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Dupuytren's disease susceptibility gene, *EPDR1*, is involved in myofibroblast contractility

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ABSTRACT

Background: Dupuytren's Disease is a common disorder of the connective tissue characterized by progressive and irreversible fibroblastic proliferation affecting the palmar fascia. Progressive flexion deformity appears over several months or years and although usually painless, it can result in a serious handicap causing loss of manual dexterity. There is no cure for the disease and the etiology is largely unknown. A genome-wide association study of Dupuytren's Disease identified nine susceptibility loci with the strongest genetic signal located in an intron of *EPDR1*, the gene encoding the Ependymin Related 1 protein.
Objective: Here, we investigate the role of EPDR1 in Dupuytren's Disease.
Methods: We research the role of EPDR1 by assessing gene expression in patient tissue and by gene silencing in fibroblast-populated collagen lattice (FPCL) assay, which is used as an in vitro model of Dupuytren's contractures.
Results: The three alternative transcripts produced by the *EPDR1* gene are all detected in affected Dupuytren's tissue and control unaffected palmar fascia tissue. Dupuytren's tissue also contracts more in the FPCL paradigm. Dicer-substrate RNA-mediated knockdown of EPDR1 results in moderate late stage attenuation of contraction rate in FPCL, implying a role in matrix contraction.
Conclusion: Our results suggest functional involvement of EPDR1 in the etiology of Dupuytren's Disease.
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1. Introduction

Dupuytren's Disease (Dupuytren) or Dupuytren's Contracture (OMIM: 126900) is a common disorder of the connective tissue characterized by progressive and irreversible fibroblastic proliferation affecting the palmar fascia. Shortening of the thickened palmar fascia results in progressive digital flexion deformity. The earliest sign of Dupuytren is the formation of fibrous nodules in the palm. Progressive flexion deformity may appear over several months or years, typically affecting the ring finger and the little finger [1]. Dupuytren is often bilateral and, although usually painless, it can result in a serious handicap causing loss of manual dexterity and the inability to touch, stroke, or shake hands in a normal manner. The average age of onset is 60 years and the incidence increases with increasing years. Standard treatment consists of surgical excision of pathologic nodules and cords or percutaneous division but other treatment modalities are emerging [2–4]. However, there is no cure for the disease, the origin of the disease is largely unknown, and there is a high recurrence rate after surgery [5,6].

The reported prevalence of Dupuytren varies between 0.2% and 56% depending on methods used [7,8]. A clinic-based study highlighted the gender differences of the disease with a fourfold male preponderance, especially for those with a relatively early onset (age <45 years) or for patients treated surgically [9]. The sex difference in prevalence diminishes with increasing age. In the UK, the incidence of new consultations with a family physician for Dupuytren is 34/100,000 men annually [10].

The histological and biochemical alterations in Dupuytren-affected tissue are similar to those in the active stages of connective tissue wound repair; high numbers of fibroblasts, increased deposition of extracellular matrix proteins (especially collagen) and the presence of contractile myofibroblasts. The latter

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is a population of cells involved in the granulation stage of wound healing responsible for wound contraction [11], and are recognized by α-smooth muscle actin (α-SMA) expression. The expression of α-SMA can be increased by Transforming Growth Factor, Beta 1 (TGFβ1), by stimulating the conversion of fibroblasts to myofibroblasts [12–14]. Histological analysis of disease tissue shows a corresponding decrease in the amount of type III collagen as a percentage of the total collagen with disease progression [15]. To measure the contractility of (myo)fibroblasts, fibroblast-populated collagen lattice (FPCL) contraction assays are used in functional Dupuytren research [16–19] to assess the contractility of fibroblasts to particular stimuli, including inflammatory cues [20]. Despite these efforts, the pathophysiological basis of Dupuytren is incompletely understood.

A genome-wide association study (GWAS) for Dupuytren identified nine susceptibility loci, showing robust genetic evidence with relatively high odds ratios (ranging from 0.72–1.98) compared to other complex human traits [21]. The results further implicated a potential involvement of Wnt signalling in disease and demonstrate that genetic variants play an important role in the etiology of Dupuytren in patients with European ancestry [22]. The most significant finding was at single nucleotide polymorphism (SNP) rs16879765, located in an intron of *EPDR1*, the Ependymin Related 1 gene, with $p = 5.6 \times 10^{-39}$ and an odds ratio of 1.98 (CI 1.78–2.18). This finding was confirmed in an independent study [23].

Little is known about *EPDR1*. The gene encodes the Ependymin Related 1 protein, and is additionally known as *MERP1,* mammalian ependymin related protein 1, and as *UCC1,* upregulated in colorectal cancer 1. Ependymins were first described as the predominant constituent of the cerebrospinal fluid of teleost fish, such as gold fish and rainbow trout (reviewed in [24]), produced by leptomeningeal fibroblasts (reviewed in [24]). It is reported that a calcium-induced conformational change in ependymin is important for its interaction with the extracellular matrix [25], particularly by association with collagen [26,27]. It was therefore subsequently hypothesized that ependymin is essential for cell-to-cell contact as a (anti) cell adhesion molecule [25]. In humans *EPDR1* gene expression is broadly expressed throughout tissues, with a particular high expression in brain, in addition to contractile tissues, such as muscle tissue and the heart [28–30].

