SCIENTIFIC ARTICLE

Genome-Wide High-Resolution Screening in Dupuytren's Disease Reveals Common Regions of DNA Copy Number Alterations

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Purpose Dupuytren's disease (DD) is a familial disorder with a high genetic susceptibility in white people; however, its etiopathogenesis remains unknown. Previous comparative genomic hybridization studies using lower-resolution, 44-k oligonucleotide-based arrays revealed no copy number variation (CNV) changes in DD. In this study, we used a higher-resolution genome-wide screening (next-generation microarrays) comprising 963,331 human sequences (3 kb spacing between probes) for whole genome DNA variation analysis. The objective was to detect cryptic chromosomal imbalances in DD.

Methods Agilent SurePrint G3 microarrays, one million format (Agilent Technologies, Santa Clara, CA), were used to detect CNV regions (CNVRs) in DNA extracted from nodules of 4 white men with DD (age, 69 ± 4 y). Reference samples were from the DNA of 10 men who served as control patients. Copy number variations that were common to greater than 3 assessed DD individuals ($p < .05$) were selected as candidate loci for DD etiology. In addition, quantitative polymerase chain reactions (qPCR) assays were designed for selected CNVRs on DNA from 13 DD patients and 11 control patients. Independent *t*-tests and Fisher's exact tests were carried out for statistical analysis.

Results Three novel CNVs previously unreported in the phenotypically normal population were detected in 3 DD cases, located at 10q22, 16p12.1, and 17p12. Nine polymorphic CNVRs potentially associated with DD were determined using our strategic selection criteria, locating to chromosomes 1q31, 6p21, 7p14, 8p11, 12p13, 14q11, 17q21 and 20p13. More than 3 of the DD cases tested had a CNVR located to a small region on 6p21 and 4 CNVRs within 6p21–22 of the human leukocyte antigen (HLA) genes.

Conclusions Three novel copy number alterations were observed in 3 unrelated patients with sporadic (no known family history) DD. Nine polymorphic CNVRs were found to be common among the DD cases. These variants might contain genes involved in DD formation, indicating that important gene networks expressed within the palmar fascia might contribute to genetic susceptibility of DD. *(J Hand Surg 2010;35A:1172–1183. © 2010 Published by Elsevier Inc. on behalf of the American Society for Surgery of the Hand.)*

Key words Comparative genomic hybridization, copy number variance, Dupuytren's contracture, Dupuytren's disease, genetics.

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DUPUYTREN'S DISEASE (DD) is a fibroproliferative disease that can lead to permanent flexion contracture of the digits. The molecular mechanisms for the pathogenesis of DD are currently unclear. Several observations in the DD population suggest genetic susceptibility, including familial predisposition,[1] occurrences in identical twins,[2] and high prevalence in the white population.[3] Dupuytren's disease has been proposed to be an inherited autosomal dominant trait with variable penetrance, although currently no specific causative gene has been identified.[4,5] On the other hand, DD has also been suggested to be a complex oligogenic condition, as opposed to a simple monogenic mendelian disorder.[6] Familial inherited genomic variations can involve mutated genes, genomic rearrangements, polymorphisms, or copy number variations (CNVs) (Fig. 1). Several studies have searched for genetic variations that might be associated with DD, using methods including linkage analysis, human leukocyte antigen (HLA) phenotypes, gene polymorphisms, and array-based comparative hybridization (aCGH).[1,3,4,7–10]

The genomic variations of interest in this study are CNVs, which have been defined as DNA segments of at least 1 kb that vary in copy numbers within the normal population.[11] Currently, there is limited knowledge on the functional impact of CNVs, and several groups have suggested that CNVs play a role in functional variation.[12–14] Several common CNVs have been associated with diseases or disease susceptibility through a single affected gene, sets of genes, or combinations of alleles in complex diseases.[15,16] Correlations between gene expression levels and CNVs have been observed in both humans and animal models.[14,17,18] For some genes, the expression levels correlate to CNVs in certain tissues but not in others, and some CNVs affect the expression of neighboring genes outside the region by affecting regulatory elements or genome architecture.[14,15,17,18] Although the etiology of DD is not yet defined, and no unique cytogenetic feature has been confirmed, there have been reports suggesting the involvement of structural chromosomal abnormalities, particularly trisomy 7 or 8.[19–21] Because some of these studies were on cultured cells, it has been proposed that these alterations might be a result of abnormal growth advantage in cultures rather than associations with the disease.[8,21] Kaur et al.[8] performed aCGH on 18 DD cases, using tissues samples on the 44 k oligonucleotide-based aCGH platform (Agilent Technologies, Santa Clara, CA), and reported no CNV changes. To determine whether chromosome abnormalities were involved in DD and to identify CNV regions (CNVRs) that might be associated with DD susceptibility, we carried out a more extensive genome-wide screening and used higher-resolution arrays with approximately 20 times higher probe density than that used in a previous study.[8]

MATERIALS AND METHODS

DNA sample preparation

Four DD subjects were screened by an aCGH DNA microarray platform. All subjects were white and male, with an average age of 69 years (± 4 SD). None of the patients had a family history of DD. Microdissected nodule tissue biopsies obtained at the time of DD surgery (palmar fasciectomy) were snap-frozen and stored at −80°C until they were required. For DNA extraction, 25 mg of each nodule biopsy sample was weighed, finely diced, and placed in a 2-mL round-bottom microcentrifuge tube with 80 μL phosphate buffered saline. A sterile steel ball bearing was placed in each tube, and the tissues were homogenized in a Qiagen TissueLyser II (Retsch GmbH, Haan, Germany) at 30 oscillations per second for 12 minutes. The lysed tissues were transferred to a fresh microcentrifuge tube and processed using the QIAamp DNA mini kit (Qiagen GmbH, Hilden, Germany), as recommended by the Agilent Technologies aCGH protocol.[22] DNA quality was assessed using a NanoDrop ND-1000 UV/Vis spectrophotometer (Thermo Fisher Scientific Inc, Wilmington, DE). All samples reached the required purity specification of A260/A280 ratio of 1.8 to 2.0 and an A260/A230 ratio of >2.0. The integrity for the extracted genomic DNA was assessed by agarose gel electrophoresis (1% gels) and visualized using ethidium bromide. The average molecular weight for the DNA samples were all >10 kb. DNA used for quantitative polymerase chain reaction (qPCR) was collected and extracted as described by Brown et al.[9]

Array-based comparative genomic hybridization

The aCGH was carried out using the Agilent oligonucleotide array-based CGH microarray kit (SurePrint G3 Human CGH Microarray Kit 1x1M; Agilent Technologies) according to the manufacturer's protocol. Commercial male human genomic DNA (G1471; Promega Corp, Madison, WI), isolated from multiple donors, was used as the control DNA. Following hybridization, washing, and drying steps, the microarray slides were scanned at 2 μm resolution, using the G2539A microarray scanner (Agilent Technologies). Features were extracted from the scanned images using Feature Extraction software (version 10.5; Agilent Technologies). The extracted features were analyzed using DNA Analytics software (version 4.0.85; Agilent Technologies) using

