

論文の内容の要旨

論文題目 **FUNCTIONAL ROLE OF *EFCAB7* AS A CANDIDATE GENETIC
MODIFIER OF ELLIS-VAN CREVELD SYNDROME**
(Ellis-van Creveld症候群の修飾遺伝子の候補*EFCAB7*の機能解析)

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Introduction/Objective

Ciliopathies are highly heterogeneous multi-organ defects affecting kidney, brain, limb, retina, liver and bone, caused by functional and structural abnormalities of cilia in human development. Primary cilia project from the cell surface like an antenna. Various proteins accumulate in primary cilia to orchestrate Hedgehog (Hh) signaling pathway. Ellis-van Creveld syndrome (EVC, MIM ID 225500), a rare ciliopathy, is an autosomal recessive, congenital disorder occurring in about 1 in 60,000 live births. EVC is characterized by disproportionate short stature, polydactyly, dystrophic nails and specific oral defects. More than half of EVC cases have severe congenital heart defects, which lead to early childhood mortality. Causative mutations of either the *EVC* or *EVC2* gene are detected in two-thirds of EVC. *WDR35* mutation has also been reported in some cases. The clinical manifestations of EVC are overlapping with other ciliopathies, which hampers accurate diagnosis. Both the *EVC* and *EVC2* proteins are localized to the basal bodies of the primary cilia and are considered to positively regulate Hh signaling with other molecules. Recently, *EFCAB7* and *IQCE* reportedly co-localized with *EVC*-*EVC2* complex and interact with one another. Based on this finding, I hypothesized that mutations in *EFCAB7* and *IQCE* may also contribute to EVC phenotype. In this study, I conducted clinical and genetic studies of EVC in Vietnam, where no data on EVC have been available so far. Seven unrelated Ellis-van Creveld families within 3 generations were recruited at Children's Hospital 2, Ho Chi Minh City, Vietnam.

Genetic analysis of *EVC* and *EVC2*, as well as other possible candidate genes, is important not only for the diagnosis but also for clarifying the pathogenesis in EVC. I detected novel *EVC*, *EVC2* mutations in two Vietnamese families with various severities of EVC phenotype. Interestingly, heterozygous *EFCAB7* c.1171T>C (p.Y391H) co-existed with *EVC* mutations in a proband and his father showing atypical cardiac defect, whereas the sibling in the same family with only *EVC* mutations had common phenotypes. Motivated by these results, I tested whether *EFCAB7* 391H modifies the interaction with *EVC*-*EVC2* complex and the regulation in Hh signaling. The objective of this study is to detect causative mutations in patients with EVC and to evaluate functional role of mutated *EFCAB7* on regulation in Hh signaling.

Materials and Methods

I recruited ten EVC patients in 7 unrelated families and 200 healthy controls (100 are Vietnamese and 100 are Japanese). After written informed consent was obtained, peripheral blood and buccal mucosa samples from all patients as well as their parents (EVC families) were collected and stored on filter papers. This study was approved by the Ethical Committee of Children's Hospital 2, Ho Chi Minh City, Vietnam No. CS/N2/13/26 and The University of Tokyo, Japan No. G1001. Genomic DNA was extracted using manufactures' protocols. I analyzed entire coding region, exon-intron boundaries of *EVC*, *EVC2*, *IQCE*, *EFCAB7* and *WDR35* by Sanger sequencing. For patients and their relatives with novel mutations, I did quantitative RT-PCR to evaluate the mRNA expression of *EVC* or *EVC2*. To detect heterozygous deletion of the causative gene in a possible carrier, who had no mutations in Sanger sequencing, single nucleotide polymorphism (SNP) array analysis was performed. I evaluated whole genome copy number aberrations in patient C4 and the mother. I validated a heterozygous deletion harboring exons 9, 10 and 11 of *EVC* in the patients and their mother by long range PCR.

