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The Genetic Basis Of Dupuytren's Disease

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THE GENETIC BASIS OF DUPUYTREN'S DISEASE.

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Dupuytren's disease is a common heritable connective tissue disorder of poorly

understood etiology. It is thought that oxidative stress pathways may play a critical role

in the development of Dupuytren's disease, given the various disease associations that

have been observed. We sought to sequence the mitochondrial and nuclear genomes of

patients affected with Dupuytren's disease using next-generation sequencing technology

to potentially identify genes of potential pathogenetic interest. Additionally, we sought to

compare the genomes between diseased tissue and blood to look for potential somatic

mutations in the diseased tissue. Upon sequencing and subsequent bioinformatics

analysis, no differences were observed in the mitochondrial and nuclear genomes

between diseased tissue and blood. However, when we compared the nuclear genome of

our patient cohort to a control cohort, we observed some significant genetic differences.

We identified a number of single nucleotide polymorphisms (SNPs) and mutations that

are potentially associated with Dupuytren's disease. Additionally, 2 novel mutations

leading to amino acid changes were present in all patients in the disease cohort but not in

the controls. These were mutations in MUC4, encoding mucin 4, and PPP1R32, encoding

protein phosphatase 1 regulatory subunit 32. These genes warrant further investigation in

the pathophysiology of Dupuytren's disease.

ACKNOWLEDGEMENTS

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INTRODUCTION

Dupuytren's disease is a common heritable connective tissue disorder characterized by fibrosis of the palm, causing flexion deformities of the digits that ultimately impair hand function. The disease progression is characterized classically by the initial appearance of palmar nodules characterized by high cellularity and cell proliferation followed by formation of cords in the palm and digits (Figure 1). Dupuytren's disease has a propensity for affecting Caucasian populations, especially those of northwestern European origin, as well as the male gender, heavy smokers, and those having manual labor as an occupation.[1] The average age of onset of disease is 60 years, with an increasing incidence with increasing age.[2] A strong genetic predisposition for this disease has also been well established, as demonstrated by concordance rates in twin studies and a predilection for familial clustering.[3] Even though this disease has been known since at least the 1830s, when Baron Guillaume Dupuytren described the clinical features of the disease in a famous lecture,[4] the etiology of this disease remains poorly understood.

Dupuytren's disease affects the fascia, or aponeurosis, of the palm. Histologically, the diseased tissue contain abundant contractile myofibroblasts,[5] which are connected intimately with the surrounding extracellular matrix.[6] Myofibroblasts are cells involved in the granulation stage of wound healing that cause wound contraction[7] and the alignment of the myofibroblasts along major lines of stress is thought to be the physical mechanism underlying the digital contractures observed in this disease.
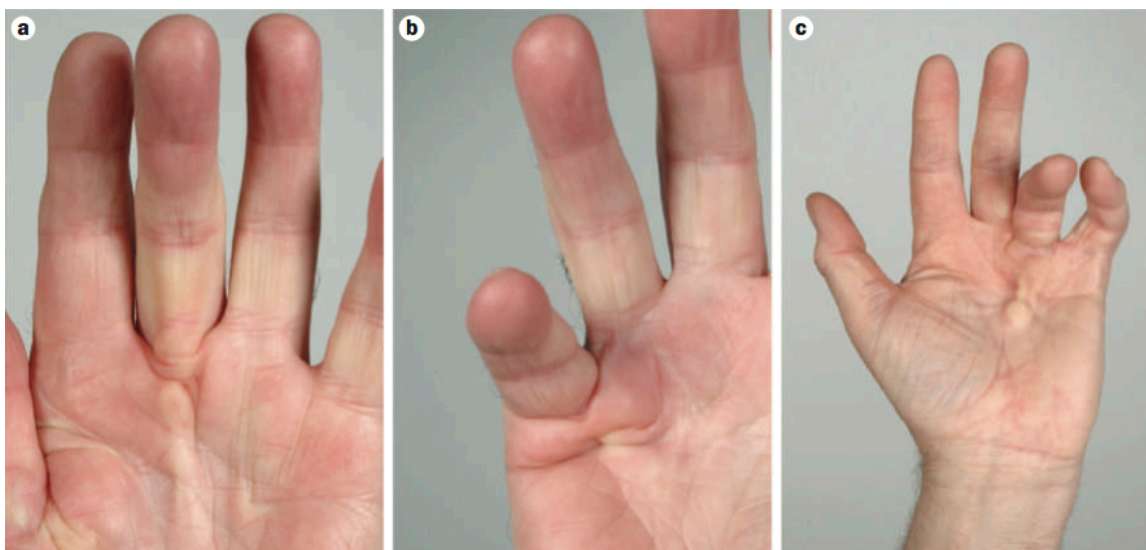
Figure 1. The clinical presentation of Dupuytren's disease. Early stage disease (a) with prominent cord and small nodules, followed by more advanced stage (b) with dimpling at the base of the digit, and subsequently advanced stage disease (c) with severe deformity. (From Shih B, Bayat A. Scientific understanding and clinical management of Dupuytren disease. *Nature reviews. Rheumatology.* Dec 2010;6(12):715-726)

Many treatment options exist for the treatment of symptomatic Dupuytren's disease, which typically involves at least a 40° digital flexion contracture. Surgery is currently the mainstay of treatment for Dupuytren's disease and, in increasing invasiveness, include fasciotomy, fasciectomy, and dermofasciectomy. Fasciotomy is performed percutaneously and is a surgical division of the cord causing the contracture. Fasciectomy involves resection of the diseased cord. Dermofasciectomy is the most invasive of these procedures and involves a fasciectomy with additional resection of the overlying skin, requiring skin grafting to cover the resulting defect. Nonsurgical interventions include collagenase injections,[8] radiotherapy,[9] and steroids.[10] Regardless of treatment modality, the main complication of treatment for Dupuytren's disease is the

high rate of disease recurrence, which is the reappearance of disease in the affected region following treatment. Given the unknown etiology of this disease, the treatment remains palliative and is directed by the severity of symptoms. A more complete understanding of the etiology of Dupuytren's disease could potentially lead to the development of targeted therapies.