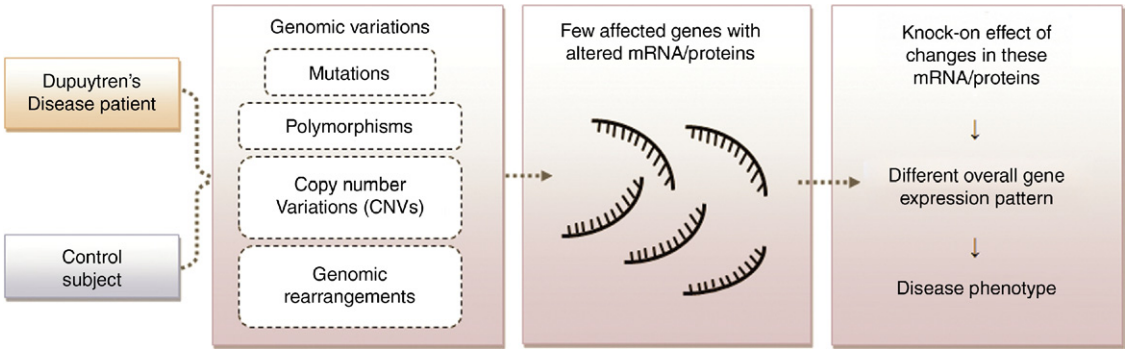

FIGURE 1: Importance of studying genomic variations for familial disease. Familial diseases, such as Dupuytren's disease, might involve inheritance of genomic variations, which could be mutated genes, genomic rearrangements, copy number variations, or polymorphisms. These genomic variations might affect a small number of genes, resulting in altered messenger RNA or protein expression. However, this could in turn have downstream effects on other genes and pathways, resulting in the observed phenotypic variation, such as a disease symptom.

the aberration detection method 2 (ADM-2) algorithm. Thresholds were set at a minimum of 6 probes and 0.25 average log ratios.

Candidate gene selection for large-scale studies

Common aberrations present in the 4 patients with DD were identified using *t*-tests in DNA Analytics software (Version 4.0.85; Agilent Technologies), with the input p value and overlap threshold set as 0.05 and 0.9, respectively. The CNVRs present in 3 or more patients were selected for the initial candidate CNVR follow-up list. Overlapping CNVRs were treated as the same CNVR. The selection of candidate genes was carried out through 2 methods: direct analysis of the regions and combined analysis of the CNVR data with our previous microarray data on Dupuytren's disease, in which nodule, cord, and fascia adjacent to the DD site from 4 DD subjects were compared to 4 external control fascia from 4 individuals unaffected by DD.[23] The selection of CNVRs was carried out using 5 criteria (Appendix A can be viewed at the *Journal's* Web site, http://www.jhandsurg.org): (1) genes within CNVR with a fold change >1.5, (2) CNVR with a significantly high portion of dysregulated genes in DD, (3) flanking genes with a fold change >2, (4) genes within CNVR with functions that might be of relevance to DD based on the literature, and (5) CNVRs covering large regions or demonstrating consistent deletions or amplifications.

Genes present within and flanking the CNVRs were identified using UCSC Genome Bioinformatics table browser (University of California Santa Cruz, Santa Cruz, CA; http://genome.ucsc.edu/cgi-bin/hgTables), and the gene symbols were converted to EntrezGene ID before functional annotation using the Database for Annotation, Visualization and Integrated Discovery (DAVID) Bioinformatics Resources (National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD; http://david.abcc.ncifcrf.gov/). A threshold of 250 k was set for the size of the region flanking the CNVRs for analysis to cover position effects based on observations in mice models in which increased expression variation was detected in regions 50 to 250 kb away from CNV boundaries.[14] Our previously published microarray data on DD[23] were also used to determine whether the genes around and within these regions have an average fold change that was significantly different from one (determined through one sample *t*-test), when comparing external control to DD tissues. One sample *t*-test was used to determine whether genes around and within the CNV regions have a fold change that significantly deviates from 1 (fold changes are a comparison made between external control and DD tissues from our previously published microarray data).

Primer design for quantitative polymerase chain reaction assays

Primers were designed for one gene within each selected CNVR. Priorities were given to genes that show gene CNV across a larger number of DD subjects. In cases in which more than one gene met the criteria, genes with higher fold change from previously published expression microarray data were used.[23] The sequences of the selected genes were determined through the UCSC Genome Bioinformatics table browser (University of California Santa Cruz, Santa Cruz, CA; http://genome.ucsc.edu/cgi-bin/hgTables) and used for the designing of primers through the Universal ProbeLibrary Assay Design Center (Roche Ap-

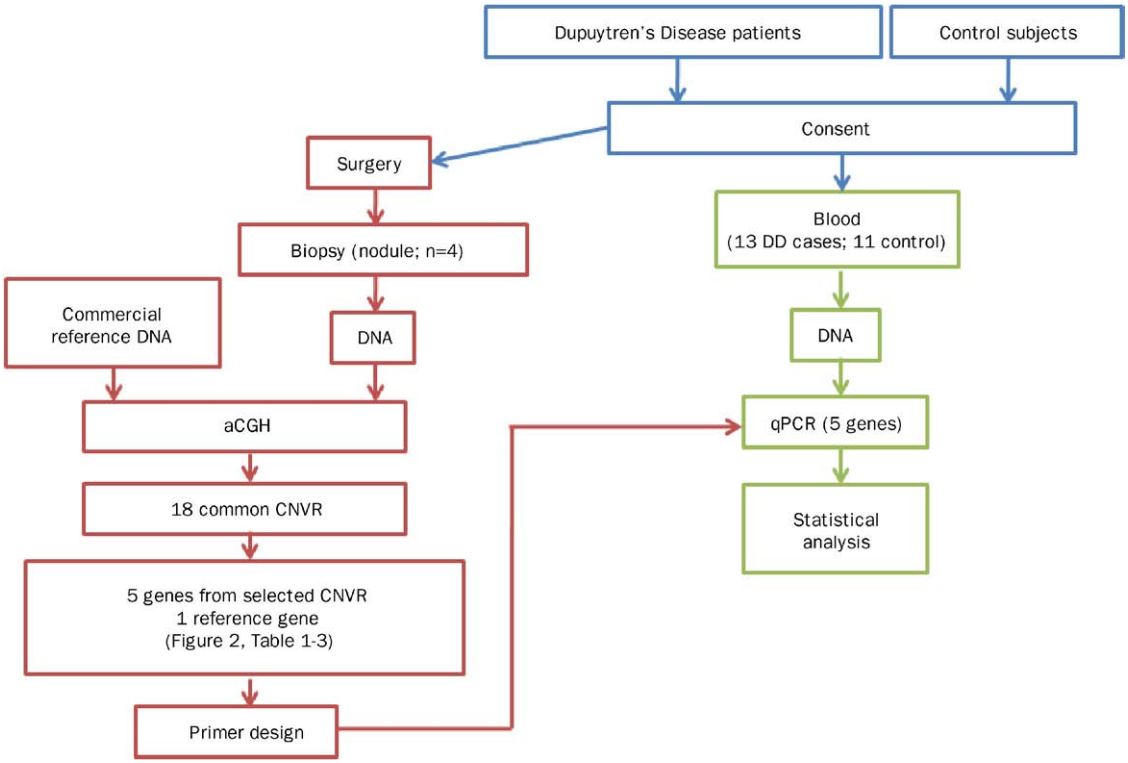

FIGURE 2: Summary of steps taken to identify CNVRs that might be associated with Dupuytren's disease. The flowchart summarizes the methods used and the results obtained in this study.

