FOXC2 Mutations in Familial and Sporadic Spinal Extradural Arachnoid Cyst

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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. Most cases are sporadic; however, three familial SEDAC cases have been reported, suggesting genetic etiological factors. All familial cases are associated with lymphedema-distichiasis syndrome (LDS), whose causal gene is FOXC2. However, FOXC2 mutation analysis has been performed in only 1 family, and no mutation analysis has been performed on sporadic (non-familial) SEDACs. We recruited 17 SEDAC subjects consisting of 2 familial and 7 sporadic cases and examined FOXC2 mutations by Sanger sequencing and structural abnormalities by TaqMan copy number assay. We identified 2 novel FOXC2 mutations in 2 familial cases. Incomplete LDS penetrance was noted in both families. Four subjects presented with SEDACs only. Thus, SEDAC caused by the heterozygous FOXC2 loss-of-function mutation should be considered a feature of LDS, although it often manifests as the sole symptom. Seven sporadic SEDAC subjects had no FOXC2 mutations, no symptoms of LDS, and showed differing clinical characteristics from those who had FOXC2 mutations, suggesting that other gene(s) besides FOXC2 are likely to be involved in SEDAC.

Introduction
Spinal extradural arachnoid cyst (SEDAC) is a cyst in the spinal canal that protrudes into the epidural space via defects in the dura mater [Fig. 1]. It commonly occurs in the posterior thoracic area,[1] predominately affects males,[2] and is relatively rare, representing only 1% of all primary spinal tumors.[3] The cyst expands due to retention of cerebrospinal fluid that collects via a pedicle connecting the intra- and epi-dural subarachnoid spaces, in response to changes in spinal pressure. An expanding cyst may compress the spinal cord and cause neurological disturbances.[4] SEDAC is surgically curable; however, early diagnosis is important because delayed treatment leads to irreversible neurological defects.[4]

The etiological factors of SEDAC remain unclear. Its origin has been attributed to congenital dural defects, arachnoid proliferation and inflammation, previous surgery, and closed spinal trauma.[5] A few reports have suggested genetic etiological factors, since 3 families with SEDAC have been reported, including a pair of siblings,[6] 3 siblings,[7] and a large pedigree.[8] Some members from the 3 families showed coexisting lymphedema in their lower extremities and distichiasis (double rows of eyelashes arising from the Meibomian glands).[6–8] These observations suggest that SEDAC is associated with lymphedema-distichiasis syndrome (LDS) (OMIM 153400);[7–9] LDS is an autosomal dominant disorder with variable expressivity. Its major features are lymphedema and distichiasis. The penetrance of lymphedema or distichiasis is 70% to 80%.[9] Its minor features include ptosis, cleft palate, renal abnormalities, congenital heart disease, vertebral anomalies, and SEDAC.[8,10–13] The minor features have lower penetrance and their details are unclear. The causal gene of LDS is FOXC2, a forkhead family transcription factor (OMIM 153400); in fact, molecular screening of 81 probands resulted in the detection of FOXC2 mutations in 100% of LDS patients.[8,9,14–17] Therefore, FOXC2 is a good candidate gene for SEDAC; however, FOXC2 mutation analysis has been performed in only 1 SEDAC family associated with LDS, and no mutation analysis has been performed on sporadic SEDACs or SEDACs unrelated to LDS (solitary SEDACs). The relationship between SEDAC and FOXC2 mutations remains unclear.

To gain insight into the genetic etiology of SEDAC, we examined FOXC2 mutations in 2 familial and 7 sporadic cases of SEDAC.

Materials and Methods
Ethics statement
The study was approved by the institutional review boards of RIKEN Center for Integrative Medical Sciences, Keio University and Fukushima Medical University. A written informed consent
The FOXC2 syndrome had heterozygous c.733delG or c.354C mutation, cyst, distichiasis and lymphedema. All affected subjects of the considered as a syndrome consisting of spinal extradural arachnoid cyst and co-segregation of the FOXC2 mutations.

