

1. Yabuki S, Kikuchi S, Ikegawa S. Spinal extradural arachnoid cysts associated with distichiasis and lymphedema. Am J Med Genet A. 2007; 143A(8):884–7. Epub 2007/03/17. doi:10.1002/ajmg.a.31669 PMID: 17366853.

2. Cilluffo JM, Gomez MR, Reese DF, Onofrio BM, Miller RH. Idiopathic (“congenital”) spinal arachnoid diverticula. Clinical diagnosis and surgical results. Mayo Clin Proc. 1981; 56(2):93–101. Epub 1981/02/01. PMID: 6780735.

3. Chang IC, Chou MC, Bell WR, Lin ZI. Spinal cord compression caused by extradural arachnoid cysts. Clinical examples and review. Pediatr Neurosurg. 2004; 40(2):70–4. Epub 2004/08/05. doi:10.1159/000078911 PMID: 15292636.

4. Miravet E, Sinisterra S, Birchansky S, Papazian O, Tuite G, Grossman JA, et al. Cervicothoracic extradural arachnoid cyst: possible association with obstetric brachial plexus palsy. J Child Neurol. 2002; 17(10):770–2. Epub 2003/01/28. PMID: 12546433.

5. Sanchez-Carpintero R, Dominguez P, Nunez MT, Patino-Garcia A. Spinal extradural arachnoid cysts in lymphedema-distichiasis syndrome. Genet Med. 2010; 12(8):532–5. Epub 2010/06/11. doi:10.1097/GIM.0b013e3181e5c7ea PMID: 2055019.

6. Ogura Y, Yabuki S, Iida A, Kou I, Nakajima M, Kano H, et al. FOXC2 Mutations in Familial and Sporadic Spinal Extradural Arachnoid Cyst. PLoS One. 2013; 8(11):e80548. Epub 2013/11/28. doi:10.1371/journal.pone.0080548 PMID: 24278289.

7. Schymkowitz J, Borg J, Stricher F, Nys R, Rousseau F, Senano L. The FoldX web server: an online force field. Nucleic Acids Res. 2005; 33(Web Server issue):W382–8. Epub 2005/06/28. doi:10.1093/nar/gki387 PMID: 15980494; PubMed Central PMCID: PMC1160148.

8. Ogura Y, Yabuki S, Iida A, Kou I, Nakajima M, Kano H, et al. Identification of FOXC2 mutations in familial and sporadic spinal extradural arachnoid cyst. PLoS One. 2012.

9. Popperl H, Featherstone MS. An autoregulatory element of the murine Hox-4.2 gene. EMBO J. 1992; 11(10):3673–80. Epub 1992/10/01. PMID: 1356763; PubMed Central PMCID: PMCP565627.

10. van Scherpenzeel Thim V, Remacle S, Picard J, Cornu G, Gofflot F, Rezsohazy R, et al. Mutation analysis of the HOX paralogous 4–13 genes in children with acute lymphoid malignancies: identification of a novel germline mutation of HOXD4 leading to a partial loss-of-function. Hum Mutat. 2005; 25(4):384–95. Epub 2005/03/19. doi:10.1002/humu.20155 PMID: 15776434.

11. Krumlauf R. Hox genes in vertebrate development. Cell. 1994; 78(2):191–201. Epub 1994/07/29. PMID: 7913880.
12. McGinnis W, Krumlauf R. Homeobox genes and axial patterning. Cell. 1992; 68(2):283–302. Epub 1992/01/24. PMID: 1346368.

13. Fang J, Dagenais SL, Erickson RP, Arlt MF, Glynn MW, Gorski JL, et al. Mutations in FOXC2 (MFH-1), a forkhead family transcription factor, are responsible for the hereditary lymphedema-distichiasis syndrome. Am J Hum Genet. 2000; 67(6):1382–8. Epub 2000/11/15. doi:10.1086/316915 PMID: 11078474; PubMed Central PMCID: PMC1287915.

14. Kaestner KH, Bleckmann SC, Monaghan AP, Schlondorff J, Mincheva A, Lichter P, et al. Clustered arrangement of winged helix genes fkh-6 and MFH-1: possible implications for mesoderm development. Development. 1996; 122(6):1751–8. Epub 1996/06/01. PMID: 8674414.

15. Miura N, Wanaka A, Tohyama M, Tanaka K. MFH-1, a new member of the fork head domain family, is expressed in developing mesenchyme. FEBS Lett. 1993; 326(1–3):171–6. Epub 1993/07/12. PMID: 8325367.

16. Mark M, Rijli FM, Chambon P. Homeobox genes in embryogenesis and pathogenesis. Pediatr Res. 1997; 42(4):421–9. Epub 1997/10/06. doi:10.1203/00006450-199710000-00001 PMID: 9380431.

17. Mavilio F, Simeone A, Giampaolo A, Faiella A, Zappavigna V, Acampora D, et al. Differential and stage-related expression in embryonic tissues of a new human homeobox gene. Nature. 1986; 324 (6098):664–8. Epub 1986/12/18. doi:10.1038/324664a0 PMID: 2879245.