plied Science, West Sussex, UK; https://www.roche-applied-science.com/sis/rtpcr/upl/ezhome.html). The specificity of designed assays was determined using Primer-Blast (National Center for Biotechnology Information (NCBI); http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi). The primers designed for the selected genes were purchased through Sigma-Aldrich (St. Louis, MO) (Appendix B can be viewed at the *Journal's* Web site, http://www.jhandsurg.org). Albumin was used as the control reference based on previous studies.[24]

Quantitative polymerase chain reactions and their data analysis

Quantitative polymerase chain reaction (qPCR) assays were carried out in 96 multi-well plates on a LightCycler 480 platform (Roche Diagnostics GmBh, Germany). Two replicates of 10 μL reactions were carried out; each consisted of 0.2 μM of each primer, 0.1 μL probe from Universal Probe Library (Roche Diagnostics GmBh, Germany), 25 ng DNA, 5 μL PerfeCTa qPCR FastMix, UNG (Quanta Biosciences, Gaithersburg, MD), and nuclease-free water (Ambion, UK). The conditions used for qPCR were as described previously by Shih et al.[25] The threshold cycle (C_T) values for the reference gene, Albumin, is deducted from C_T values obtained for each target gene to obtain ΔC_T. Albumin is used as a reference because no known copy number difference has been reported in this gene. The target genes include signal-regulatory protein beta 1 (SIRPB1), proline rich 4 (PRR4), cell division cycle 2-like 1 (CDC2L1), T-cell receptor gamma alternate reading frame protein (TARP) and a disintegrin and metalloproteinase domain 3a (ADAM3A). The ΔC_T for each gene from each patient is deducted from the ΔC_T for the same gene from one control patient to obtain $\Delta\Delta C_T$. The amplification and deletion relative to the single control patient is represented by the equation $2^{-\Delta\Delta CT}$. Independent sample *t*-tests were used to determine whether there is a significant difference between the average relative copy number in the test and control groups. For SIRPB1, the relative copy number method is not used because several individuals present low or no amplification due to homozygous deletion of the gene. Whether qPCR amplification had taken place was noted and Fisher's exact tests were carried out to determine whether the frequency of SIRPB1 deletion was significantly different between DD and control subjects. A flowchart of the study strategy is presented in Figure 2.

RESULTS

Array-based comparative genomic hybridization

Through aCGH, 31 significant ($p < .05$) CNVRs were determined to be common in 4 patients with DD (Appendix C can be viewed at the *Journal's* Web site, http://www.jhandsurg.org). Using the Database of Genomic Variants (The Centre for Applied Genomics, Toronto, Ontario, Canada; http://projects.tcag.ca/variation/), all 31 CNVRs have been previously reported in phenotypically normal populations, suggesting that these are common polymorphic CNVRs. However, because DD is not observed until a later age of onset, it is possible that some of these control individuals with overlapping CNVs might go on to develop DD. Three of 4 patients show copy number alterations in regions that have not previously been reported as polymorphic in the Database of Genomic Variants (Appendix D and Appendix E can be viewed at the *Journal's* Web site, http://www.jhandsurg.org). Each of the 3 copy number alterations was observed in a different patient with DD. The 3 genomic alterations are located on chromosomes 10q22.3 (82,084,886 bp to 82,105,011bp), 16p12.1 (22,979,208 bp to 23,025,167 bp) and 17p12 (13,124,272 bp to 13,171, 228 bp), with the former 2 regions covering partial sequences for DPY30 domain containing 1 (DYDC1) and ubiquitin specific peptidase 31 (USP31), respectively. The chromosome 16 CNV also falls within a larger region deleted in patients with 16p11.2–p12.2 microdeletion syndrome.[26] The chromosome 17p12 CNV does not contain any genes, and the closest flanking genes are elaC homolog 2 (ELAC2), which has been linked to tRNA 3′ processing and heparan sulfate (glucosamine) 3-O-sulfotransferase 3A1 (HS3ST3A1), which might be involved in the biosynthesis of an entry receptor for herpes simplex virus 1. Single-nucleotide polymorphisms associated with ELAC2 have also been associated with risk of prostate cancer.[27]

Selection of candidate copy number variation regions for Dupuytren's disease

Using the UCSC Genome Bioinformatics Table Browser (http://genome.ucsc.edu/cgi-bin/hgTables; human genome, March 2006 assembly), 154 genes were identified within the 31 CNVRs and an additional 330 genes located in the flanking regions (up to 250 kb upstream and downstream). For the genes within the CNVRs, 38 of the 154 genes were converted to EntrezGene identifiers for functional annotation, and 25 were found to be present in our previously published gene expression microarray data.[23] When more than one probe was present for a gene, the microarray values for probes with the highest fold change were used. Examination of expression patterns of genes from these CNVR regions in previous microarray data[23] showed that one or more probes from 5 genes demonstrated a fold change of >1.5 when comparing external fascia (control subjects) to the disease tissues from patients with DD (Table 1). These include T-cell receptor gamma constant 2 (TRGC2), T-cell receptor gamma variable 9 (TRGV9), signal-regulatory protein beta 1 (SIRPB1) on 20p13, HLA-A (major histocompatibility complex, class I, A) and HCG4P6 (HLA complex group 4 pseudogene 6). The TRGC2 and TRGV9 overlap in the CNVR at 7p14. The HLA-A and HCG4P6 are located on 6p21, where a common CNVR was identified. Using one sample *t*-test on the expression changes of genes within and flanking the CNVRs, 2 regions, 12p13 and 6p21, were determined to show an average fold change that deviates from an expected value of 1 (Table 1). As described earlier, 2 genes within the CNVR at 6p21 also demonstrated a fold change >1.5 or -1.5. The 12p13 region includes the gene proline rich 4 (PRR4). Nine genes from the CNVRs demonstrated a fold change >2 on previous DD expression arrays (Table 1).[23] Only the CNVR on 17q21 has 2 or more flanking genes showing an expression fold change >2. Two CNVRs containing genes with high expression differences in tissue from patients with DD were selected for further analysis based on their biological function; these include the CNVR on chromosome 1p36, which contains matrix metallopeptidase (MMP)21/22B, and the CNVR located on chromosome 8p11 (which contains ADAM3A) (Table 1). Both MMPs and ADAMs have been implicated in DD pathology,[23,28,29] and the CNVRs we detected were also in close proximity to other MMP and ADAM genes. Finally, CNVRs 1q31, 14q11, and 20p13 were selected for their consistently large DNA changes (amplification or deletion) seen in more than 2 patients with DD (Table 1; Fig. 3).