Figure 1. Spinal extradural arachnoid cyst. T1- (a) and T2- (b) weighted sagittal plane images of MRI (magnetic resonance imaging) scan. Subject III-2 of Family 1, 13 years old. There are multiple cysts dorsal to the spinal cord at the thoracolumbar spine.

Figure 2. Pedigrees of the families with spinal extradural arachnoid cyst and co-segregation of the FOX2 mutations. a) Family 1 with c.733delG, b) Family 2 with c.354C>G. Some family members had distichiasis and/or lymphedema. Autosomal dominant mode of inheritance is definite when the disorder of the pedigree was observed from a previously reported family.[3] (Family 1; Fig. 2a), three were from another family (Family 2; Fig. 2b) and 7 were sporadic SEDAC cases with no family history. All subjects had no history of infection, trauma and previous surgery of the spine. All but one proband had received surgery for SEDAC. There were no operative findings suggestive of infection and trauma. Ten subjects without SEDAC from the familial SEDAC pedigrees were also recruited for the DNA analysis. Magnetic resonance imaging (MRI) scans of the thoracic and lumbar spines were obtained for all subjects. The T1- and T2-weighted images in the sagittal plane were used for evaluation of SEDACs (Fig. 1).

Mutation detection
DNA was extracted from saliva using the Oragene DNA self-collection kit (DNA Genotek, Ottawa, Canada) according to the manufacturer’s protocol. The single coding exon of FOXC2 (NM_005251.2) was PCR-amplified using KOD FX (TOYOBO, Osaka, Japan) and the primers 5’-TCTGCCCTCTGGGCTTCT-3’ (forward) and 5’-TCTGCAGCCCCTTAATTGTC-3’ (reverse). PCR products were sequenced by the dideoxy termination method, using an ABI 3730 automated sequencer (Applied Biosystems, Foster City, USA), and screened for mutations.

In silico structural analysis
To evaluate the effect of the N118K FOXC2 variant on its ability to bind DNA, we used FoldX as previously described.[16] FoldX is an algorithm that calculates the free energy of proteins and nucleic acids based on a high-resolution 3D model of their structure, in order to predict the effect of mutations on their stability, folding, and dynamics. Because the structure of FOXC2 has not been identified, we analysed the FOXK2 protein, since both have similar primary sequences in their forkhead DNA binding domains.

Quantitative PCR
The TaqMan real-time quantitative PCR (qPCR) method was used to examine the copy number of FOXC2. The primers and probes for the qPCR were designed using Primer Express software v3.0 (Applied Biosystems). The RNase P gene was used as a reference gene. All assays were performed with the TaqMan Universal PCR Master Mix according to the manufacturer’s protocol (Applied Biosystems).

Plasmid construction for western blot and the luciferase assay
The wild-type, c.354C>G, and c.733delG FOXC2 genes were PCR-amplified using the primers 5’TATGATTTCAATGTCAGGCGGCTACTC-3’ (forward) and 5’TATAGATTTCAATGTCAGGCGGCTACTC-3’ (reverse). The PCR products were cloned into the EcoRI and BglII sites of the pFLAG-CMV-4 expression vector (Sigma Aldrich, St. Louis, USA). The nucleotide sequences of the clones were confirmed by Sanger sequencing.

Cell culture and transfection
COS-7 and HeLa cells were grown in Dulbecco’s modified Eagle’s medium and 10% foetal bovine serum. Transfection was performed using FuGENE6 transfection reagent (Promega, Fitchburg, USA) according to the manufacturer’s protocol. For protein expression, COS-7 cells were transfected in a 12-well plate using 500 ng of the FOXC2-pFLAG construct. For luciferase assays, HeLa cells were transfected in a 24-well plate using 500 ng of the FOXC2-pFLAG construct, 50 ng of the FOXC2 luciferase reporter (a gift from M. Walter), and 1 ng of the pRLTK control vector (Promega).[19] Transfected cells were grown for 48 h under 5% CO2 at 37°C.

