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Short report

Delineation of a new fibrillino-2-pathway with evidence for a role of *FBN2* in the pathogenesis of carpal tunnel syndrome

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Abstract

Background: Although carpal tunnel syndrome (CTS) is the most common form of peripheral entrapment neuropathy, its pathogenesis remains largely unknown. An estimated heritability index of 0.46 and an increased familial occurrence indicate that genetic factors must play a role in the pathogenesis.

Methods and Results: We report on a family in which CTS occurred in subsequent generations at an unusually young age. Additional clinical features included brachydactyly and short Achilles tendons resulting in toe walking in childhood. Using exome sequencing, we identified a heterozygous variant (c.5009T>G; p.Phe1670Cys) in the fibrillin-2 (*FBN2*) gene that co-segregated with the phenotype in the family. Functional assays showed that the missense variant impaired integrin-mediated cell adhesion and migration. Moreover, we observed an increased TGF- β signaling and fibrosis in the carpal tissues of affected individuals. A variant burden test in a large cohort of CTS patients revealed a significantly increased frequency of rare (6.7% versus 2.5-3.4%, $p<0.001$) and high-impact (6.9% versus 2.7%, $p<0.001$) *FBN2* variants in patient alleles compared to controls.

Conclusion: The identification of a novel *FBN2* variant (p.Phe1670Cys) in a unique family with early-onset CTS, together with the observed increased frequency of rare and high-impact *FBN2* variants in sporadic CTS patients, strongly suggest a role of *FBN2* in the pathogenesis of CTS.

Introduction

Carpal tunnel syndrome (CTS) is the most common entrapment neuropathy. The disease is caused by an elevated pressure in the carpal tunnel resulting in compression and traction on the median nerve at the level of the wrist. This results in pain and paresthesia, eventually leading to severe functional impairment of the corresponding fingers. CTS is estimated to occur in about 4% of the general population and most often affects people between the ages of 40 and 60¹. Although CTS is common and widely recognized, its precise etiology still remains largely unknown. Several environmental, occupational and anthropometric factors have shown to contribute to the disease risk^{2,3}. CTS can be observed secondary to a variety of conditions including diabetes mellitus, hypothyroidism, amyloidosis, obesity and rare metabolic disorders such as lysosomal storage disorders⁴. In most cases, however, no specific cause or underlying condition can be found. The most characteristic histological finding in idiopathic CTS is non-inflammatory fibrosis and thickening of the subsynovial connective tissue in the carpal tunnel^{5,6}. Moreover, increased expression levels of TGF- β related proteins have been found in SSCT of patients with idiopathic CTS⁷. An estimated heritability index of 0.46 and a familial occurrence in 24-39% of the CTS patients indicate that genetic factors must play a role in the pathogenesis^{8,9}. Targeted and genome-wide association studies revealed a significant association between CTS and common variants (MAF>0.01) in several loci, including genes encoding extracellular matrix proteins (*COL11A1*, *ADAMTS10*, *COL5A1*, *BGN*, *ADAMTS17*) and genes involved in the TGF- β signaling pathway (*LTBP1*, *TGFB3*, *SMAD6*)¹⁰⁻¹². Only the association with *COL11A1* and *ADAMTS10* have been validated in independent patient cohorts. We report here on a family in whom CTS occurred in three subsequent generations at an unusually young age. Additional clinical features included brachydactyly and short Achilles tendons resulting in toe walking in childhood. We show that in this family the phenotype is caused by a pathogenic missense variant in *FBN2* and provide evidence that this gene may also play a role in the pathogenesis of the more common forms of this mononeuropathy.

Methods

Ethical approval and informed consent

This study was reviewed and approved by the Committee for Medical Ethics of the Antwerp University Hospital. The study was also approved by the institutional review boards of each collaborating hospital. The patients provided signed informed consents to participate in the study.

Genetic and functional analysis of the study family

Exome sequencing and genome-wide linkage analysis was performed as described in the methods section in the Supplementary Appendix. Sanger sequencing was used for variant confirmation and co-segregation analysis within the family. Biopsies of the transverse carpal ligament, subsynovial connective tissue and the skin were collected during carpal tunnel release surgery. Immunohistochemical staining, the generation and purification of recombinant FBN2 fragments, multi-angle light scattering (MALS) and cell adhesion- and spreading assays were performed as described in the Methods section in the Supplementary Appendix.

Genetic analysis of sporadic CTS patients

We performed molecular genetic analysis of *FBN2* in a cohort of 216 adult-onset CTS patients. A variant burden test was performed to determine differences in frequencies of *FBN2* variants between the patients and two independent control groups. More details are provided in the Methods section in the Supplementary Appendix.