Assessing candidate gene dosage in patients with Dupuytren's disease by quantitative polymerase chain reaction assays

Nine CNVRs were selected from the aforementioned selection criteria, but quantitative PCR assays were only designed for 5 genes. Primers for genes present in CNVRs at 14q11, 6p21, and 1q31 could not be designed due to high homology to other genes at different loci. Region 17q21 was excluded because no genes reside within this CNVR. Five unique genes from 5 of the DD CNVRs were

TABLE 1. Summary of Results for the Copy Number Variation Regions Common to the Patients With Dupuytren's Disease

Selection Method (Fig. 1)	Copy Number Variation Region (hg18 Genome Built)			Gene Symbol	Function	Relevance to Dupuytren's Disease	Microarray Fold Change[25]
	Chromosome Position	Start Position	End Position				
(a)	7p14	38256971	38347360	TRGC2	T-cell receptor	Possible involvement of immunity has been suggested in DD[43]	1.6 (fascia) 1.5 (nodule)
(a)	7p14	38256971	38347360	TRGV9	Antigen binding	Possible involvement of immunity has been suggested in DD[43]	1.6 (fascia) 1.5 (nodule)
(a)	6p21	29966136	30005538	HCG4P6	Antigen processing and presentation	Possible involvement of immunity has been suggested in DD[43]	1.5 (fascia) 1.5 (nodule)
(a)	6p21	29966136	30005538	HLA-A	Antigen processing and presentation	Possible involvement of immunity and HLA molecules has been suggested in DD[22,43]	−1.7 (fascia)
(a)	20p13	1506179	1532633	SIRPB1	It is part of the immunoglobulin superfamily; involved in the regulation of receptor tyrosine kinase–coupled signaling processes	Possible involvement of immunity has been suggested in DD[43]	1.5 (fascia)
(b)	6p21	29966136	30005538	n/a	n/a	n/a	n/a
(b)	12p13	11110535	11140621	n/a	n/a	n/a	n/a
(c)	17q21	36675787	36684819	KRT15	Responsible for the structural integrity of epithelial cells	KRT14 is down-regulated in all DD tissues[23]	−2.9 (fascia) −3.1 (cord)
(c)	17q21	36675787	36684819	KRT19	Responsible for the structural integrity of epithelial cells	KRT14 is down-regulated in all DD tissues[23]	−2.6 (fascia) −4.7 (cord)
(c)	19q13	58211913	58244212	ZNF160	May function in transcription regulation	Other	4.8 (nodule)
(c)	6p21	29966136	30005538	HCG2P7	Antigen processing and presentation	Possible involvement of immunity in DD[43]	2.5 (nodule)
(c)	5p15	325994	1323565	ZDHHC11	Unknown	Gain of ZDHHC11 is observed in high grade bladder cancer	
(c)	20p13	1506179	1532633	FKBP1A	Immune regulation, protein folding and trafficking	Interact with TGF-beta receptor, previously implicated in DD[44]	−2.5 (nodule)
(c)	17q12	31457689	31889766	CCL18	Chemotactic activity for T cells	Possible involvement of immunity in DD[43]	−2.2 (nodule)
(c)	14q11	21423730	22041771	DAD1	Apoptosis regulator	Possible involvement of apoptotic pathways have been suggested in DD[23,45]	−2.11 (nodule)
(c)	1p36	1575354	1640762	GNB1	Signal transduction		−3.3 (nodule)
(d)	1p36	1575354	1640762	MMP21/22B	Metalloprotease	Other matrix metalloproteases suggested to be involved in DD pathology[28,29,46]	n/a
(d)	8p11	39350598	39505456	ADAM3A	Metalloprotease-like protein	Other matrix metalloproteases suggested to be involved in DD pathology[46]	n/a
(e)	1q31	195009158	195066067	n/a	n/a	n/a	n/a
(e)	14q11	18864361	19494616	n/a	n/a	n/a	n/a
(e)	20p13	1506179	1532633	n/a	n/a	n/a	n/a

Genes that reside in these regions and show considerable expression changes in DD tissue on Affymetrix Human Genome U133A (HG-U133A) are listed alongside.[23] Probes with the highest fold change value for the genes are used. n/a, not applicable (because the information is not relevant to the selection of the CNVR).