Western blot analysis
Transfected COS-7 cells were washed with PBS and harvested by scraping after 48 h of incubation. The cell lysates were resolved by SDS-PAGE. The N-terminal FLAG epitope was detected by...
immunoblot analysis, using a mouse anti-FLAG monoclonal antibody (Sigma Aldrich).

**Luciferase assay**

Transfected HeLa cells were washed with PBS and dissolved in 150 ml of passive lysis buffer after 48 h of incubation. The dual-luciferase assays were performed in triplicate, using the Promega Dual Luciferase Assay kit (Promega), according to the manufacturer’s protocol.

**Results**

**Mutation analysis of FOXC2**

We performed direct sequencing to analyse the PCR products of DNA obtained from familial and sporadic SEDAC subjects. We found 2 novel FOXC2 mutations in 2 familial cases. In Family 1 (Fig. 2a), all the affected subjects had a heterozygous guanine deletion (c.733delG) (Fig. 3a). This mutation leads to an early stop codon at amino acid 257 (p.A245P*32), which is unlikely to cause nonsense-mediated mRNA decay, since FOXC2 is a single exonic gene. This mutation was not found in any database or in the general population of 100 randomly sampled Japanese people. Furthermore, no FOXC2 mutations were found in 6 subjects who belonged to the family and did not have any symptoms of SEDAC, distichiasis, or lymphedema (Fig. 2a).

Family 2 (Fig. 2b) had a c.354C>G (p.N118K) heterozygous mutation (Fig. 3b), which was also not found in any database and in the general population of 100 Japanese people. N118 is located in the forkhead domain of the FOXC2 protein. We investigated the effect of N118K by using FoldX. The interaction energy ΔG of the mutation was 1.28±0.40 kcal/mol. This finding indicated that a hydrogen bond between the side chain of N118 and the adenine base of DNA is lost, suggesting that c.354C>G is a disease-causing mutation (Fig. 4).

No mutation was found in the other 7 subjects with sporadic SEDACs. Structural abnormalities such as deletion or duplication of FOXC2 were investigated using the TaqMan copy number assay; however, no such abnormality was found (data not shown).

**Clinical features of the subjects with FOXC2 mutations**

The familial cases with FOXC2 mutations were also diagnosed as LDS, since the family members had lymphedema and/or distichiasis (Fig. 2 and Table 1). However, the penetrance of the LDS features was incomplete. While FOXC2 mutations were found in a total of 13 subjects, lymphedema was found in 4 and distichiasis in 9 subjects. Only 3 SEDAC subjects had both lymphedema and distichiasis. Three subjects had no SEDAC and 4 subjects had only SEDAC. SEDAC subjects without FOXC2 mutations had no features of LDS.

The age of diagnosis of the SEDAC patients with FOXC2 mutations was significantly lower than those without FOXC2 mutations (Table 1): the mean ages of SEDAC subjects with and without FOXC2 mutations were 23.2 (range: 7–51) and 40.3 (range: 36–64) years, respectively. Male/female ratios of SEDAC with and without FOXC2 mutations were 4/6 and 4/3, respectively. Furthermore, the number and location of the cysts were different. All SEDAC subjects without FOXC2 mutations had a single cyst, which occurred in the thoracolumbar junction, whereas 7 of 10 SEDAC subjects with FOXC2 mutations had multiple cysts that occurred in various areas, ranging from the upper thoracic to the sacral regions.

**Analysis of FOXC2 mutations in vitro**

COS-7 cells were transfected with vectors encoding the wild-type and mutant FOXC2. Whole cells were resolved by SDS-PAGE and subjected to immunoblot analysis. Detection of the N-terminal vector-encoded FLAG epitope demonstrated a stable product, 56 kDa in size, for both wild type and c.354C>G FOXC2. It also demonstrated a product, 31 kDa in size, for the c.733delG FOXC2 protein (Fig. 5a). The effect of each FOXC2-FLAG construct, on the ability to drive transactivation was tested in HeLa cells. The FOXC2 constructs with c.733delG and c.354C>G mutations had reduced transactivation activities of 68% and 42%, respectively, when compared to the wild type (Fig. 5b).

