DNA Copy Number Variations at Chromosome 7p14.1 and Chromosome 14q11.2 Are Associated with Dupuytren's Disease: Potential Role for MMP and Wnt Signaling Pathway

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Background: Dupuytren's disease is a common fibroproliferative disorder with an unknown etiology. Emerging evidence suggests a strong genetic component involved in the manifestation of the disease. This study aims to investigate the potential involvement of copy number variations in Dupuytren's disease pathogenesis.
Methods: Array-based comparative genomic hybridization (NimbleGen Human CGH 2.1 M) was utilized to compare DNA from (1) nodules versus internal control (patient's blood; $n = 4$) and (2) nodules ($n = 4$) versus external control (commercial reference DNA pooled from 10 donors). Analysis was carried out using Nexus 5.1 (BioDiscovery, El Segundo, Calif.) with the inclusion of additional results from previously published array-based comparative genomic hybridization. Copy number variations were considered to be common in Dupuytren's disease if the overlap was statistically significant and they were present in the majority (75 to 87.5 percent when compared with controls) of Dupuytren's disease nodules. The copy number variations loci were also compared with recently published genome wide-association studies. Common copy number variations were further validated using quantitative polymerase chain reaction. DNA from 25 Dupuytren's disease cases and 30 external controls were used in the quantitative polymerase chain reaction validation. In addition, gene expression was compared between Dupuytren's disease nodules and internal controls (transverse palmar fascia; $n = 7$).
Results: Five common copy number variations, on chromosome 17q12, 1p31.1, 20p13, 7p14.1, and 14q11.2, were identified by array-based comparative genomic hybridization. Significantly higher copy numbers of copy number variations at chromosome 7p14.1 and 14q11.2 in Dupuytren's disease were confirmed in quantitative polymerase chain reaction validation. Matrix metalloproteinase-14 and secreted frizzled-related protein 4 (near a polymorphism recently associated with Dupuytren's disease) were significantly up-regulated in nodules.
Conclusion: This study demonstrated an association between Dupuytren's disease and copy number variations at chromosomes 7p14.1 and 14q11.2. (*Plast. Reconstr. Surg.* 129: 921, 2012.)

Dupuytren's disease is a fibroproliferative disorder affecting the palm of the hand and often leads to permanent flexion contracture of the affected digits.[1] The molecular mechanism of the disease pathogenesis is currently unclear.[1] Strong genetic components have been suggested to be involved in Dupuytren's disease formation.[1,2] Observations supporting this assertion include its prevalence in certain ethnic groups,[3,4] disease severity for those with a positive family history,[5,6] and observations in twin studies.[2] A number of inheritance modes for Dupuytren's disease have been described; Ling[7] proposed a Mendelian

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dominant pattern of inheritance. However, as no single causative gene has been determined to date, Dupuytren's disease has been suggested to be a complex oligogenic disorder with variable penetrance.[8,9]

The heritable genetic components that may contribute to the development of Dupuytren's disease have been explored in several ways, including assessment of selected single nucleotide polymorphism,[8,10–12] human leukocyte antigen typing,[13] multiplex denaturing high-performance liquid chromatography (for mutations),[14] family linkage,[15] whole genome association,[9,16] and copy number variants using array-based comparative genomic hybridization.[17,18] These different approaches studied different types of genetic variations. This study focuses on copy number variations, which are defined as DNA segments of between 1 kb to several megabases that are present in variable copy numbers within the population. As much as 12 percent of the human genome is variable in copy numbers within the normal population.[19] Copy number variations have been suggested to be in involved in complex trait disorders, such as psoriasis,[20] human immunodeficiency virus type 1/acquired immune deficiency syndrome susceptibility,[21] and the onset or progression of neoplasia.[22]

In addition to inherited genetic variations, de novo mutations at the disease site have been suggested to be involved in Dupuytren's disease.[1,23–27] Several numerical and structural aberrations have been observed in Dupuytren's disease fibroblast cultures.[23–27] There, however, has not been a specific change that is associated with Dupuytren's disease, and the changes observed in cytogenetic studies have not been confirmed in array-based comparative genomic hybridization. This may due to the low presence of aberrant cells, which are not detected in this hybridization with biopsy samples.[17,18] Three novel copy number variations have previously been identified in Dupuytren's disease, but it is unclear if they are de novo or inherited, as internal controls or familial data were not available.[18]

Genetic alteration in diseases, such as cancer, may result in inactivation of tumor suppressor genes or activation of oncogenes, resulting in uncontrolled cell growth.[28] The presence of acquired genomic changes at Dupuytren's disease sites may account for the high recurrence after surgery; there may be small numbers of aberrant cells remaining at the disease site after surgical removal, which would then proliferate, and the disease would redevelop.