Results

Clinical findings

We present a family in which early-onset CTS was diagnosed in 10 relatives in 3 subsequent generations, compatible with a pattern of autosomal dominant inheritance with high penetrance. The pedigree of the family is shown in Figure 1A. The affected individuals presented with symptoms of

pain, numbness, and/or decreased grip strength in both hands and fingers. Atrophy of the thenar muscles was observed in most of the affected individuals. The youngest affected individual was 10 months of age (IV-2). The diagnosis of CTS in each individual was based on the clinical symptoms, nerve conduction studies and electromyography. Magnetic resonance imaging of the wrist in patient III.2 did not show evidence for external or anatomical causes for compression of the median nerve. During carpal tunnel release surgery in patients III.2, III.4. and III.5, excessive fibrosis and hypertrophy of the transverse carpal ligament and the subsynovial connective tissue was observed. All affected individuals had short hands (Supplementary figure S1) and feet (brachydactyly), a face with rather short palpebral fissures and in some of them (7/13) short Achilles tendons resulted in toe walking during childhood. Moreover, the affected individuals were rather short in comparison to their unaffected relatives. The mean adult height SDS of the affected individuals was -1.17 SDS. The affected individuals were otherwise healthy.

Identification of a disease-causing missense variant in FBN2

Exome sequencing in two sibs (III.2 and III.5) revealed a novel heterozygous variant (c.5009T>G; p.Phe1670Cys; ENST00000262464.9) in the *fibrillin-2 (FBN2)* gene. This variant co-segregated with CTS in the family (Figure 1A, Figure 1B) with the exception of individual II.6 who has not developed CTS by the age of 55 years but did have the brachydactyly and short palpebral fissures observed in the other affected family members. The variant was also present in IV.1 and IV.7 but these children were still too young (respectively 3 years and 4 years of age) to develop signs of CTS. Genome-wide linkage analysis supported the causal role of the *FBN2* gene by showing significant evidence of linkage with a maximum LOD score of 3.6 for the locus (Supplementary figure S2). In the linkage region, only one other rare variant in the chondroitin sulfate synthase 3 (*CHSY3*) gene was identified (Supplementary table S2). Since most diseases caused by “enzymatic” deficiencies are inherited in an autosomal recessive manner, we believe that a heterozygous variant is reflecting a carrier status rather than disease causality.

The *FBN2* variant is absent in 141,456 genomes and exomes available in the Genome Aggregation Database (GnomAD version 2.1, March 2020) and is predicted to have a deleterious effect on the function of the protein (Supplementary table S1). It replaces a highly conserved phenylalanine with a cysteine residue in the 23rd calcium-binding EGF-like (cbEGF) motif of FBN2 (Figure 1C). Cysteine residues are important for correct folding of fibrillins through the formation of disulfide bonds. Variants that introduce or replace cysteine residues in fibrillin-1 (FBN1), a protein highly homologous to FBN2, are deleterious and can cause various connective tissue disorders, including Marfan syndrome (MFS, [MIM: 154700]).

Functional characterization of the FBN2 variant

FBN2 plays a role in the assembly of elastic fibers during embryonic development and is responsible for regulating the bioavailability of growth factors including transforming growth factor beta (TGF- β)¹³. Tissue fibrosis and increased expression levels of TGF- β related genes have been reported in subsynovial connective tissue of patients with idiopathic CTS⁷. Therefore, we further investigated the effect of the identified *FBN2* variant on extracellular matrix (ECM) structure and function. In affected individuals III.2 and III.5, histological analysis was done on carpal tissues obtained during surgery. Fibrosis was mainly observed in the transverse carpal ligament but was also present in the subsynovial connective tissue (SSCT) where it was more diffuse and less consistent across the images (Figure 2A; Supplementary figure S3). Staining for phosphorylated SMAD2 (pSMAD2) and phosphorylated ERK1/2 (pERK1/2), two direct effectors of the TGF- β pathway, demonstrated an increased TGF- β signaling in the carpal tissues compared to controls. This again was most prominent in the transverse carpal ligaments (Figure 2A). Moreover, quantification revealed a higher percentage of pERK1/2 and pSMAD2 stained cells in the ligament of CTS patients harboring the p.Phe1670Cys variant compared to two CTS patients without a rare FBN2 sequence variant (Supplementary figure S4). To further study the effect of the *FBN2* variant on protein folding and secretion, we produced recombinant human wild-type and mutant FBN2 (PF14-2) fragments (indicated with a curly bracket in Figure 1C). A normal secretion into