**Discussion**

We identified 2 novel FOXC2 mutations in 2 familial SEDACs. To our knowledge, only 1 study on genetic analysis of SEDAC has been reported previously.[8] Our study reinforced the hypothesis that familial SEDAC is caused by FOXC2 mutations. The FOXC2 mutations in the family studies co-segregated with LDS, but not with SEDAC alone. The FOXC2 mutation was also found in family members who had no SEDAC but had lymphedema or distichiasis; the penetrance of SEDAC was 70%; distichiasis, 70%; and lymphedema, 20% (Fig. 2). All previously reported familial cases of SEDAC have been associated with LDS.[6-8] However, FOXC2 was analysed in only 1 pedigree.[8] Sanchez-Carpintero et al. examined a family of 48 members and identified a FOXC2 c.298C>T (p.Q100X) mutation in 12 of them. This mutation co-segregated with LDS: distichiasis, lymphedema and SEDAC were observed in 12, 11, and 7 of the 12 subjects, respectively. Taken
together, these data suggest that familial SEDAC occurs as a feature of LDS caused by FOXC2 mutation, although symptomatic SEDAC is a rare complication of LDS.[20,21]

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

| Subject ID | Age at diagnosis (years) | Sex | Cyst Number | Location | Surgery | Associated feature | FOXC2 mutation |
|------------|--------------------------|-----|-------------|----------|---------|--------------------|---------------|
| Family 1   |                          |     |             |          |         |                    |               |
| II-6       | 38                       | F   | 1           | T9       | --      | Distichiasis, Lymphedema | c.733delG     |
| III-1      | 15                       | M   | 3           | T11-S1   | +       | Distichiasis       | c.733delG     |
| III-2      | 13                       | F   | 3           | T5-10/L4-5 | +       | Distichiasis       | c.733delG     |
| III-5      | 12                       | F   | 3           | T5-12    | --      | --                 | c.733delG     |
| III-6      | 7                        | F   | 3           | T7-10/L4-5 | --      | Distichiasis       | c.733delG     |
| III-7      | 10                       | M   | 1           | T8-9     | --      | --                 | c.733delG     |
| III-8      | 7                        | F   | 1           | T2-5     | --      | --                 | c.733delG     |
| Family 2   |                          |     |             |          |         |                    |               |
| II-1       | 51                       | F   | 3           | T3/T4-7  | +       | Distichiasis, Lymphedema | c.354C>G     |
| II-2       | 50                       | M   | 4           | T3-8/L1  | --      | --                 | c.354C>G     |
| III-2      | 29                       | M   | 5           | T4-7/T11-L/L2-L5 | --      | Distichiasis, Lymphedema | c.354C>G     |
| Sporadic   |                          |     |             |          |         |                    |               |
| S-1        | 64                       | M   | 1           | T12-L2   | --      | --                 |               |
| S-2        | 36                       | M   | 1           | T11-L3   | +       | --                 |               |
| S-3        | 45                       | M   | 1           | T12-L2   | +       | --                 |               |
| S-4        | 60                       | F   | 1           | T12-L2   | +       | --                 |               |
| S-5        | 50                       | F   | 1           | T12-L2   | +       | --                 |               |
| S-6        | 38                       | M   | 1           | T12-L1   | +       | --                 |               |
| S-7        | 45                       | F   | 1           | T11-L3   | +       | --                 |               |