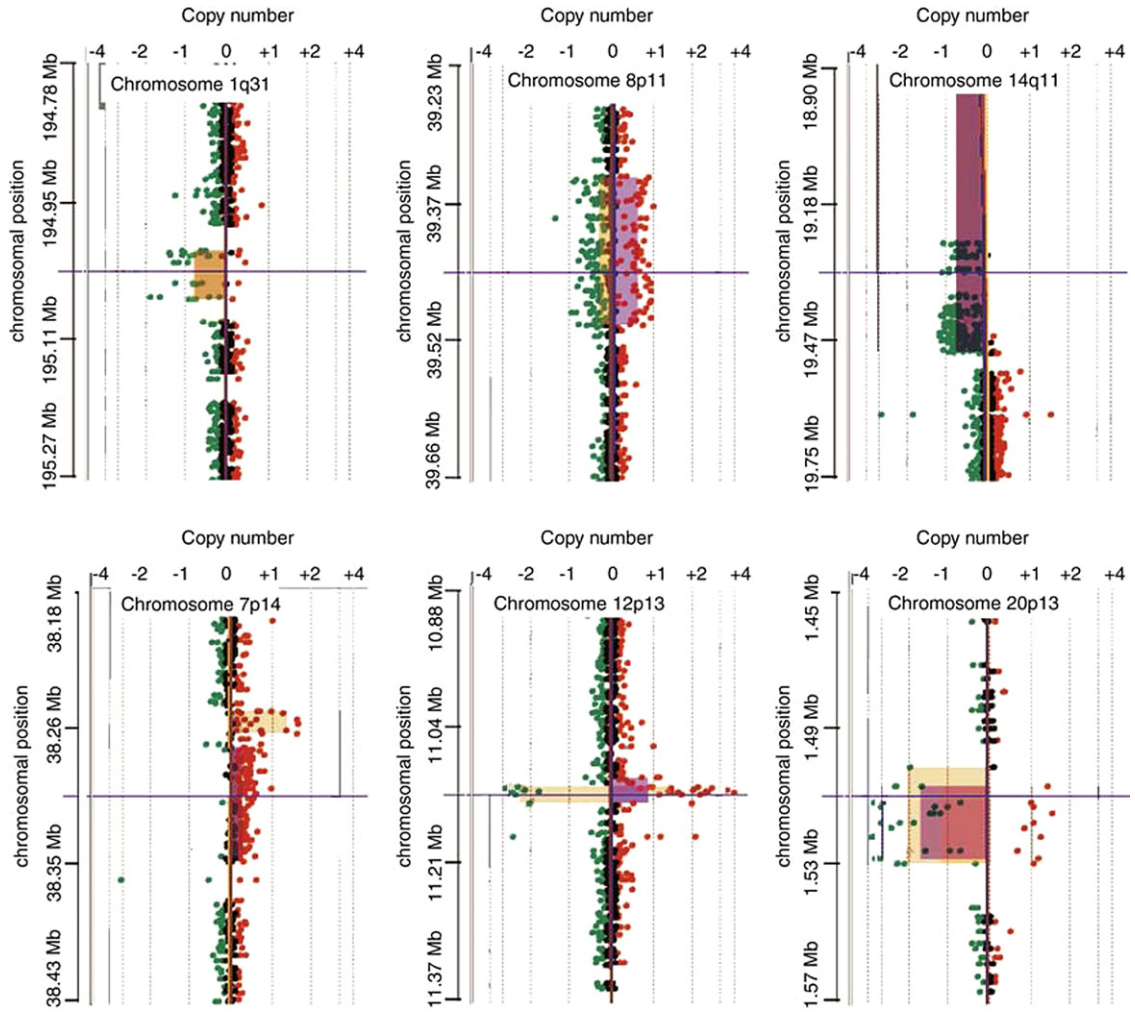

FIGURE 3: Selected Copy Number Variation Regions (CNVRs) identified in Dupuytren's disease. The figure shows the CNVRs (in shaded boxes; each shade represents the dataset of one patient) detected with SurePrint G3 2 X 400 k CNV (Agilent Technologies). The figures also indicate the chromosomal position of each CNVR. The copy number changes (in patients with DD in relation to controls) are indicated in the x-axis, as well as by the shift of large numbers of red dots (copy number gain) or green dots (copy number loss) toward the positive or negative ends of the x-axis. For example, when there is a copy number gain in the DNA of a patient with DD in comparison to the control DNA, there will be a mass of red dots that shift toward the positive end of the axis. DNA Analytics settings: ADM-2; threshold, 6; filter, 6 probes, 0.25 log2 ratio, centralization threshold, 6; centralization bin size, 10; *t*-test p value threshold, 0.05; overlap threshold, 0.9; 2 Mb moving average.

selected for qPCR analysis (PRR4, CDC2L1, SIRPB1, ADAM3A, and TARP on 7p14, 12p13, 1p36, 8p11 and 20p13, respectively; Table 2). In the set of 13 subjects with DD screened against 11 white control subjects, we found no significant ($p > .05$) difference between the relative copy numbers for any of these 5 genes (Table 3), indicating that the significance of common CNVRs observed in the initial 4 DD patients were not replicated in this small cohort of DD patients.

DISCUSSION

This study has identified 31 CNVRs that are common across the DD cases investigated using high-resolution aCGH technology. This is in contrast to previous findings that reported no gene copy number changes in a DD cohort.[8] By comparing the identified CNVRs detected in this study to our previous gene expression array data,[23] several CNVRs were assessed to have potential involvement in the biological processes that might contribute to DD development.

To date, there are approximately 29,133 common CNVs, observed in more than 5% of the population, reported in around 8,410 regions, based on the Database of Genomic Variants.[12,14] These regions cover more than 12% of the human genome and involve hundreds of genes.[30] Specific CNVs might contribute to disease

TABLE 2. Selected Copy Number Variation Regions for In-Depth Investigation

Copy Number Variation Region (hg18 Genome Built)			Potential Genes for Quantitative Polymerase Chain Reaction Assay Design	Disease Association
Chromosomal Position	Start Position	End Position		
7p14	38256971	38347360	TARP	Expressed with breast and prostate cancer cells[47,48]
6p21	29966136	30005538	HLA-A	HLA types have been implicated in several familial diseases, including DD[22]
12p13	11110535	11140621	PRR4	None
17q21	36675787	36684819	No genes in the region	n/a
1p36	1575354	1640762	CDC2L1	Loss of one CDC2L1 allele is associated with skin cancer susceptibility in mice[49]
8p11	39350598	39505456	X89654 (ADAM metallopeptidase domain 3A)	Several other ADAM, MMPs, and ADAMTs have been demonstrated to be dysregulated in DD[23,28,29]
1q31	195009158	195066067	CFHR3 CFHR1	Deletion of CFHR3 is associated with age-related macular degeneration[50] Deletion of CFHR1 is associated with atypical hemolytic uremicsyndrome[51]
14q11	18864361	19494616	OR4K5	Unknown
20p13	1506179	1532633	SIRPB1	Involved in migration and phagocytosis of leukocytes[52,53]

ADAM3A, a disintegrin and metalloproteinase domain 3a; CDC2L1, cell division cycle 2-like; CFHR3, complement factor H-related; HLA-A, human leukocyte antigens A; OR4K, olfactory receptor, family 4, subfamily K, member 5; PRR4, proline rich 4; chain reaction; SIRPB1, signal-regulatory protein beta 1; TARP, TCR gamma alternate reading frame protein; n/a, not applicable.

susceptibility. For instance, low copy number of the Chemokine (C-C motif) ligand 3-like 1 gene is associated with susceptibility to enhanced human immunodeficiency virus-1/acquired immune deficiency syndrome.[31] Low copy numbers of the complement component 4 (C4) gene at 6p21 is also associated with a higher risk of developing systemic lupus erythematosus.[32] Changes in gene copy numbers have also been associated with malignancy of certain cancerous cells, through inherited or acquired means.[33]

The functional impact of CNVs is currently not clearly understood. Some studies have associated copy number differences to gene expression alterations and disease.[24] In addition to having a direct effect on genes located within the CNVs, CNVs might also have an indirect effect on downstream pathways or networks, as well as positional effects on neighboring genes.[14,15,18,34] The CNVs might play an important role with respect to risk of complex diseases.[14]

In this study, we found 3 novel copy number alterations (microduplications) in the nodules of 3 different patients with sporadic (no known family history) DD that have not previously been reported in other subjects with DD or in the normal population. These CNVs might be a result of acquired genomic variation that occurred at the tumor site, resulting in DD. Only the CNVRs on 10q22.3 and 16p12.1 contain known genes. Gene DYDC1 resides within the CNV found on 10q22.3, a region previously linked to myodegenerative disorders.[35] In the microarray study by Forsman et al.,[36] muscle-related genes were the largest category with the greatest differential, usually reduced, expression in patients with DD.[36] The copy number gain at 16p12.1 includes the gene USP31. Gene USP31 interacts with p65/RelA, a subunit of nuclear factor kappa beta (NF-$\kappa\beta$), and it appears to have a role in the regulation of NF-$\kappa\beta$ activation by members of the tumor necrosis factor receptor superfamily.[37] Constitutive activation of NF-$\kappa\beta$ has been reported in cancer cells, and its activation appears to play a role in maintaining a malignancy or survival of tumor cells.[38] Increased cancer incidences have been previously reported in patients with DD.[38,39] The chromosome 16p12.1 CNV falls within a larger region deleted in patients with 16p11.2-p12.2 microdeletion syndrome who have clinical features including facial dysmorphology and mental retardation.[26] No duplications have been reported involving this region.

TABLE 3. Quantitative Polymerase Chain Reaction Results on Selected Genes

Gene	p Value	Average Relative Gene Copy Number		Standard Deviation	
		Control Subjects	DD Cases	Control Subjects	DD Cases
ADAM3A	.47	1.12	1.03	0.18	0.34
CDC2L1	.21	1.01	0.85	0.25	0.34
PRR4	.36	1.13	1.03	0.21	0.31
TARP	.90	1.10	1.11	0.09	0.14
SIRPB1	1.00	n/a	n/a	n/a	n/a

For ADAM3A, CDC2L1, PRR4, and TARP, independent *t*-tests were used to assess whether the relative gene copy numbers from control subjects and subjects with DD are significantly different, and the p values from the *t*-tests are indicated in the table. The SIRPB1 was analyzed using Fisher's exact test, because many individuals have complete deletion of the gene, resulting in no amplification for qPCRs. The SIPPB1 was not amplified in 11 of 13 (85%) subjects with DD and 9 of 11 (82%) control subjects. The difference between the percentage of individuals positive for the SIRPB1 qPCR were not significantly different ($p = 1.00$). ADAM3A, a disintegrin and metalloproteinase domain 3a; CDC2L1, cell division cycle 2-like; PRR4, proline rich 4; SIRPB1, signal-regulatory protein beta 1; TARP, TCR gamma alternate reading frame protein; n/a, not applicable.