The leading hypotheses on the etiologic mechanism of Dupuytren's disease include altered immune response causing abnormal wound repair and abnormalities in the oxidative stress pathway.[11] Autoantibodies against collagen molecules are detected at higher levels from patients with Dupuytren's disease compared to those without the disease.[12,13] Certain HLA haplotypes also appear to be associated with the disease.[14,15] Additionally, an increased number of macrophages[16] and Langerhans cells[17] in Dupuytren's-affected tissue compared to controls have been observed. Together, these observations suggest a role for immune dysregulation in the development of Dupuytren's disease.

Many observations also suggest a role for oxidative stress pathway dysregulation in the development of this disease, with some recent reports suggesting that mitochondrial abnormalities are associated with Dupuytren's disease. In particular, susceptibility to disease has been associated with a single nucleotide polymorphism (SNP) in the mitochondrial gene encoding Zf9, a transcription factor,[18] and with a heteroplasmic mutation in the mitochondrial 16S ribosomal RNA region.[19] Mitochondria play a critical role in regulating reactive oxygen species generation, with defective mitochondria capable of generating excess reactive oxygen species due to electron leak.[20] Dupuytren's disease has also been clinically associated with comorbidities that include

epilepsy[21] and diabetes,[22] both of which interestingly have been associated with mutations of mitochondrial DNA.[23,24] Social factors including smoking and alcohol use have been associated with Dupuytren's disease as well,[25] which have also been associated with mitochondrial abnormalities.[26,27] Dupuytren's tissue has an increased propensity for oxygen free radical formation compared to control tissue.[28] Oxygen free radicals can in turn stimulate proliferation of fibroblasts.[29]

In addition to mitochondrial studies, a limited number of nuclear genomic studies have been performed. However, the interpretation of these preliminary genetic studies remains difficult and the lack of a clear inheritance pattern of Dupuytren's disease argues against a monogenic cause but rather suggests a multifactorial etiology. A recent meta-analysis combining data from both RNA expression profiling and genome-wide association studies has highlighted two potential susceptibility genes for development of Dupuytren's disease: MAFB and PRKX.[30] To our knowledge, complete genomic sequencing has yet to be utilized in the study of this disease.

Given that oxidative stress pathways appear to play a critical role in the development of Dupuytren's disease, we sought to better characterize the role of the mitochondria in the development of disease. Variations in mitochondrial DNA are thought to predispose to common diseases.[31] Due to the recent advances in genomic sequencing, giving rise to the so-called "next-generation sequencing" making high-throughput sequencing a new reality, we sought to utilize these novel genomic analytical tools to elucidate the genetic basis of Dupuytren's disease. Among patients with Dupuytren's disease, we sought to sequence both mitochondrial DNA as well as nuclear DNA, given the interactions between the cell nucleus and mitochondria. Additionally,

most proteins involved in mitochondrial metabolism are nuclear-encoded. As such,

mutations in certain nuclear genes can mimic features seen in the presence of

mitochondrial DNA defects.

STATEMENT OF PURPOSE

The broad objectives of this research are to better characterize the etiology of

Dupuytren's disease with the ultimate goal of optimizing treatment for this disease. Our

specific research goal is to identify genetic mutations that play a critical role in the

development of Dupuytren's disease.

HYPOTHESIS

Due to prior studies suggesting the crucial role of oxidative damage in the development

of Dupuytren's disease and the well-characterized role of mitochondria in regulating

biological oxidation, we hypothesize that there may be unique mitochondrial mutations or

nuclear mutations present in tissue affected with Dupuytren's disease.

RESEARCH AIM

We seek to sequence and analyze the mitochondrial and nuclear genomes of patients

affected with Dupuytren's disease to identify genes of pathogenetic interest. In addition,

we seek to identify potential differences in the genomes between diseased tissue

compared to cells circulating in venous blood.

METHODS

Patient Cohort

We identified 23 consecutive patients undergoing open fasciectomy for treatment of symptomatic Dupuytren's disease at the Yale-New Haven Hospital from 2012-2013 (Figure 2). Informed consent was obtained from patients involved in this study, which was approved by the Human Investigation Committee at the Yale University School of Medicine. We collected the diseased palmar fascia that was resected during the standard fasciectomy. All collected fascia were deemed to be disease-affected, as indicated by experienced hand surgeons under visual examination with magnifying surgical loupes. All fascial specimens were collected in RNAlater RNA Stabilization Reagent (QIAGEN, Venlo, Netherlands) and stored immediately in a -80° freezer until DNA extraction. Approximately 1 mL of venous blood were collected from all patients at the time of the surgical fasciectomy in lavender Vacutainer Blood Collection Tubes (BD, Franklin Lakes, NJ) and were stored immediately in a -80° freezer until DNA extraction.

DNA Extraction

DNA from all tissue and blood samples were extracted using the DNeasy Blood & Tissue Kit (QIAGEN, Venlo, Netherlands) per manufacturer's protocol.
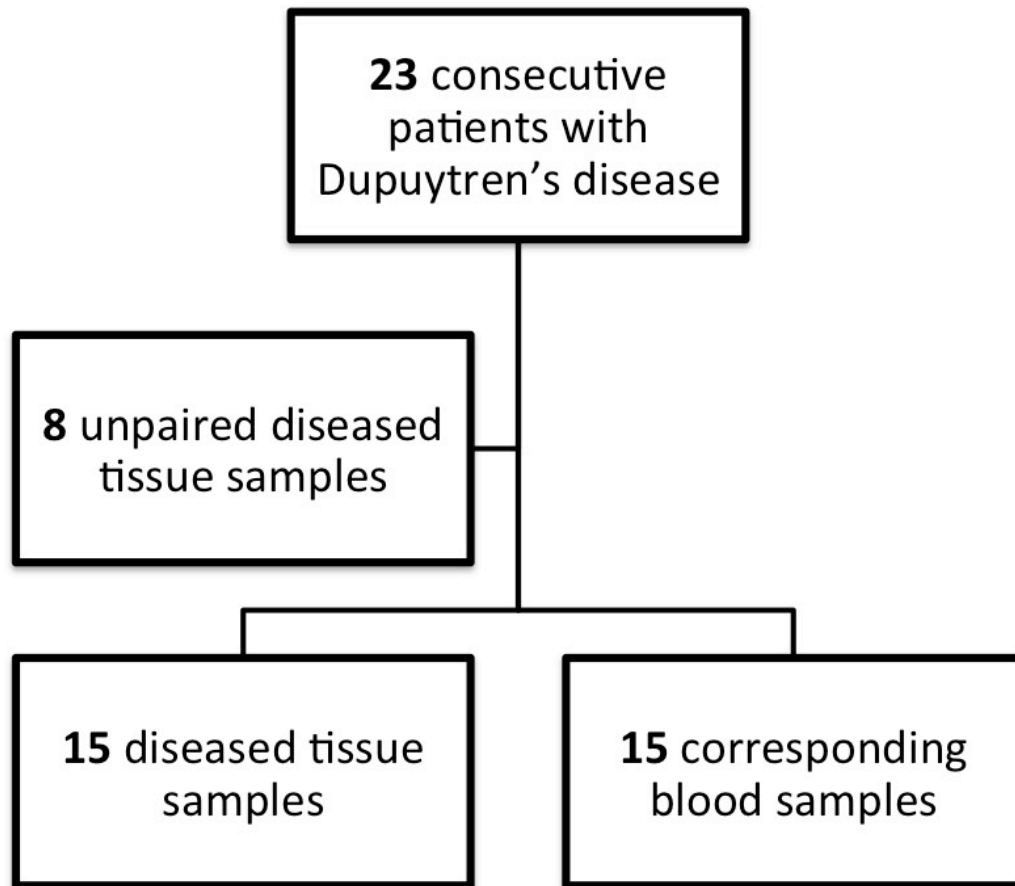
Figure 2. Distribution of samples from patient cohort; all samples underwent DNA extraction and subsequent mitochondrial sequencing and whole-exome sequencing.