To assess the functional role of mutated EFCAB7, I constructed ECH1 391H (one of the domains in EFCAB7 protein) and tagged with FLAG. The binding partner of EFCAB7, WEYER, which consists of 87 amino acids located in C-terminal of EVC2 was fused with GST. After transformation of constructed vectors to *E.coli*, IPTG stimulation was done to obtain fused proteins. The binding capacity of WEYER-ECH1 was assessed after pull-down assay. Next, I transfected full-length *EFCAB7* 1171C/wild-type into HEK 293T cells. I compared *EVC*, *EVC2* and *IQCE* mRNA expression level between *EFCAB7* 1171C and wild-type transfected cells. Finally, I co-transfected *EFCAB7* 1171C and shRNA *EVC* plasmids into HEK 293T cells to evaluate the effect on Hh signaling regulation. Hh signaling is initiated by the binding of Hh molecules to their receptor and results in GLI transcriptional activation. Cleavage of full-length GLI3 (GLI3FL) to GLI3R, a transcriptional repressor, negatively regulates Hh signaling. Therefore, I analyzed GLI3R/GLI3FL ratio by Western Blotting after stimulation of SAG, a Hh analogue, in co-transfected cells.

Results

All the cases shared common EVC manifestations: moderate to severe short stature, oral abnormalities, nail dystrophy and CHD. Eight of them had polydactyly/syndactyly of hands and feet. Six patients had atrio-ventricular septal defect (AVSD), 2 of them had pulmonary stenosis associated with AVSD. Common atrium, the most severe CHD in EVC, were detected in two cases. Interestingly, two patients in one EVC family had short chordae, an extremely rare CHD, which was never reported in EVC patients.

Patient C2 had compound heterozygous *EVC2* mutations: a novel mutation c.769G>T-p.E177X in exon 6 inherited from mother, and another previously reported c.2476C>T-p.R826X mutation in exon 14 inherited from father. The *EVC2* mRNA expression level was 15~50% in the patient and her parents, respectively, compared to the mean values of 10 normal controls. Two cases (C4, C5) had a novel heterozygous *EVC* mutation (c.1715C>G-p.S572X) in exon 12, and a heterozygous deletion of 16.4 kb in *EVC*, inherited from their father and mother, respectively. The relative expression of *EVC*

mRNA was lower in the patient C4, father and mother than those in controls. This patient C4 and his father also had a novel heterozygous variant in exon 9 of *EFCAB7* c.1171T>C (p.Y391H), who had short chordae of atrioventricular valves in the heart. Thus, I hypothesized that *EFCAB7* 391H may contribute to EVC phenotype. I conducted the functional analysis of *EFCAB7* 391H.

The binding amount of FLAG-ECH1 391H to GST-WEYER was three more times higher than that of FLAG-ECH1 wild-type ($P < 0.001$). The relative *EVC* mRNA expression level in transfected *EFCAB7* 1171C cells (1.236 ± 0.050) was significantly higher than in transfected *EFCAB7* wild-type cells (0.616 ± 0.009) ($P=0.004$). On the other hand, the relative *EVC2* mRNA expression levels between mutated and wild-type of *EFCAB7* were not statistically different ($P=0.159$). In addition, the relative *IQCE* mRNA expression level of *EFCAB7* 1171C transfected cells (3.562 ± 0.450) was significantly lower than in *EFCAB7* wild-type transfected cells (5.660 ± 0.599) ($P=0.005$).

I examined GLI3FL, GLI3R expression level by Western Blotting with GLI3 antibody in cells under three different combinations of transfection: full length *EFCAB7* 1171C/shRNA *EVC*, full length *EFCAB7* wild-type/shRNA *EVC* and full length *EFCAB7* wild-type/shRNA-mock. *EFCAB7* 1171C/shRNA *EVC* transfection mimicked the genetic background of patient C4 in family 3 (typical EVC phenotype with short chordae of AV valves), whereas *EFCAB7* wild-type and shRNA *EVC* co-transfection that of patient C5 (typical EVC phenotype). *EFCAB7* wild-type and shRNA control were used as normal control.

Relative GLI3R expression level in full length *EFCAB7* 1171C/shRNA *EVC* transfected cells (0.529 ± 0.096) was higher than in full length *EFCAB7* wild-type/shRNA *EVC* transfected cells (0.374 ± 0.071) with statistical significance ($P=0.008$). The GLI3R/GLI3FL ratio in the former (0.617 ± 0.072) was markedly higher than that in the latter (0.333 ± 0.112), as well as in the control (0.437 ± 0.063) ($P<0.05$).