Eighteen CNVRs were found to be common in the DD cases investigated in this study. Using a combination of selection criteria, including looking for differential expression of genes located within or flanking the CNVRs in DD samples in our previous expression microarray data,[23] 9 CNVRs were selected for investigation. Of these, 5 CNVRs were assessed on a larger patient sample size. A qPCR analysis was carried out on a gene residing within the region for each CNVR, using 13 DD cases and 11 controls; however, no significant DD association was observed for these particular genes. Although not meeting the selection criteria for further investigation, several genes within or flanking the CNVR at 6p21 demonstrate altered gene expression. Altered gene transcription might result in aberrant levels of proteins coded by the genes, thus affecting the characteristics and phenotypes of the involved cells. The 6p21 region contains a large number of genes involved in HLA complexes and antigen processing, which are involved in the immune system. Between the regions 6p21–6p22, there were 4 distinctive common CNVRs that were common in at least 2 DD cases (Fig. 4). In our expression microarray data, we observed reduced expression for several HLA genes located adjacent to or within these CNVRs (Fig. 4). Although this region is highly polymorphic due to the nature of HLA genes, it is possible that DD susceptibility is associated with 6p21–22. Recent published evidence suggests a positive association between DD and HLA-DRB1*15.[9] Apart from the possible association of HLA to DD suggested by the prevalence of common CNVRs at 6p21, the other CNVRs identified do not coincide with the observations from previous genomic variation studies in DD. Interestingly, the regions defined in this study do not coincide with the linkage region mapped to 16q in a Swedish family by Hu et al.,[1] or to mutations in the Zf9 gene on 10p15.[3] Cytogenetic chromosomal abnormalities previously reported include trisomies of chromosome 8 (in 5 of 9 DD early passage cell cultures),[19] trisomies of chromosome 7 or 8 and loss of the Y chromosome in 22 of 40 nodule short-term cultures,[20] chromosome 7 and 8 numerical abnormalities in 4 and 5 of 8 DD cultures,[21] and trisomy 7 and 8 in patients with DD (1 and 6 of 26 patients, respectively).[40] We did not detect any such chromosome abnormalities in this aCGH study; only small copy number gains were found in 7p14 and 8p11.

Balanced translocations and chromosome rearrangements such as inversions cannot be detected by aCGH, and various abnormal karyotypes, mainly insertions and translocations, have been seen in some cells from early passage DD cultures (4 of 6 DD cases).[41] We identified a CNVR at 1p36 that might be involved in DD, and Bowser-Riley et al.[41] described an inverted insertion between chromosome 15q26 and 1p11/1p36 in one DD case. These numerous observed chromosomal changes in DD cell cultures must be interpreted with caution because they are likely to be a consequence of abnormal fibroblastic growth or selective growth advantage for abnormal cells in the *in vitro* environment, rather than being the cause for DD development.[8,21,42] Kaur et al.[8] suggested that when tissue biopsies, rather than cultures, are used for studying chromosomal changes, alterations present in aberrant DD causative cells may be overlooked due to low numbers present in the tissues.[8] These authors reported no chromosomal imbalances in any of the 18 patients with DD investigated when DNA was extracted from DD tissue samples directly.[8] This contrasts with our findings; this might be due to differences in the microarray resolution. The aCGH technology used in our study has a probe density of one million, which is approximately 20 times that of previous microarray studies.

This study identified 3 novel copy number alterations in 3 different patients with sporadic DD. These results might indicate that DD is a heterogeneous disease caused by different genes in different DD clinical subgroups. In addition, because 9 common CNVRs