This study aims to determine the possible involvement of copy number variations in Dupuytren's disease through two different approaches: microarray screening for copy number variations using (1) case versus external control (Dupuytren's disease cases versus control subjects) samples and (2) case versus internal control (Dupuytren's disease nodules versus blood from the same patient) samples. The aim of the study was to identify potential copy number variations that may be involved in the pathogenesis of Dupuytren's disease.

PATIENTS AND METHODS

Participant Selection and Recruitment

Dupuytren's disease patients for this study were recruited from a cohort of Dupuytren's disease patients undergoing routine elective surgery (fasciectomy or dermofasciectomy). All subjects gave written, informed consent for the study. The local research ethics committee gave approval for the study protocol before commencement of the study. This study was conducted in accordance with the ethical principles of Good Clinical Practice and the Declaration of Helsinki.

Samples

All cases included in the study were of Northern European Caucasian origin. Four male Dupuytren's disease cases with an average age of 58 ± 13 years (mean ± SD) were used in array-based comparative genomic hybridization comparing DNA from palmar fascia nodules with DNA from blood. Four male Dupuytren's disease cases (age, 59 ±14 years) were used in array-based comparative genomic hybridization comparing DNA from nodules with external commercial DNA from controls. In the downstream quantitative polymerase chain reaction validation assays, 10 male Dupuytren's disease cases were used to compare DNA from nodules and blood samples (age, 61 ± 11 years). A further 25 Dupuytren's disease cases were used to compare DNA from nodules or blood (age, 65 ± 11 years; two women and 23 men) with DNA from blood of 30 external controls (age, 38 ± 13 years; 21 women and nine men). In addition, gene expression in nodules was compared with gene expression in the transverse palmar fascia in seven male Dupuytren's disease cases (age, 61 ± 11 years; five of these cases were the same as those used in the quantitative polymerase chain reaction validation for common copy number variations).

DNA Extraction

Extraction of DNA samples used for array-based comparative genomic hybridization was car-

ried out as previously described.[18] Blood and/or tissues were taken from individuals with and without Dupuytren's disease. Blood was collected in ethylenediaminetetraacetic acid, whereas tissue biopsies were placed in RNAlater (Ambion, Cambridge, United Kingdom), kept in 4°C fridge overnight, and stored at −80°C until used. Tissues were washed with phosphate buffered saline for 5 minutes. DNA was extracted using QIAmp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacture's protocol. The eluted DNA was further purified using ethanol precipitation overnight with sodium acetate (final concentration, 0.3 M; pH, 5.2).

The purity and quality of DNA was assessed using the NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, Inc., Wilmington, Del.) and by running on an 0.8 percent agarose gel with SYBR Safe DNA gel stain (Invitrogen, Eugene, Ore.). Only DNA samples with a 260/280 nm value greater than or equal to 1.8 and 260/230 nm value greater than or equal to 1.9 and a prominent band greater than 10 kb were used in array-based comparative genomic hybridization assays and DNA samples with a 260/280 nm value greater than or equal to 1.8 and 260/230 nm value greater than or equal to 1.8 for quantitative polymerase chain reaction.

RNA Extraction and Complementary DNA Synthesis

For RNA extraction, approximately $3 \times 2\text{-mm}^3$ tissues were finely diced and placed in three 2-ml round-bottom microcentrifuge tubes, each with a sterilized steel ball bearing and 1 ml of Trizol. Qiagen TissueLyser II was used to mechanically lyse the tissues. Protocol for RNA extraction has been described elsewhere.[29] Complementary DNA was synthesized using qScript's Complementary DNA Synthesis Kit with 192 ng of starting RNA template (Quanta Biosciences, Gaithersburg, Md.).

Array-Based Comparative Genomic Hybridization

Eight Roche-NimbleGen CGH 2.1M Whole-Genome Tiling v2.0D arrays (comprising 2.1 million genome-wide probes with 1.1-kb median probe spacing; Roche Diagnostics GmBH, Mannheim, Germany) reactions were carried out; four with Dupuytren's disease nodules compared with external DNA and four Dupuytren's disease nodules compared with internal control blood DNA. Control samples [female genomic DNA (Promega, Corp, Madison, Wisc.) and DNA from the blood of Dupuytren's disease patients] were labeled using Cy5 dye, whereas DNA extracted from nodules was labeled with Cy3 dye. The reactions were carried out according to the manufacturer's protocol. The use of the female external control against male Dupuytren's disease samples can serve as a quality control for array-based comparative genomic hybridization; however, it would mask potential copy number changes in the sex chromosomes.