Mitochondrial Genome Sequencing

The extracted DNA samples were subjected to agarose gel and NanoDrop (Thermo Scientific, Waltham, MA) OD ratio tests to confirm the purity and concentration prior to amplification of mtDNA by proprietary methods (Otogenetics Corporation, Norcross, GA). The purified mtDNA was subjected to fragmentation using Covaris (Covaris, Inc., Woburn, MA) and profiled using 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Illumina libraries were made from qualified fragmented mtDNA using NEBNext reagents (New England Biolabs, Ipswich, MA) and the resulting libraries were tested for size distribution and concentration by the 2100 Bioanalyzer. The samples were then sequenced on an Illumina HiSeq2000 which generated paired-end reads of 100 nucleotides. Data was analyzed for data quality, coverage, SNP, insertions, and deletions using the platform provided by DNAnexus (DNAnexus, Inc., Mountain View, CA) using default settings and the mutation percentage set at 1% to include low-frequency variants.

Nuclear Whole-Exome Sequencing

High-Throughput Barcoded Sample Preparation:

For each sample, 1 μg of genomic DNA was sheared to a mean fragment length of 140 base pairs using focused acoustic energy (Covaris E210, part #5000003). Fragmented DNA samples were then transferred to a 96-well plate and library construction was completed using a liquid handling robot (Caliper

Sciclone, part #SG3-11020-0100). Magnetic AMPure XP beads (Beckman Coulter, part #63882) were used to purify the sheared DNA samples and remained with the sample throughout library construction. Following each process step, DNA was selectively precipitated by weight and re-bound to the beads through the addition of a 20% polyethylene glycol, 2.5 M NaCl solution. Following fragmentation, T4 DNA polymerase and T4 polynucleotide kinase blunt ended and phosphorylated the fragments. The large Klenow fragment then added a single adenine residue to the 3' end of each fragment and custom adapters (IDT) were ligated using T4 DNA ligase. Adapter-ligated DNA fragments were then PCR amplified using custom-made primers (IDT). During PCR, a unique 6 base index was inserted at one end of each DNA fragment. Sample concentration and insert size distribution were determined using the Caliper LabChip GX system (Caliper, part #122000/B). Samples yielding at least 1 μg of amplified DNA were used for capture.

Automated Sample Exome Capture:

500 ng of prepared genomic DNA library was lyophilized with Cot-1 DNA and custom adapter blocking oligos (IDT). The dried sample was reconstituted according the manufacturer's protocol (Roche/Nimblegen), heat-denatured, and mixed with biotinylated DNA probes produced by Nimblegen (Nimblegen, SeqCap EZ Exome version 2, part #05860504001). Hybridizations were performed at 47°C for 68 hours. Once the capture was complete, the samples were mixed with streptavidin-coated beads and washed with a series of stringent

buffers to remove non-specifically bound DNA fragments. The captured

fragments were PCR amplified and purified with AMPure XP beads. Capture

efficiency was evaluated by quantitative PCR (Roche Light Cycler 480, part

#5015243001). Equal amounts of pre- and post-capture libraries were evaluated at

4 sites to confirm successful exome enrichment and at 2 other sites to show non-

exome de-enrichment in the captured sample relative to the pre-capture library.

Samples that met appropriate cut-offs for both were quantified by qRT-PCR using

a commercially available kit (KAPA Biosystems, part #KK4601) and insert size

distribution determined with the LabChip GX. Samples showing a yield of at least

0.5 ng/μl were used for sequencing.

Flow Cell Preparation and Sequencing:

Sample concentrations were normalized to 2 nM, combined accordingly

for the number of samples to be sequenced per lane, and loaded onto Illumina

version 3 flow cells at a concentration that yielded 170-200 million passing filter

clusters. The samples were sequenced using 75 base pair paired end sequencing

on an Illumina HiSeq 2000 according to Illumina protocols. The 6 base pair index

was read during an independent sequencing read that automatically followed the

completion of read 1 and used an additional sequencing primer (Illumina, part

#15019606). Data generated during sequencing runs were simultaneously

transferred to a high performance computing cluster.

In-Run Sequencing Quality Control:

A positive control (a prepared bacteriophage Phi X library) provided by Illumina was spiked into every lane at a concentration of 0.3% to monitor sequencing quality in real time via information displayed by the instrument's Sequence Analysis Viewer. Signal intensities, Q30 values (an estimate of the number of errors per thousand bases), and Phi X error rates were monitored periodically to assess the quality of the ongoing run.

Data Analysis and Storage:

Signal intensities were converted to individual base calls on the machine during each run using the system's Real Time Analysis (RTA) software. Base calls were transferred from the machine's dedicated personal computer to the Yale High Performance Computing cluster via a 1 Gigabit network mount for downstream analysis. Primary analysis - sample de-multiplexing and alignment to the human genome - was performed using Illumina's CASAVA 1.8 software suite. The data were accepted if the sample error rate was less than 2%.