the media of mammalian cells was observed for the mutant PF14-2 fragments (Supplementary figure S5). However, size exclusion chromatography revealed an increased disulfide mediated dimer formation of mutant PF14-2 fragments (31% dimer: 69% monomer) compared to wild-type fragments (5% dimer: 95% monomer) (Supplementary figure S6). Molecular weight analysis of the separated peak samples by MALS confirmed the presence of mutant dimers (Figure 2B). These results supported our hypothesis that the variant results in abnormal disulfide bridging. This may interfere with correct protein folding but may also affect cell adhesion and spreading since the variant is located next to a motif that contains an Arg-Gly-Asp (RGD) sequence which is important for integrin-mediated cell adhesion. Indeed, we observed a reduced fibroblast adhesion to both the monomeric and dimeric forms of the mutant PF14-2 fragments compared to wild type PF14-2 fragments (Figure 2C). Analysis of the shape and size of the attached fibroblasts indicated a reduced cell spreading resulting in a more rounded shape on mutant dimer PF14-2 fragments compared to wild-type PF14-2 fragments (Supplementary figure S7).

Role of FBN2 in more common forms of CTS

In a further step, we investigated the role of *FBN2* in the pathogenesis of CTS in a larger population. We sequenced *FBN2* in a cohort of 216 patients of Belgian or Dutch origin with adult-onset CTS using a targeted gene panel. We identified 12 different rare variants ($MAF \leq 0.01$) in 26 unrelated CTS patients (Supplementary table S2). No cysteine or RGD-disrupting variants were identified. We then compared the frequency of *FBN2* variants in the CTS patient cohort to the frequency in an unscreened control dataset of 913 samples that were collected in the context of a study on cardiovascular disorders at the Department of Medical Genetics (Antwerp University Hospital, Belgium) (coverage in dataset was at least 20x for 99.7% of the coding regions of *FBN2*). A variant burden analysis revealed a significantly increased occurrence of *FBN2* variants ($p < 0.001$) in CTS patient alleles (6.7%; 29/432) compared to the control cohort (2.5%; 46/1826). To prove the robustness of this association, we repeated the analysis using 10 randomly selected subsets ($n=216$) of the control group. The association was consistently

significant across all 10 tests (with p -values <0.05). Upon removing the three most common variants (Arg347His, His1381Asn, Ileu2394Thr) from the burden test, we still observed a nominally significant p -value (0.049) for this test. Although this p -value would not withstand multiple testing, it suggests that the significance of the initial burden test is partly, but not entirely, attributable to these three more common variants.

To further validate our findings, we compared the CTS variant frequencies to those of a second independent (and again unscreened) control dataset of 714 WES samples (with 99.13% of the coding regions of *FBN2* at least 20x covered) from healthy individuals (available in the Department of Medical Genetics, Antwerp University Hospital). In this dataset only 48 variants in 1428 alleles (3.4%) were identified, which is also significantly different ($p<0.01$) from the *FBN2* variants in the CTS patient alleles (6.7%; 29/432) (Supplementary table S2). A robustness test again confirmed the statistical consistency of the association (with p -values <0.05). No significant differences were observed between both control groups ($p=0.16$).

Because also more common, high-impact variants (MAF between 0.01-0.05 AND with a CADD score >20) may contribute to CTS, we performed a second variant burden test. Again, a significantly increased occurrence of these variants (depicted in Supplementary table S3) was found within the patients (6.9%; 30/432) compared to the controls (2.7%; 50/1826 (TAAD) and 2.7%; 38/1428 (WES), p -values <0.001). All p -values withstood multiple testing.

Discussion

We identified a rare pathogenic variant in the *FBN2* gene in a three-generation family with early-onset carpal tunnel syndrome (CTS) and brachydactyly as major features. The *FBN2* variant is absent in GnomAD and replaces a highly conserved phenylalanine with a cysteine residue in the 23rd cbEGF domain of the FBN2 protein. Cysteine substitutions or additions that disrupt one of the three disulfide bonds within the cbEGF domains of fibrillin proteins have previously been shown to be pathogenic and disease-causing by affecting normal protein function^{14,15}. Heterozygous pathogenic *FBN2* variants have