*Roman numbers represent the family numbers in the pedigrees (Fig. 2).
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The diagnosis of LDS in our subjects was difficult in the absence of any family history, since its major features have low penetrance. Four among 13 subjects with FOXC2 mutations presented with SEDAC alone and only 3 subjects with SEDAC had both of the 2...
major features of LDS (lymphedema and distichiasis). Consequently, the mode of inheritance of the disease in the family was sometimes unclear (Fig. 2a). Indeed, we were able to infer an autosomal dominant mode of inheritance in Family 1 only by viewing the pedigree as LDS associated with SEDAC, and not SEDAC alone. In contrast, a previous study described that the penetrance of distichiasis was 100%; lymphedema, 92%; and SEDAC, 58%. It also reported that there were no SEDAC-only subjects.[8] The difference in the penetrance of these features may be due to the variable expressivity of LDS or due to differences in the effect of the FOXC2 mutation on the different features. In addition, these features are difficult to detect in the early stage of life. SEDAC has no symptoms in the early stage and can be diagnosed only by using MRI. Distichiasis usually has no symptoms and tends to be missed unless physicians examine the eyelids of patients carefully with this possibility in mind. Lymphedema is not usually apparent before adolescence and is not specific to LDS. Therefore, SEDAC associated with LDS is likely to be diagnosed as a sporadic SEDAC.

We showed that the FOXC2 mutation is one of the etiological factors of SEDAC. FOXC2 encodes a regulatory transcription factor and plays a role in the development of the mesodermal mesenchyme.[22,23] Mice with heterozygous Foxc2 deficiency uniformly display distichiasis and also exhibit hyperplasia, as well as incomplete valve formation in their lymphatic vessels.[24] Heterozygous FOXC2 loss-of-function mutations like those in our subjects have been found in a number of LDS subjects.[17,25-31] These findings indicate that FOXC2 haploinsufficiency is the disease-causing mechanism of LDS. Notably, SEDAC has not been reported in the Foxc2 heterozygous deficient mice.[24,32] It is not found even in Foxc2 homozygous deficient mice, although these mice show overt spinal abnormalities.[23] However, we have a theory to explain the occurrence of SEDAC as a result of FOXC2 mutation. FOXC2 is expressed in the developing mesodermal mesenchyme. The dura mater originates from the mesoderm,[22,23] and small defects in the dura mater play an important role in SEDAC pathology.[5] We propose that dura mater development is inhibited by FOXC2 mutations in the fetus, and that as a result, the arachnoid mater protrudes from the dural defect, forming an expanding cyst.

Our study suggests that additional etiological factors of SEDAC, other than the FOXC2 mutation, may exist because most of the sporadic subjects we studied had no FOXC2 mutation. Interestingly, the 2 groups of SEDAC cases, with and without FOXC2 mutations, had significantly different clinical features (Table 1). The age at diagnosis was significantly younger in SEDAC cases with a FOXC2 mutation. The location of cysts was different between the 2 groups; SEDAC without FOXC2 mutations occurred in the thoracolumbar junction while SEDAC with FOXC2 mutations occurred at various sites ranging from the upper thoracic to sacral areas. Furthermore, there were differences in the cyst numbers; all SEDAC subjects without FOXC2 mutations had a single cyst, while SEDAC subjects with FOXC2 mutations frequently had multiple cysts. These data indicate that there could be other SEDAC causal gene(s). The identification of new SEDAC associated genes will help not only in early detection but also in clarifying the pathology of SEDAC.

In summary, we showed that SEDAC is caused by heterozygous loss-of-function mutations in FOXC2. As a result of our study, SEDAC patients and their family members will be able to use genetic screening to evaluate their risk and undergo close examination and surgery before developing irreversible neurological defects. It is easy to screen for the FOXC2 mutation since FOXC2 is a small 1-exon gene that can be covered by a single PCR reaction. To date, we have observed that SEDAC caused by FOXC2 mutations is also associated with LDS. Hence, examination of family history and clinical investigation for the features of LDS are important for the early detection of the disease. However, most cases of SEDAC are not caused by the FOXC2 mutation and show different clinical features from those with FOXC2 mutations. Further studies aimed at identifying new SEDAC causal gene(s) are necessary to clarify their etiological factors and pathogenesis.

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Author Contributions

Conceived and designed the experiments: SI. Performed the experiments: YO AI IK. Analyzed the data: YO SI. Contributed reagents/materials/analysis tools: SY M. Nakajima HK SK YT M. Nakamura MM. Wrote the paper: YO SI. In silico computational analysis: MS KO.

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