Bioinformatics Analysis

Analysis of the sequencing data was performed using open-source software. Data were pre-processed using Btrim to remove low quality regions and trim off adaptors.[32] Mapping was then performed using the Burrows-Wheeler Alignment tool[33] and alignment performed using SAMtools.[34] Subsequent annotations were performed using personally developed software. For both the mitochondrial genome and whole-exome

analyses, each patient's diseased tissue and blood samples were directly aligned and

compared to each other to evaluate for any genetic differences. For the whole-exome

analyses, an additional comparison was made between the diseased tissues from the

Dupuytren's disease cohort and the control tissues. The mitochondrial consensus

sequences were also aligned to the reference mitochondrial genome, the revised

Cambridge Reference Sequence (National Center for Biotechnology Information

reference sequence NC_012920). The nuclear genomes were aligned to the reference

human genome hg19 from the University of California Santa Cruz (National Center for

Biotechnology Information reference sequence GRCh37).

RESULTS

Paired Comparisons Between Mitochondrial Diseased Tissue and Blood

Upon comparing the consensus sequences of the paired tissue and blood samples, we did not observe any differences in the mitochondrial genomes for all patient pairs. Additionally, no significant differences in degree of heteroplasmy were observed between the paired samples.

Identification of Unique Mitochondrial Genome Mutations

When we compared the mitochondrial consensus sequences of diseased tissue from each individual patient to the reference genome, we identified several differences common to patients in our cohort (Table 1). Most of these mutations are common polymorphisms, including A263G, A750G, A1438G, A4769G, A8860G, and A15326G. Excluding common polymorphisms, the only notable difference in the genomes between our patient cohort and the reference sequence is the insertion of either a C or CC at position 309 in the mitochondrial genome. This was observed in all patients in our patient cohort.

Mutation	Mitochondrial region
A263G	Control region
Insertion C/CC at 309	Control region
A750G	12S RNA
A1438G	12S RNA
A4769G	NADH dehydrogenase 2
A8860G	ATP synthase F0 subunit 6
A15326G	Cytochrome b

Table 1. Common mitochondrial genomic changes observed patient cohort.

Comparisons to Prior Mitochondrial Genome Studies

We did not observe among our cohort mitochondrial mutations in either the transcription factor Zf9 or the 16S ribosomal RNA region, both of which have been previously reported to be associated with the Dupuytren's disease.

Paired Comparisons of Nuclear Exomes Between Diseased Tissue and Blood

No nuclear genomic mutations were consistently present in the diseased tissue group and were not present in the corresponding blood samples. No distinct patterns of mutations were identifiable when comparing nuclear DNA of tissue to blood.

Identification of Unique Nuclear Genome Mutations

Upon comparison of the nuclear genome of the 23 diseased fascial tissues to the 2 controls, we identified a set of 13 heterozygous SNPs causing amino acid changes present in all samples of the disease cohort and not present in the control group (Table 2). We also identified 4 SNPs leading to synonymous substitutions that also were present in all disease samples but not in control samples: PRB1 gene amino acid Q168, CCDC66 amino acid T115, MCPH1 amino acid V76, and CA2 amino acid L188.

Comparisons to Prior Nuclear Genome Studies

We observed 15 unique heterozygous mutations in the MAFB gene among our cohort (Table 3), though no single mutation was observed in two or more patients in the cohort. These 15 mutations were observed in 10 different patients, with one patient accounting for 4 mutations, two patients with 2 mutations, and seven patients with 1 mutation each.

Additionally, we observed 9 unique heterozygous mutations in the PRKX gene among our cohort (Table 4), and again no single mutation was observed in two or more patients in the cohort. These 9 mutations were observed in 8 different patients, with one patient accounting for 2 mutations and the remaining seven accounting for 1 mutation each.

Gene	Gene name	Chr[1]	Base sub[2]	MAF[3]	AA sub[4]
OR2T12	Olfactory receptor, family 2, subfamily T, member 12	1	T310A	0.413	T104S
OR2M7	Olfactory receptor, family 2, subfamily M, member 7	1	C571T	0.404	D191N
OR2T1	Olfactory receptor, family 2, subfamily T, member 1	1	A74G	0.334	H25R
SSX2IP	Synovial sarcoma, X breakpoint 2 interacting protein	1	A2058G	0.444	C578R
MUC4	Mucin 4	3	T11164G	n/a	T3670P
MCPH1	Microcephalin 1	8	G1016C	0.316	D314H
CA3	Carbonic anhydrase 3	8	G174A	0.489	V31I
SH2D3C	SH2 domain containing 3C	9	G209A	0.447	L23F
HEPN1	Hepatocellular carcinoma, down-regulated 1	11	T610C	0.210	W37R
HEPACAM	Hepatic and glial cell adhesion molecule	11	T1058C	0.216	M218V
PPP1R32	Protein phosphatase 1, regulatory subunit 32	11	C234A	n/a	T34S
PRB1	Proline-rich protein BstNI subfamily	12	G405T	0.490	P123Q
PRB1	Proline-rich protein BstNI subfamily	12	C483T	0.374	R149Q

[1]Chr = chromosome; [2]base sub = base substitution; [3]MAF = minor allele frequency (source: 1000 Genomes); [4]AA sub = amino acid substitution

Table 2. Thirteen nuclear SNPs leading to amino acid changes observed in all diseased tissue that are not present in control tissue.

MAFB mutations observed
P63Q
P68T
M95I
P97H
T103M
S114→stop codon
D125N
T245N
V284L
L287M
A293S
V296L
K297N
F306Y
G310R

Table 3. List of mutations in MAFB gene among patient cohort.

PRKX mutations observed
V43A
K67E
E68→stop codon
H73N
Y128C
G142W
T148S
D329E
W341C

Table 4. List of mutations in PRKX gene among patient cohort.

DISCUSSION

Our study of the mitochondrial and nuclear genome in patients with Dupuytren's disease clarifies the role of genetics in the development of this disease. Firstly, we did not observe differences in the mitochondrial or nuclear genomes of the diseased tissue when compared to the blood. This is a significant finding that has previously not been reported. This finding suggests that the disease does not develop due to a local accumulation of novel mutations in the hand that are not observed in the germline, for which we used circulating venous blood as a proxy. Secondly, our genetic analysis did not corroborate the reported mitochondrial genetic mutations from prior studies, suggesting that the previously reported mutations may be false positive findings or perhaps that the disease pathophysiology is heterogeneous and multifactorial.