mainly been identified so far in individuals with congenital contractural arachnodactyly (CCA, [MIM: 121050]). This rare connective tissue disorder is characterized by joint contractures, arachnodactyly, severe kyphoscoliosis, crumpled ears and a long and slender build. It shares overlapping features with Marfan syndrome (MFS, [MIM 154700]), which is caused by pathogenic variants in the paralogous *FBN1* gene. Other *FBN1*-related disorders such as acromicric dysplasia (ACMICD, [MIM: 102370]), geleophysic dysplasia 2 (GPHYSD2, [MIM: 614185]) and Weill-Marchesani syndrome type 2 (WMS2, [MIM: 608328]) have opposite phenotypic features with short stature and brachydactyly. Pathogenic *FBN1* variants that cause these “brachydactyly and/or short stature” phenotypes are all clustered in or nearby the cell adhesion region of *FBN1* (TB4-TB5)¹⁶. Interestingly, the variant that we identified in our family resides in the same, corresponding region of the *FBN2* protein. The remarkable observation that heterozygous variants in *FBN1* can cause opposite phenotypes (short stature with brachydactyly versus tall stature with arachnodactyly) is now also true for the *FBN2* gene where CCA represents “the arachnodactyly phenotype” and the family delineated in this study the “brachydactyly phenotype”. Early-onset CTS is an important feature in our family but it has also been reported in *FBN1*-related disorders¹⁷⁻²⁰. The high incidence and early onset of CTS in our family is remarkable. To better understand the role of the identified *FBN2* variant in the pathogenesis of CTS, we evaluated the carpal tissues collected during surgery and performed additional assays on the mutant protein. Histological analysis revealed fibrosis and increased collagen deposition, which was most apparent in the transverse carpal ligament. Immunohistochemical staining of carpal tissues indicated increased TGF- β signaling in comparison to both controls and unrelated individuals with only CTS. For study of the protein we made mutant *FBN2* (PF14-2) fragments. We did observe a normal secretion into the media of mammalian cells but noticed an increased disulfide-mediated dimer formation of the mutant PF14-2 fragments. Because the p.Phe1670Cys variant is localized next to a motif that contains an RGD sequence, which is important for integrin-mediated cell adhesion, we also investigated the effect of the mutant fragment on cell adhesion. A reduced cell adhesion and spreading of fibroblasts to both

the monomeric and dimeric forms of the mutant PF14-2 fragments in comparison to wild-type fragments was observed.

All these experiments together indicate that the p.Phe1670Cys variant affects the normal structure and function of the protein. The observation of increased dimerization of the mutant PF14-2 fragment suggests that the variant creates new disulfide-mediated intermolecular links, however, electron microscopy studies should be performed to confirm that the *FBN2* variant does affect microfibril formation and/or aggregation *in vivo*. We could not find evidence that the variant interferes with normal secretion of FBN2 into the extracellular matrix. However, we found evidence that the mutant protein affects normal integrin-mediated cell adhesion in the ECM and observed increased TGF- β signaling, resulting in fibrosis of the carpal tissues with entrapment of the median nerve as a consequence. Interestingly, a direct link between disrupted RGD-integrin interactions and TGF- β signaling has been reported³⁸. Moreover, the mechanism of abnormal RGD-integrin interactions triggering TGF- β -related tissue fibrosis has also been hypothesized for the development of fibrotic skin in patients with stiff skin syndrome (SSKS, [MIM 184900]), another connective tissue disorder caused by (most often cysteine replacing or introducing) variants in the same cell-adhesion region of the FBN1 protein^{19,21}.

Since TGF- β related tissue fibrosis is the most common histopathological finding in carpal tissues of idiopathic CTS patients⁷, we hypothesized that *FBN2* may be (one of the) the missing link(s) between these (immuno)histological findings and the occurrence of common or so-called idiopathic CTS in sporadic patients. We therefore decided to sequence *FBN2* in a cohort of patients with idiopathic CTS. This analysis revealed a significant association between rare (and high-impact) *FBN2* variants and CTS. Interestingly, some *FBN2* variants that were present in the patient cohort were also found in individuals in the control groups. This can be explained by the fact that we used a study design with unscreened controls²². It is therefore possible that also individuals in the control cohort carry *FBN2* variants that contribute to the development of CTS. The *FBN2* variants that we identified in the cohort of individuals with common CTS were not clustered but dispersed all over the gene. They most likely

do not have the same mechanistic effect as the p.Phe1670Cys variant. One may speculate that these variants cause small changes in the conformation of the microfibril, predisposing to TGF- β related tissue fibrosis. Together with other risk factors, such as work-related repetitious heavy load on the wrists, this may eventually lead to CTS later on in life. However, we do recognize that functional studies are necessary to prove this hypothesis.