2. Materials and methods

2.1. Cell line collection

Primary fibroblasts derived from Dupuytren's tissue (D), normal palmar fascia tissue (PF) and control tissue (CT) were obtained at the Lawson Health Research Institute, London, Ontario Canada. We collected 4 allogeneic isolates from unrelated individuals per group. All tissues were surgically resected from patients who were undergoing primary surgery at St. Joseph's Hospital, London, Ontario, as described previously [31]. Dupuytren's cells were derived from fibrotic palmar fascia, whereas PF and CT fibroblasts were both derived from visibly unaffected, palmar fascia. PF fibroblasts are derived from the palmar fascia of an individual with Dupuytren's in an adjacent digit, whereas CT fibroblasts are from the palmar fascia of an individual with Carpal Tunnel syndrome. All of these fibroblasts are derived from the same tissue source, palmar fascia. Patient-derived tissues were cultured up to a maximum of 8 passages in all protocols. Primary human neonatal foreskin fibroblasts (PCS-201-010) were purchased from ATCC (Manassas, VA, USA) and were cultured up to 9 passages. All cells were grown in Fibroblast Basal Medium (FBM, PCS-201-030; ATCC, Manassas, VA, USA) supplemented with Fibroblast Growth kit-Low Serum (PCS-201-041; ATCC, Manassas, VA, USA) and 10 units/mL penicillin-streptomycin (15140-122; Life Technologies, Carlsbad, CA, USA) at 37 °C and 5% (vol/vol) CO_2 in a humidified incubator.

2.2. Gene expression results from Genevar database

In order to consider genetic effects of the disease locus (SNP rs16879765) on local (*cis*) gene expression levels we used the available Genevar database version 3.3.0 [32] with the GenCord dataset [33], in which gene expression was determined by Illumina HumanWG-6 v3. We restricted our analyses to the expression data of fibroblasts (GenCord-F) as being the most-related cell type to Dupuytren affected tissue.

2.3. RNA isolation and cDNA conversion

Adherent fibroblast cultures were trypsinized, re-suspended in FBM and pelleted prior to cell lysis with buffer RLT from the RNeasy Mini Kit (74104; Qiagen, Venlo, The Netherlands). Lysed samples were homogenized with QIAshredder columns (79654; Qiagen, Venlo, The Netherlands) and stored at −80 °C or processed immediately per manufacturer's protocol. Purified RNA was quantified using the Quant-iT Ribogreen assay (R11490; Life Technologies, Carlsbad, CA, USA) and RIN values were assessed using the Agilent Bioanalyzer 2100 (G2940CA; Agilent Technologies, Santa Clara, CA, USA). Collagen gels from select FPCL experiments were suspended directly in TRIzol (15596-026; Life Technologies, Carlsbad, CA, USA) and incubated at room temperature until completely dissociated. Samples were stored at −80 °C until ready for extraction. After thawing, extraction occurred by adding 0.2x volume of chloroform to the TRIzol suspension. Samples were mixed and centrifuged at 12,000*g* for 15 min at 4 °C. The top aqueous phase was combined with 1 vol of 70% ethanol and loaded onto RNeasy spin columns. RNA purification with RNeasy was performed as described above. 100–250 ng of RNA for each sample was used in cDNA synthesis with the High-Capacity RNA-to-cDNA Kit (4387406; Life Technologies, Carlsbad, CA, USA) per manufacturer's protocol.

2.4. RT-PCR gene expression

EPDR1 transcript presence was assessed via RT-PCR. Custom cDNA primers were generated using the NCBI primer blast tool and subsequently synthesized by IDT DNA, (Coralville, Iowa, CA USA). The GAPDH positive control primer pair was obtained from Walker et al. [34] (forward: 5′-AATCCCATCACCATCTTCCA-3′, reverse: 5′-TGGACTCCACGACGTACTCA-3′). Three EPDR1 mRNA variants were detected by two primer pairs: pair 1 (forward: 5′-TCAGATTGACCAAGCCACCAA-3′, reverse: 5′-ACGTGCTTG-GAGGGGTAAAC-3′) detects mRNA variant 1 and 3, and pair 2 (forward: 5′- GATCTCAAAAGCGGCAGAGG-3′, reverse: 5′- CGTGCTTGGAGGGG TAAACA-3′) detects mRNA variant 1 and 2. Synthesized cDNA was used in conventional touchdown PCR with 1uM of the above primers for gene expression analysis. The PCR occurred with 1x MyFi PCR Master Mix (BIO-25049, Bio Line, London UK) and the BioRad C1000 thermocycler (185-1048, BioRad, Hercules, CA USA). Select PCR templates were treated with Exo-Sap-IT (78200, Affymetrix, Santa Clara, CA USA) and were subsequently Sanger sequenced to ensure that the proper product was detected.

2.5. Quantitative real time-PCR

Relative *EPDR1* gene expression was assessed with Primetime qPCR Assay Hs.PT.58.38898209 and Hs.PT.58.20021550 (IDT DNA, Coralville, Iowa, USA), IDT DNA Primetime qPCR assays for *GAPDH*

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