Our interest in the mitochondrial genome was borne out of the reported associations between oxidative stress pathway dysregulation and the development of Dupuytren's disease. We read with particular interest two studies by Bayat et al suggesting that the disease is associated with a SNP in mitochondrial gene Zf9[18] and a heteroplasmic mutation in the mitochondrial 16S rRNA.[19] In the former study, the authors used a polymerase chain reaction-restriction fragment polymorphism method to genotype the novel SNP in the 3' untranslated region of the Zf9 gene. A case-control study comparing 138 patients with Dupuytren's disease to 255 control patients without disease demonstrated a difference in the genotype and allele frequencies between these two groups. Presence of the G allele compared to the A allele was associated with an increased risk of developing Dupuytren's disease (odds ratio 1.9, 95% confidence

interval 1.2 to 2.9). This was the first study reporting a positive association with a presence of a SNP in Dupuytren's disease. In the latter study, the authors utilized a multiplex denaturing high-performance liquid chromatography approach to sequence 100 to 600 base pair fragments of the mitochondrial genome. 20 patients with apparent maternally inherited Dupuytren's disease were compared to 20 matched control patients, yielding the discovery of a novel heteroplasmic mutation in the mitochondrial 16S rRNA present in 90% of the disease cohort and absent from all control patients (P < 0.001). This was the first study reporting an association between a mitochondrial heteroplasmic mutation and presence of Dupuytren's disease. However, the findings presented in both studies have yet to be corroborated. Subsequent studies by Hu et al and Ojwang et al, with Dupuytren's disease patient cohort sizes of 26 and 40, respectively, failed to demonstrate the presence of either results. Additionally, among our patient cohort, we also did not find significant differences in the Zf9 gene SNP or identify presence of the 16S rRNA gene heteroplasmy. Our study benefits from the use of next-generation sequencing compared to the Sanger sequencing methods used in the other studies, which should theoretically provide a higher level of reliable detection of mitochondrial mutations.[35]

One unique mitochondrial mutation that we observed in all patients in our cohort is the insertion of cytosine bases at position 309 in the mitochondrial genome. The significance of this finding remains unclear. This may be a sequencing artifact, given that this cytosine base insertion is located in the midst of a chain of cytosine nucleotides from positions 303-308. However, the insertions could presumably also be a true finding and potentially represent either a cause of mitochondrial instability or reflect a result of

mitochondrial instability. Additional follow-up studies on mitochondrial function in patients with Dupuytren's disease could potentially shed more light on the significance of this mutation. One potential study that we intend to pursue is a metabolomics analysis of diseased Dupuytren's tissue, which would be able to quantify abnormalities in the mitochondrial metabolites present in diseased tissue.

In our study, we did not identify any significant difference in the mitochondrial genome of diseased tissue compared to blood samples taken from the same patient. This is a critical negative finding. In light of this finding, it is unlikely that de novo mutations in the mitochondrial genome are the causative factor in the development of Dupuytren's disease. Similar to nuclear DNA, mitochondrial DNA constantly undergo mutation, with studies suggesting that random processes alone are sufficient to give rise to mitochondrial DNA mutations.[36,37] Studies have even suggested that the accumulation of mutations in the mitochondrial genome can result in a significant biochemical defect in their progeny cells.[37] It is therefore thought that the high mutation rate in mitochondria is due to an environment in which there is a relatively high production rate of free radicals by the mitochondrial respiratory chain. Our finding that the mitochondrial sequences of diseased tissue and the blood within all paired samples showed no differences is significant, and suggests that the mitochondria and their associated processes within the diseased tissue is appropriately functional.

Limited nuclear genomic studies have been performed to date to analyze the genetic contribution in the development of Dupuytren's disease. To date, no whole-exome sequencing studies of Dupuytren's disease have been reported in the literature. However, a variety of related studies have been performed, which include global RNA

expression profiling and genome-wide association studies. A recent meta-analysis of seven unique sets of global messenger RNA expression microarray studies identified 16 genes that demonstrate consistently statistically significant dysregulation in patients with Dupuytren's disease (Table 5).[30] Additionally, case-control whole genome association studies have highlighted 2 genes located within significantly associated loci that have also been observed to be differentially expressed in prior microarray studies: MAFB,[38] a transcription factor that regulates the differentiation of hematopoietic stem cells, and PRKX,[39] a stimulator of endothelial cell proliferation and migration. However, we did not find significant mutations in either of these genes. Only a subset of patients had mutations in either gene, and none of the mutations observed were in more than a single patient. Therefore, it is unlikely that mutations in these particular genes are associated with development of Dupuytren's disease. However, it remains possible that nearby regulators of these two genes may play a role in pathogenesis, which is consistent with the previously reported findings from both the whole genome linkage studies and microarray analyses.

Gene	Meta-analysis fold change
ADAM12	+2.9
ADH1B	-4.1
AKR1C2	-1.9
ALDH2	-1.7
ANGPTL7	-1.9
CDO1	-2.2
CLU	-4.6
COL1A1	+1.9
COL4A2	+2.4
COL5A1	+2.7
COL5A2	+2.9
LRRC17	+4.4
MMP2	+1.8
POSTN	+5.0
RGS3	+2.5
TNC	+1.9

Table 5. 16 genes have been consistently reported to be dysregulated by mRNA expression microarray studies in patients with Dupuytren's disease.