In conclusion, we have identified a new fibrillino-2-pathway that together with CCA recapitulates the theme of opposite phenotypes seen in the fibrillino-1-pathies. If CCA can be considered as the mild counterpart of Marfan syndrome, then the disorder in this family can be viewed as the mild equivalent of acromicric dysplasia and geleophysic dysplasia. Identification of additional families or individuals with the same “disorder” would further reinforce this statement. But we suspect that the ascertainment will be difficult because of the rather mild phenotype in “affected” individuals who therefore may not come to the attention of clinical geneticists dealing with rare and complicated heritable disorders. Screening a cohort of individuals with brachydactyly of unknown cause, may be a more successful approach. Our study not only identified a rare condition characterized by early-onset CTS but also provided evidence for a role of FBN2 in the pathogenesis of common forms of CTS. Whether all these new insights will result in the development of new therapeutic and preventive measures for CTS, such as the (local) application of TGF- β antagonists or integrin-modulating substances, remains an open question, warranting further research.

Author contributions

G.M. devised the project, the main conceptual ideas and proof outline. S.P. contributed to the design of the study, worked out almost all of the technical details, and performed all experiments. E.B., W.V.H., B.L., C.B and S.C. supervised the work, helped with working out technical details and contributed to the interpretation of the results. E.F, A.K, G.V. and S.C. performed and supervised (bioinformatics) data analysis and statistical analyses. C.H., M.L., W.J. were involved in the sample preparation, optimization of histopathological staining procedures, technical support and interpretation of the data. A.D., P.H.,

F.V., J.N. collected clinical data and provided blood and tissue samples of the patients. All authors discussed the results and implications and commented on the manuscript.

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Competing Interests Statement

The authors have declared that no conflict of interest exists.

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Legends to figures

Figure 1. Pedigree of the Family with early-onset Carpal tunnel syndrome and Identification of the Fibrillin-2 (*FBN2*) Variant. A) pedigree of the family. The presence or absence of the *FBN2* variant is indicated with respectively a (+) or (-) symbol. B) chromatograms of part of the DNA sequences of *FBN2*. The heterozygous T to G transversion (black arrowhead) is only observed in affected family members. C) the domain structure of *FBN2* and a schematic representation of the 23rd calcium binding epidermal growth factor domain (cbEGF). The variant replaces a phenylalanine by a cysteine residue at amino acid position 1670 (indicated with a red star) in the 23rd cbEGF. The three intradomain disulfide bridges of cbEGF23 are organized in a 1-3, 2-4, 5-6 arrangement and are indicated with red dotted lines. A wild-type and mutant *FBN2* protein fragment (PF14-2) containing the 23rd cbEGF domain, the fourth TGF- β -binding like domain (TB4), which possesses an Arg-Gly-Asp (RGD) site for cell adhesion, and five adjacent cbEGF domains was produced for functional studies. The domains of the PF14-2 fragment are indicated with a curly bracket.

Figure 2. Functional Characterization of the *FBN2* Variant. A) Masson's trichrome stain (scale bar=200 μ m), pSMAD2-staining and pERK1/2 staining (scale bars=100 μ m) of the transverse carpal ligament (TCL) in a patient (II.6) and a control. Black arrowheads in the magnification boxes indicate positively stained fibroblasts. In the graphs below, the quantification results of the pERK1/2 and the pSMAD2 staining are depicted. Percentage of positively stained cells in each individual (dots) and the mean percentages of individuals within the same group (lines) are shown. A two-way mixed effects model measured a high interobserver agreement between observer 1 (blue) and observer 2 (red) (ICC =0.887, 95% confidence interval 0.545-0.972). B) the combined results of the light scattering traces (left y-axis) and molecule mass traces (right y-axis) of the multi-angle light scattering (MALS) analysis of the wild-type (blue), mutant monomer (red) and mutant dimer (yellow) PF14-2 samples. C) adhesion of human dermal fibroblast to wild-type, mutant monomer, and mutant dimer PF14-2 fragments at different concentrations (0-10 μ g/ml) after 2 hours of adhesion. Values were normalized to wild-type binding at 10 μ g/ml (100%). The maximum binding capacities (Rmax) of the fibroblasts to wild-type, mutant monomer and mutant dimer PF14-2 fragments are depicted in the right graph. Statistical analysis, using one-way ANOVA followed by a Tukey's multiple comparison test, indicates a significant reduction in Rmax to both mutant monomer (Tukey's adjusted p-value=0.0078, mean diff=22.87, 95% CI=8.012-37.3) and mutant dimer PF14-2 fragments (Tukey's adjusted p-value<0.0001, mean diff=51.55, 95% CI=36.69-66.41) compared to wild-type PF14-2 fragments. In both graphs, values represent means \pm s.e.m. (T bars) of three biologic replicates.