Discussion

I screened EVC- complex genes and *WDR35* for the first time in patients with Ellis-van Creveld syndrome in Vietnam. Two compound heterozygous mutations (c.769G>T and c.2476C>T) in *EVC2* were found in patient C2. Both of them were truncated mutations, one was inherited from father and another from mother. The parents showed variable phenotypes; her father had mild EVC phenotype while the mother had only short stature. One of the possible explanations for the difference in severity between the parents is somatic mosaicism. However, both the parents showed the same mutation in genomic DNA samples from the different tissues. The modulating factors determining the disease severity in each mutation remains unknown.

I identified another family with *EVC* with or without *EFCAB7* mutations. The genotype-phenotype expression in each family member raised a question whether *EFCAB7* mutation played a role on the pathogenesis of EVC. RNA expression of *EFCAB7* is recognized in various organs, however, protein expression is only detected in fetal heart. It was suggested that *EFCAB7* expression is spatiotemporally regulated and important for normal cardiac development. So far, *EFCAB7* mutations

have neither been reported as the cause of congenital human diseases, nor as somatic mutations detected in such as neoplastic tissues. A previous study reported that in vitro depletion of *EFCAB7* downregulates expression of *EVC2* and *IQCE* mRNA. However, the role of missense mutation in *EFCAB7* has not yet been studied. *EFCAB7* recruits the EVC-EVC2 complex by interactions between mainly ECH2, a domain of *EFCAB7*, and the WEYER peptides of *EVC2*. In this study, a missense mutation located in ECH1 domain, which also binds to WEYER, resulting in a stronger binding to WEYER compared to wild-type ECH1. The complex of WEYER and *EFCAB7* activates Hh signaling. The alteration of binding capacity might affect the Hh signaling regulation. Previous studies have observed the binding of *EFCAB7* to *EVC2*, and a notable association of expression levels of EVC-EVC2 and *EFCAB7*-*IQCE*. In addition, the presence of interaction partner (EVC to *EVC2* and *EFCAB7* to *IQCE*) induces expression of another. Based on these findings, I tested whether mRNA expression of *EVC*, *EVC2* and *IQCE* were altered by transfection of mutated *EFCAB7*. A possible explanation for the significant lower expression of the binding partner *IQCE* with mutated *EFCAB7* than with wild one, was that the mutated *EFCAB7* may be less stable than wild-type *EFCAB7*. On the contrary, *EVC* mRNA expression was upregulated more in the presence of mutated *EFCAB7* compared with wild-type. Recent report revealed that *SUFU* missense variant transfection increased the basal expression levels of the key Sonic Hh signaling - target genes *BCL2*, *GLI1*, and *PTCH1* compared to control. It is suggested that missense variant in a gene could modulate other genes in the same signal transduction pathway. Transfection of shRNA *EVC* together with wild-type *EFCAB7* severely perturbed the expression of *EVC2*. Whereas *EVC2* expression was not altered in mutated *EFCAB7* cells, the *EVC2* mRNA expression level was reassessed (20% of normal level) in co-transfection shRNA *EVC* and *EFCAB7* 1171C. It suggested that mutated *EFCAB7* 1171C might increase *EVC2* mRNA expression in *EVC* knockdown cells. Taken together, both *EFCAB7* and *EVC* may be required for normal *EVC2* transcription. Further in vitro experiments are necessary to understand this mechanism.

The ratio of GLI3R/GLI3FL in co-transfected cells (shRNA *EVC* and mutated *EFCAB7*) was significantly different from that in shRNA *EVC* and wild-type *EFCAB7* also imply that mutated *EFCAB7* modulate Hh signaling together with *EVC* depletion.

Conclusions

I found compound heterozygous mutations of *EVC* and *EVC2* genes in Vietnamese families diagnosed with EVC. The relative expression of *EVC/EVC2* mRNA was reduced in these mutation carriers, which revealed that these mutations were disease-causative. Moreover, *EFCAB7* c.1171T>C (p.Y391H) mutation was firstly co-detected in positive-*EVC* cases, which modified *EFCAB7*-*EVC2* interaction and GLI3 activation in Hh signaling. It suggested that *EFCAB7* is a genetic modifier of *EVC*.