We identified a set of 13 SNPs that give rise to amino acid changes that were observed in all diseased tissue but not observed in the control tissue. Most of these SNPs are natural variants with relatively high minor allele frequency (MAF) as per the 1000 Genomes Project. Three of the SNPs are located in three different olfactory receptor genes in the G-protein coupled receptor 1 family. The SNP causing the T104S amino acid change in OR2T12 has a MAF of 0.413, D191N in OR2M7 has a MAF of 0.404, and H25R in OR2T1 has a MAF of 0.334. The SNP in the SSX2IP gene, which encodes for a widely expressed protein involved in the mediating cell-cell adherens junctions, is also a natural variant with a MAF of 0.444. The MCPH1 gene, which encodes for the protein microcephalin, contains a SNP causing a D314H mutation with a MAF of 0.316. The SNP in the CA3 gene, for carbonic anhydrase 3, is another natural variant with a MAF of 0.489. The SNP in the SH2D3C gene, encoding SH2 domain-containing protein 3C, has a MAF of 0.447. Two additional SNPs are present in the PRB1 gene, coding for basic salivary proline-rich protein 1, leading to P123Q and R129Q mutations, with corresponding MAF values of 0.490 and 0.374. A SNP in the HEPN1 gene with a MAF of 0.210 leading to a W37R mutation is notable given that this gene encodes for the putative cancer susceptibility gene HEPN1 protein. This protein is expressed in the liver and is down-regulated in hepatocellular carcinomas.[40]

The SNP in the HEPACAM gene causing the M218V mutation has a MAF of 0.216. This gene encodes for the hepatocyte cell adhesion molecule and is involved in regulating cell-matrix interactions and inhibiting cell growth by suppressing cell proliferation.[41,42] This SNP is particularly interesting given the relevance of the extracellular matrix in the development of Dupuytren's disease. It has been postulated

that the mechanism of contracture is the result of two related processes: first, cell-mediated contraction of the extracellular matrix leads to physical deformation,[43] and subsequently continuous matrix remodeling results in the permanence of the contracture.[7] Related fibrotic diseases affecting the liver, heart, lung, and kidney have been associated with the unbalanced degradation of extracellular matrix, mediated by matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMP).[44,45] Interestingly, in one study of 12 patients with advanced inoperable gastric carcinoma treated with the MMP inhibitor Marimastat (British Biotech Ltd., Oxford, United Kingdom) daily for more than one month, half of the patients developed Dupuytren's disease or a frozen shoulder.[46] The authors of this study subsequently postulated that the development of Dupuytren's disease in this patient cohort was caused by a decrease in the MMP to TIMP ratio, leading to increased synthesis and deposition of collagen and connective tissue. Another research group subsequently analyzed both MMP and TIMP levels in patients with Dupuytren's disease. They observed that patients with Dupuytren's contracture, when compared to control patients, had significantly higher serum TIMP-1 levels (437 ng/mL vs. 321 ng/mL, $P < 0.05$) and significantly lower MMP to TIMP ratios (1.1 vs. 1.5, $P < 0.05$).[47] Additionally, TIMP-1 and TIMP-2 were detectable by immunohistochemistry in Dupuytren's tissue but were undetectable in tissue from the control group.[47] Both TIMP-1 and TIMP-2 were preferentially accumulated in proliferative areas of the palmar fascia in the diseased tissues.

The SNP in the PPP1R32 gene, encoding protein phosphatase 1 regulatory subunit 32, is a novel SNP causing a previously uncharacterized T34S mutation. This is a 425 amino acid protein that interacts with PPP1CA, the protein phosphatase 1 (PP1)

catalytic subunit.[48] PP1 is a ubiquitous and conserved protein Serine/Threonine

phosphatase that is thought to catalyze approximately one third of all protein

dephosphorylations in eukaryotic cells.[49-51] However, the specific role of the PPP1R32

protein in mediating these processes is not well understood. This novel SNP is

particularly interesting given that it has not been previously characterized, and this

particular SNP may be of significance from an etiological standpoint or as a marker of

disease susceptibility. Additional studies are needed to better clarify the associations

between this SNP and Dupuytren's disease.

Perhaps most notably, a SNP in the MUC4 gene leading to amino acid mutation

T3670P was observed. This amino acid change was observed in the long splice variant of

the MUC4 gene. This SNP has not previously been characterized and is present in all

patients in our Dupuytren's disease cohort and not observed at all in the control group.

The MUC4 gene encodes for mucin 4 glycoprotein, which is produced primarily in

epithelial cells and is thought to play a role in tumor progression.[52] A transmembrane

component of MUC4 acts as an activator for the receptor tyrosine kinase avian

erythroblastosis oncogene homolog 2 (ERBB2, also referred to as HER2), which is

overexpressed in a number of human cancers, notably breast and ovarian carcinomas.[53]

MUC4 itself has been implicated in a number of human cancers, including pancreatic,

lung, breast, salivary gland, gall bladder, biliary tract, prostate, and ovarian carcinomas,

and may potentially be a useful clinical marker for these diseases.[54] Interestingly, MUC4

has recently been characterized as a highly sensitive and specific marker for low-grade

fibromyxoid sarcoma (LGFMS).[55] LGFMS is a fibroblastic neoplasm characterized by

alternating collagenous and myxoid regions. Clinically, LGFMS is characterized by a

protracted course with a tendency for local recurrence and late distant metastases.[56]

Although the palmar fibromatosis in Dupuytren's disease is considered benign,

Dupuytren's disease shares certain properties with the malignant fibrosarcoma.

Histologically, the diseased palmar nodules show marked fibroblast proliferation.[5] The

origins of Dupuytren's disease are also similar to fibrosarcomas in that both arise from

mesenchymal cells. Both are also characterized by infiltrative growth, proliferation, and a

tendency to recur despite treatment. One critical difference, however, is that unlike

sarcomas, Dupuytren's disease does not metastasize. Of particular interest, one study has

shown that patients with Dupuytren's disease have an increased risk of developing

sarcomas of connective tissue compared to the general population.[57] This suggests that

there could potentially be co-existing risk factors for the development of both

Dupuytren's disease and connective tissue sarcomas, or possibly that they share a

common etiological mechanism. In a follow-up study of patient records of patients with

both Dupuytren's disease and sarcoma, the same research group concluded that neither

smoking nor diabetes, two established risk factors, can account for why patients with

Dupuytren's disease have a higher incidence of sarcoma.[58] This finding supports the

notion that these two diseases share a common pathophysiological pathway, although this

has yet to be verified. These findings lend additional support for the observed genetic

associations among our patient cohort with SNPs in genes that are known to play a role in

malignant tumorigenesis.