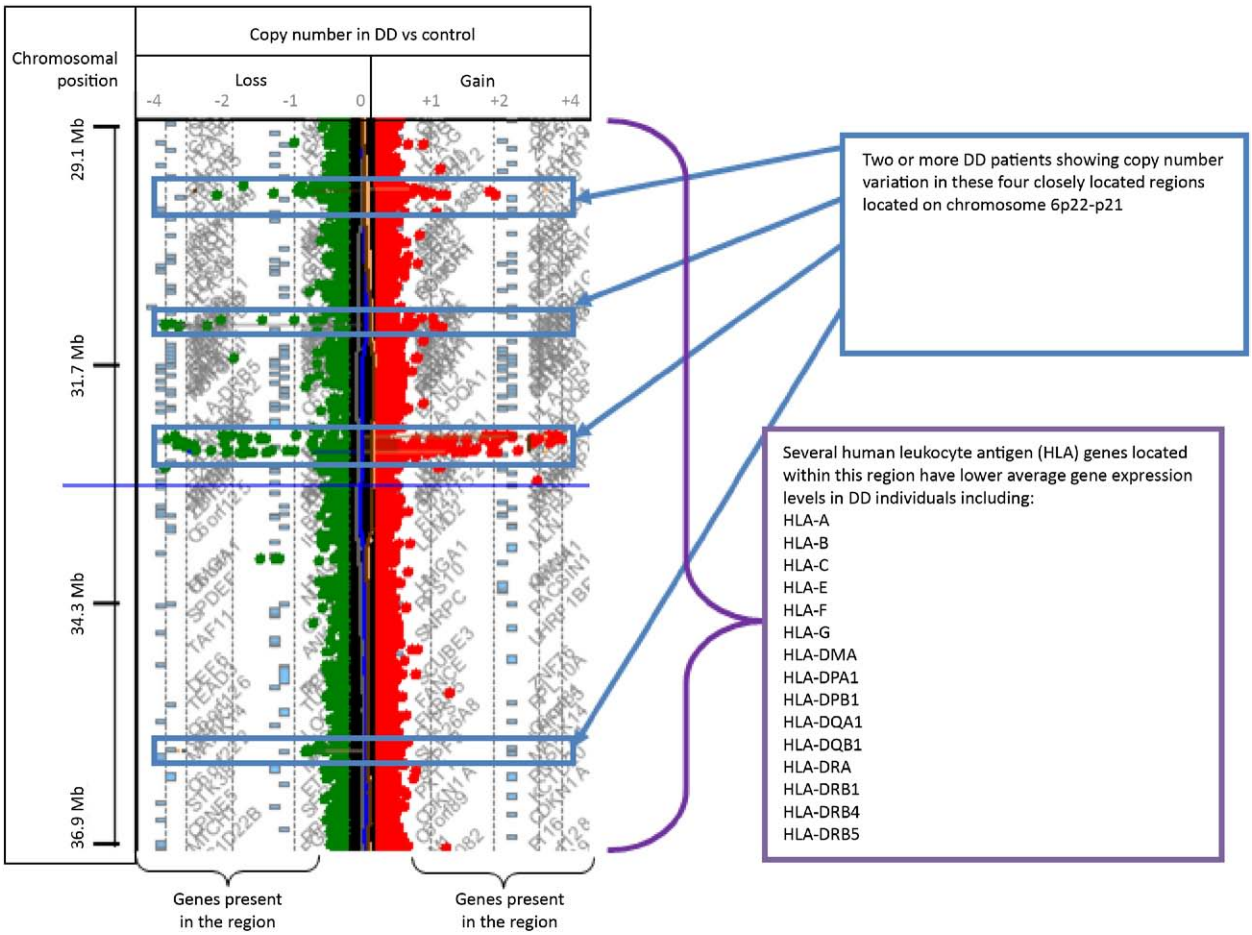

FIGURE 4: Complex Copy Number Variation Region (CNVR) identified in Dupuytren's Disease (DD) patients on chromosome 6p22–p21. Several CNVs between chromosome 6p22–p21 show amplification or deletion in 2 or more patients with DD with SurePrint G3 2x400K CNV arrays (Agilent Technologies). For the top 3 CNV regions, there is a higher average copy number among 3 of the 4 patients with DD. However, for the bottom one, significantly lower copy numbers were demonstrated in 2 patients. DNA Analytics settings: ADM-2; threshold, 6; filter, 6 probes, 0.25 log2 ratio, centralization threshold, 6; centralization bin size, 10; *t*-test p value threshold, 0.05; overlap threshold, 0.9; 2 Mb moving average.

have been identified that appear to be more prevalent among the 4 investigated cases, it is possible that DD is a complex oligogenic condition involving several different, interacting gene regions. Further CNV studies into these regions with a larger DD population size would provide more knowledge regarding the contribution of these and other genomic variations to DD development. Further detailed characterization of CNVR associated with DD and that determines whether the CNVR is developed or inherited may potentially lead to better DD diagnosis and prognosis as well as provision of possible future tailored therapeutic approaches.

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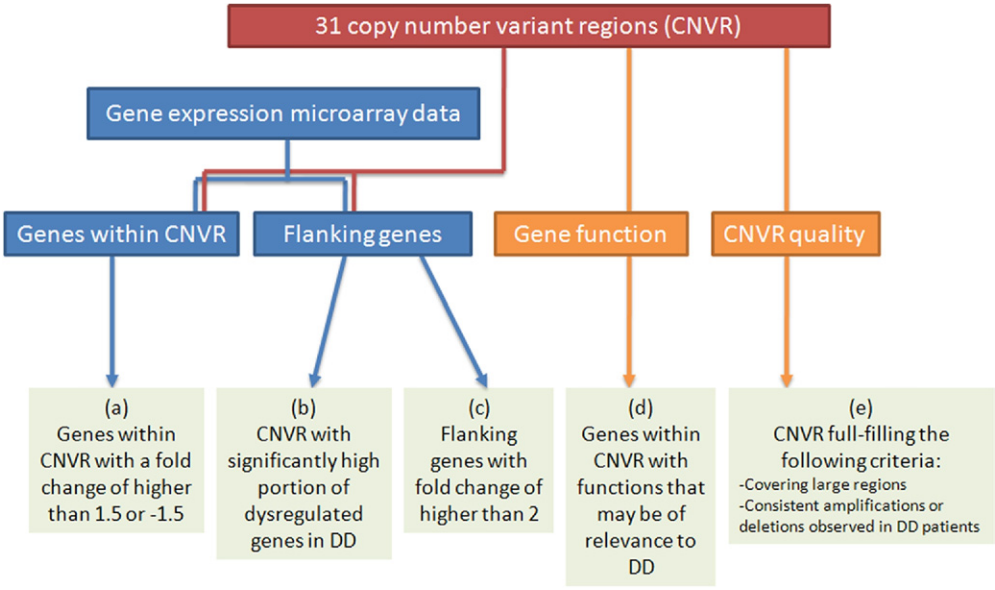
APPENDIX A: Summary of Steps Taken to Select Candidate Genes to Carry Out Further Analysis for Downstream Quantitative Polymerase Chain Reaction Confirmation

Thirty-one copy number variation regions (CNVRs) were found to be statistically significantly different in 3 or more of the DD samples (n=4). Five selection criteria, taking into account gene expression microarray data, gene function, and CNVR quality, were used to further narrow down the regions of interest.



APPENDIX B. Quantitative Polymerase Chain Reaction Assay Primers

Gene Designed for Quantitative Polymerase Chain Reaction	Forward Primer	Reverse Primer	Probe Number
TARP	TTTGGACAGTTGCGTTGACT	GCCATTGTTTCCTTTGCACT	45
PRR4	ACGAGGACACCGTCAACTCT	CTGGTCTGTCCCTCTGGAAG	4
CDC2L1	GACATGTGGTCAGTGGGTTG	CCTTGTTGATCTGATCGATTTCT	24
ADAM3A	TGCTGATGGGACAATATCCA	CCTTTGCTGCAACAGTTAGGA	76
SIRPB1	TGACCTCAGCTCATCACCAT	GTGACAACCTCGGCCAAT	10
ALB	GTGTAGCAATGTCAATTCGTGTT	GGCAAGGTCCGCCTATAAGT	31

Abbreviations: ADAM3A, a disintegrin and metalloproteinase domain 3a; ALB, albumin; CDC2L1, cell division cycle 2-like; PRR4, proline rich 4; qPCR, quantitative polymerase chain reaction; SIRPB1, signal-regulatory protein beta 1; TARP, TCR gamma alternate reading frame protein.

APPENDIX C. Common Copy Number Variation Regions That Are Present in 3 or More Subjects With Dupuytren's Disease

Loci			Copy Number Variation Size (Bp)	Number of Probes	Genes Within the Copy Number Variation Region			Reported Gain or Loss in the Copy Number Variation Database*	Patient Presenting the Copy Number Variation (Patient Identifier)			
Chromosome	Start	End				Loss	Gain		A	B	C	D
chr1	1575354	1640762	65409	13	CDC2L1 SLC35E2 KIAA0447 LOC728661 MMP21/22B CDC2L2		✓	Both	✓	✓	✓	✓
chr1	72538943	72574679	35737	13			✓	Both		✓	✓	✓
chr1	150822873	150851639	28767	12	LCE3C		✓	Both	✓		✓	✓
chr1	195009158	195091958	82801	10	CFHR3 CFHR1	✓		Both	✓		✓	✓
chr1	246928254	247186714	258461	80	LOC646627 KIAA1720 SH3BP5L ZNF672 ZNF692 DKFZp434G1221 AK309421 PGBD2	✓		Both	✓	✓	✓	✓
chr2	118006345	118094523	88179	15		✓		Loss	✓	✓	✓	
chr2	180121071	180129880	8810	6	ZNF385B	✓		Loss	✓	✓	✓	
chr2	242505061	242687145	182085	66	AK097934 FLJ38379 LOC441309		✓	Both	✓	✓		✓
chr3	174722241	174772531	50291	22	NLGN1	✓		Both	✓	✓		✓
chr4	173662854	173666272	3419	6	GALNTL6		✓	Both		✓	✓	✓
chr5	775994	873565			ZDHHC11	✓		Both	✓	✓	✓	
chr5	140203240	140216724	13485	8	PCDHA1 PCDHA2 PCDHA3 PCDHA4 PCDHA5 PCDHA6 PCDHA7 PCDHA8 PCDHA9 PCDHA10		✓	Both		✓	✓	✓
chr5	180344764	180359177	14414	8	BTNL8 LOC646227 BTNL3	✓		Both	✓	✓	✓	
chr6	29966136	30005538	39403	13	HLA-A HLA-A*0226 HCG2P7 AK097625 HCG4P6 BC035647		✓	Both		✓	✓	✓
chr7	38256971	38347360	90390	46	TCRG TRGC2 Z22690 TARP TRGV9 TCRGV AK096766 X06774 AK291611		✓	Both	✓	✓	✓	✓