Fibrosis is a hallmark of a multitude of clinical disorders, with Dupuytren's

disease being one of the more common manifestations. Fibrosis occurs due to

uncontrolled fibroblast activity that produces an overabundance of extracellular collagen. Dupuytren's disease is categorized as a superficial fibromatosis, which also includes plantar fibromatoses (Ledderhose's disease) and penile fibromatoses (Peyronie's disease).[59] Abnormal focal nuclear accumulations of β-catenin are present in these disease processes.[60] β-catenin is a dual function protein that regulates cell-cell adhesion as well as gene transcription influencing cell proliferation and survival, and is notably part of the canonical Wnt signaling pathway (Figure 3).[61] Recently, a large genome-wide association study was performed, which identified areas of the genome associated with Dupuytren's disease (Figure 4) and implicated 11 common SNPs at nine different loci in the nuclear genome.[38] Although these 11 SNPs do not correlate with any of the SNPs we have identified in our study, notably, the majority of these identified loci harbor genes encoding proteins in the Wnt signaling pathway. Wnt signaling is notable for regulating the proliferation of fibroblasts in both cancer and fibromatosis.[62] As an example, an increase in Wnt signaling in the intestine causes prolongation of the proliferation phase of crypt cells, which results in polyp formation and subsequently and increased predisposition to developing cancer.[63] All in all, the observation that there exist strong associations with multiple genes in the Wnt signaling pathway suggests both that this pathway confers susceptibility to Dupuytren's disease but also that genetic factors play a major role in the development of this disease. In particular, it appears that common genetic variants have a critical causative role in Dupuytren's disease. Our present study proposes a set of genetic variants common to our disease cohort that potentially play a causative role in development of Dupuytren's disease.
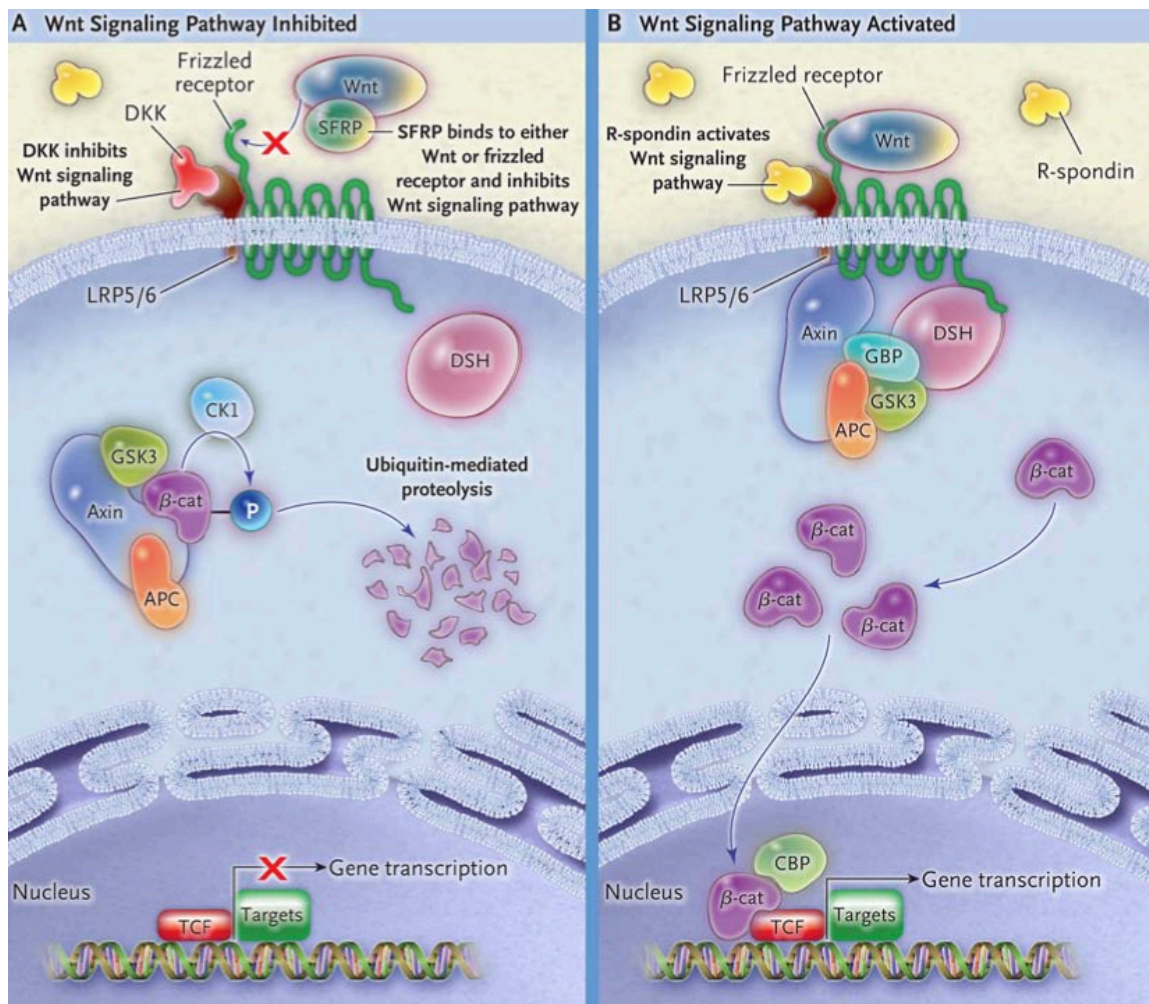
Figure 3. Signaling pathways of Wnt and β-catenin. (From Dolmans GH, Werker PM, Hennies HC, et al. Wnt signaling and Dupuytren's disease. *The New England journal of medicine.* Jul 28 2011;365(4):307-317)
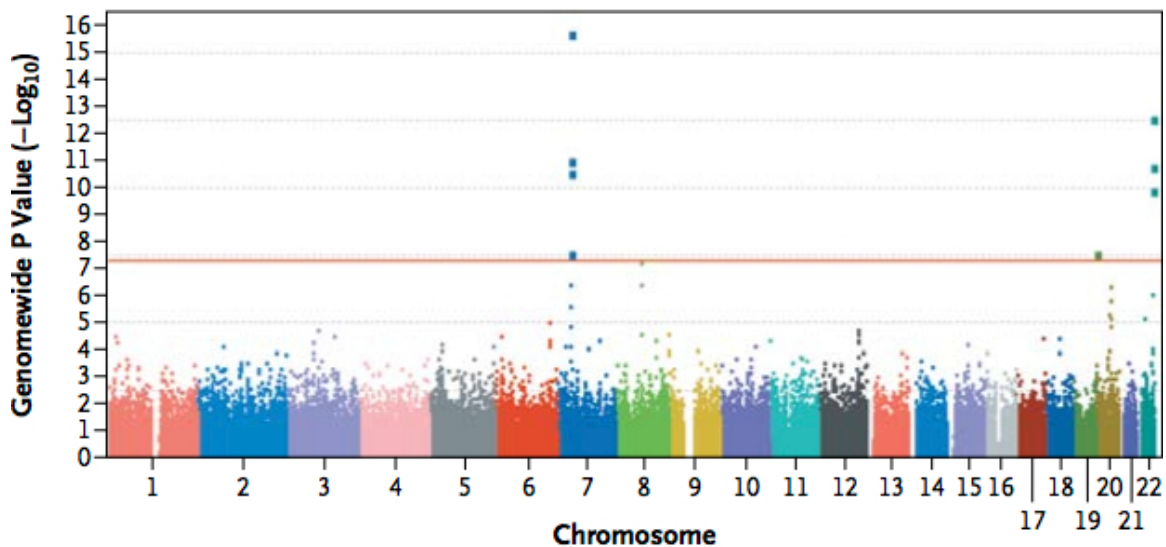
Figure 4. Manhattan plot of the genomewide P values plotted against position on each chromosome, with the bold horizontal line indicating the significance threshold. (From Dolmans GH, Werker PM, Hennies HC, et al. Wnt signaling and Dupuytren's disease. *The New England journal of medicine.* Jul 28 2011;365(4):307-317)

One particularly exciting observation from our study worth further investigation is the role of MUC4 in Dupuytren's disease. A recent study of MUC4 signaling in human pancreatic cancer cell lines demonstrated a close association with the Wnt signaling pathway.[64] Specifically, the knockdown of MUC4 led to downregulation of lysosomal degradation of E-cadherin which in turn induced the formation of the E-cadherin/β-catenin complex, resulting in downregulation of the Wnt signaling pathway.[64] The presence of mutated MUC4, as observed in our patient cohort, could potentially upregulate Wnt signaling and be one of the mechanistic links between the genetic basis of the disease and the abnormal Wnt signaling that is likely directly responsible for the fibromatosis (Figure 5). The clarification of this mechanistic pathway has potential

implications for treatment of Dupuytren's disease through the identification of additional therapeutic targets.

Figure 5. Proposed contributing mechanism in the pathogenesis of Dupuytren's disease.

One recent study has identified TNF as a potential therapeutic target in patients with Dupuytren's disease and is a testament to the utility of determining signaling pathways in disease processes.[65] In this study, diseased tissues from patients with Dupuytren's disease were collected. Fibroblasts were isolated and cultured. High levels of proinflammatory cytokines and immune cells were observed, including activated macrophages, which secrete TNF. Notably, the exogenous addition of TNF promoted differentiation of palmar dermal fibroblasts in to myofibroblasts, which is the cell directly responsible for matrix deposition and contraction in Dupuytren's disease. The authors also observed that TNF stimulation of palmar fibroblasts from Dupuytren's disease patients upregulated Wnt signaling, which notably was not observed in nonpalmar fibroblasts from Dupuytren's disease patients or palmar fibroblasts from unaffected individuals.[65] This specific action of TNF reflects the localization of disease presentation in susceptible patients, though it remains unclear what factors account for the susceptibility. Notably, the addition of exogenous DKK, an inhibitor of Wnt ligand

binding to the Frizzled receptor (Figure 3), did not affect myofibroblast contractility. This suggests that the signaling processes governing myofibroblast activity is likely not dependent on the binding of Wnt ligand to their receptor but rather more involved with the β-catenin component of the canonical signaling pathway. Lastly, with the addition of neutralizing antibodies to TNF, the contractile activity of myofibroblasts from the diseased tissue was inhibited and the contractile apparatus was observed to disassemble. This study lends credence to the theory that the Wnt/β-catenin signaling pathway is a primary mediator in the development of fibrosis. However, the upstream and downstream signaling processes remain poorly understood and are an exciting area for continued research.

In summary, with our present study, we did not detect any somatic mutations in either the mitochondrial or nuclear genomes of patients with Dupuytren's disease. However, we have identified a set of SNPs that are associated with the disease and may potentially play a causative role in this disease. Future directions for this work include confirming our findings using Sanger sequencing, as well as increasing the number of patients in both the disease and control cohorts. One limitation of this study is that because of the vast amounts of genetic data that were analyzed, it is possible that some of the SNPs identified are not true associations but are coincidental findings. Performing mitochondrial and nuclear sequencing on a larger cohort could potentially account for this limitation. Additional investigation of the proposed molecular pathways, such as the MUC4 and Wnt signaling pathway interactions in patients with Dupuytren's disease, would be of great benefit. Focused genetic studies to follow-up the mitochondrial and nuclear mutations identified by our study could also shed additional light on the disease

pathogenesis. Given that several molecular mechanisms have been observed to play a

role in Dupuytren's disease, one of the biggest challenges for researchers in this field is to

determine the relative contributions of each and to mechanistically link them together.

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FIGURE LEGENDS

Figure 1. The clinical presentation of Dupuytren's disease. Early stage disease (a) with prominent cord and small nodules, followed by more advanced stage (b) with dimpling at the base of the digit, and subsequently advanced stage disease (c) with severe deformity.

Figure 2. Distribution of samples from patient cohort; all samples underwent DNA extraction and subsequent mitochondrial sequencing and whole-exome sequencing.

Figure 3. Signaling pathways of Wnt and β-catenin.

Figure 4. Manhattan plot of the genomewide P values plotted against position on each chromosome, with the bold horizontal line indicating the significance threshold.

Figure 5. Proposed contributing mechanism in the pathogenesis of Dupuytren's disease.

TABLES

Table 1. Common mitochondrial genomic changes observed in all patients in cohort.

Table 2. Nuclear SNPs leading to amino acid changes observed in all diseased tissue that are not present in control tissue.

Table 3. List of mutations in MAFB gene among patient cohort.

Table 4. List of mutations in PRKX gene among patient cohort.

Table 5. 16 genes have been consistently reported to be dysregulated by mRNA expression microarray studies in patients with Dupuytren's disease.