APPENDIX C. Common Copy Number Variation Regions That Are Present in 3 or More Subjects With Dupuytren's Disease (Continued)

Loci			Copy Number Variation Size (Bp)	Number of Probes	Genes Within the Copy Number Variation Region			Reported Gain or Loss in the Copy Number Variation Database*	Patient Presenting the Copy Number Variation (Patient Identifier)			
Chromosome	Start	End				Loss	Gain		A	B	C	D
chr8	39350598	39505456	154859	60	ADAM5P tMDC	✓		Both	✓		✓	✓
chr12	33654	202872	169219	72	IQSEC3 AK096077 SLC6A12 SLC6A13	✓		Both	✓	✓	✓	✓
chr12	11110535	11140621	30087	10	PRR4 PROL4 PRH1 PRB4 TAS2R43		✓	Both		✓	✓	✓
chr13	68144064	68163723	19660	10			✓	Both	✓		✓	✓
chr14	18536977	19494616	957640	56	POTEG A26C2 AY458019 DQ595091 DQ591735 DQ590589 DQ583610 AY338954 AK056135 BX248778 AK022914 BC041856 BC016035 DQ786293 BC040855 BC017398 DQ583164 DQ595048 AY338952 OR4Q3 OR4M1 OR4N2 OR4K2 OR4K5 OR4K1	✓		Both	✓	✓	✓	
chr14	21423730	22041771	618042	305	TRAJ17 TCRA TCRAV8.1a AV2S1A1 TRA@ AV8S2 hADV14S1 AV22S1A2N1 T-Cell Receptor V-alpha region TCRVA2 AV1S3A1T TRD TRA AV30S1							

APPENDIX C. Common Copy Number Variation Regions That Are Present in 3 or More Subjects With Dupuytren's Disease (Continued)

Loci			Copy Number Variation Size (Bp)	Number of Probes	Genes Within the Copy Number Variation Region	Loss	Gain	Reported Gain or Loss in the Copy Number Variation Database*	Patient Presenting the Copy Number Variation (Patient Identifier)			
Chromosome	Start	End							A	B	C	D
					TCRVA13 hADV23S1 TCRD TCR-alpha TCRAVN1 hADV29S1 V alpha immunoglobulin AV4S1 AV25S1 hADV36S1 TCRAV14.1a hADV38S2 av27s1 TCR-α V 33.1 AK093552 AK125397 hDV102S1 TRD@ AK310110 TCRDV2 hDV103S1 X61074 TCR V alpha 8.1, J alpha IGRJa07 TRAC X60137 TCRAV21-J9.11		✓	Both	✓	✓	✓	✓
chr16	34306313	34602518	296206	55	BC045579 BC023607 BC038761	✓		Both	✓	✓	✓	
chr17	31457689	31889766	432078	22	TBC1D3B TBC1D3 DQ575408 DQ571391 DQ593188 DQ586142 DQ575686 CCL3L3 CCL3L1 CCL4L2 CCL4L CCL4L1 AL832615 DQ580766 TBC1D3C TBC1D3G PRC17 DQ580080 D63785 AX747639 DQ571776 DQ597784	✓		Both	✓	✓	✓	✓

APPENDIX C. Common Copy Number Variation Regions That Are Present in 3 or More Subjects With Dupuytren's Disease (Continued)

Loci			Copy Number Variation Size (Bp)	Number of Probes	Genes Within the Copy Number Variation Region			Reported Gain or Loss in the Copy Number Variation Database*	Patient Presenting the Copy Number Variation (Patient Identifier)			
Chromosome	Start	End				Loss	Gain		A	B	C	D
chr17	36675787	36684819	9033	6	No genes	✓		Both	✓	✓		✓
chr19	20422023	20474617	52595	13	No genes		✓	Both	✓	✓		✓
chr19	58211913	58244212	32300	15	No genes	✓		Both		✓	✓	✓
chr19	59420997	59434402	13406	10	LILRB3 LILRA6 LIR-3	✓		Both	✓	✓	✓	
chr20	1506179	1532633	26455	11	SIRPB1	✓		Both	✓	✓		✓

*Database of Genomic Variants (The Centre for Applied Genomics, Toronto, Ontario, Canada; http://projects.tcag.ca/variation/).

APPENDIX D. Details of the Novel Array-Based Comparative Hybridization Copy Number Variations Detected in 3 Patients With Dupuytren's Disease

Chromosome	Start Position (Bp)	Stop Position (Bp)	Copy Number Variation Size (Bp)	Probe	Number of Probes	Gain or Loss (Average Fold Change)	Genes in the Region
10q22	82,084,686	82,105,154	20,470	A_16_P02301838 A_16_P18994300 A_16_P39104095 A_16_P18994311 A_16_P18994316 A_16_P02301852 A_16_P18994318 A_14_P134967 A_16_P32704548 A_16_P18994333	10	Gain (0.62)	DYDC1
16p12.1	22,979,008	23,025,308	46,301	A_18_P12413915 A_16_P40586142 A_16_P33876740 A_16_P20427988 A_14_P136376 A_18_P20571315 A_14_P138547 A_16_P03127107 A_16_P20428014 A_16_P03127116 A_16_P40586204 A_16_P20428035 A_18_P12415869 A_16_P03127130 A_16_P20428050 A_18_P12416269 A_16_P40586244 A_16_P40586252 A_18_P20572734 A_18_P20573223 A_16_P20428080 A_16_P03127153 A_16_P20428089	23	Gain (0.72)	USP31
17p12	13,124,072	13,171,369	47,298	A_16_P20592174 A_16_P20592185 A_16_P20592189 A_16_P20592204 A_16_P20592219 A_16_P03217440 A_16_P40758864 A_16_P20592255 A_18_P12588158	9	Gain (0.53)	No genes listed. Closest flanking genes: HS3ST3A1 ELAC2

APPENDIX E: Novel Copy Number Variations

Three novel copy number variations were identified in 3 different patients with Dupuytren's disease by aCGH using the SureprintG3 microarrays (Agilent Technologies). Red spots above the baseline indicate regions in which a copy number gain is seen (duplication) on chromosomes 16p12, 17p12, and 10q22. DNA Analytics settings: ADM-2; threshold, 6; filter, 6 probes, 0.25 log2 ratio, centralization threshold, 6; centralization bin size.