Quantitative Polymerase Chain Reaction

The sequences for each copy number variation region were determined using the UCSC Genome Browser (http://genome.ucsc.edu/).[30,31] The Roche Universal ProbeLibrary Assay Design Center was used for designing quantitative polymerase chain reaction assays, and the National Center for Biotechnology Information PrimerBlast (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) was used to check the specificity of the primers. Quantitative polymerase chain reaction primer sequences are detailed in Table 1.

Quantitative polymerase chain reaction reactions were carried out in triplicate, each consisting of a 25-ng genomic DNA template, 5 μL of PerfeCTa FastMix (Quanta Biosciences), 0.2 μM forward primer, 0.2 μM reverse primer, 0.1 μM relevant probes from Universal Probe Library (Roche Diagnostics GmBH), and nuclease free water to a total reaction volume of 10 μL. For gene expression analysis, approximately 8 ng of complementary DNA was used. RPL32 and GAPDH were used as the reference genes. Secreted frizzled-related protein 4 (*SFRP4*), defender against cell death-*1* (*DAD1*), and matrix metalloproteinase-14 (*MMP14*) were the three selected target genes. The Roche LightCycler 480 System (Roche Diagnostics GmBH) was used to carry out the quantitative polymerase chain reaction cycling, and the reaction conditions were an initial activation step at 95°C for 10 minutes, followed by 45 cycles of 10 seconds at 95°C and 30 seconds at 60°C, and a final step of cooling at 40°C. Threshold cycles, the cycle numbers when the fluorescence levels become detectable, were calculated using second derivative method in the Roche LightCycler 480 software (Roche Diagnostic GmBH).

Data Analysis

Nexus 5.1 (BioDiscovery, El Segundo, Calif.) was used for data analysis. Four additional samples from published array-based comparative genomic hybridization study were also included.[1] Regions that displayed significant overlap in copy number

Table 1. Primers Used to Validate Selected Copy Number Variations/Alterations by Quantitative Polymerase Chain Reaction

Assay	Loci (hg18)	Forward Primer	Reverse Primer	Probe
Chr1A	Chr1: 72,540,188–72,540,256	tgtgggcattgggttattct	catatgtaacagcacggaactga	67
Chr1B	Chr1: 72,574,572–72,574,640	agttgcgttcccacagaagt	cattgcggtgttcactaagg	53
Chr7A	Chr7: 38,288,318–38,288,386	tcctgggggaactcaaacta	caaccgggagggtatctgta	76
Chr7B	Chr7: 38,290,844–38,290,913	ttacgaggacagacgctgaa	tttgtggtcagagttcttaactgg	23
Chr7C	Chr7: 38,301,010–38,301,081	ctttacccttctgtgactgagga	catggcagtgtaggattgga	50
Chr14A	Chr14: 21,824,761–21,824,825	tccttaccatacctgccaaatta	ggaaatatcacaaaagaagcctgt	67
Chr14B	Chr14: 21,911,024–21,911,083	tgggtctcgagtcactgttg	ctgcatccagcgaatctttc	21
Chr14C	Chr14: 22,004,507–22,004,567	caaagacaacaaacccagctc	ccttccccaggacttttgtc	78
Chr17A	chr17: 31,468,116–31,468,184	tctagcctttatcctgaaagtgc	tgtcaagagctttgtaaatctaggc	63
Chr17B	chr17: 31,480,240–31,480,299	agagtggccttgctaactgc	caactgcaggtgtaaccgttc	80
Chr20A	Chr20: 1,515,766–1,515,833	gggaacgatagacaagttcctc	gctacaactcatattccctatttgc	16
Chr20B	Chr20: 1,524,102–1,524,163	gatgtcccaggagggaagtt	atggaagatggccccagt	69
DAD1	mRNA transcript	tgcacttactgctagctctgct	gaaagttgttctgacacacagtga	2
MMP14	mRNA transcript	gacctacttcttccgtggaaac	ttgatgttcttggggtactcg	86
SFRP4	mRNA transcript	tcatgaagatgtacaaccacagc	tgtctggtgtgatgtctatcca	27

DAD1, defender against cell death-*1*; MMP14, matrix metalloproteinase-14; SFRP4, secreted frizzled-related protein 4.

changes ($p < 0.05$) in the majority of the samples (seven of eight when comparing nodules to external DNA, three of four when comparing nodules with blood) were selected for further quantitative polymerase chain reaction validation. The frequencies of these defined regions in control population were also compared with previously published data on The Database of Genomic Variants (http://projects.tcag.ca/variation/); results from bacterial artificial chromosome arrays and insertion/deletion of less than 1 kb were excluded.

The relative copy numbers from quantitative polymerase chain reaction were determined by using albumin as the reference. In the case in which DNA from Dupuytren's disease nodules was compared with internal blood controls, the relative copy numbers were obtained using the equation ($2^{-(CT_{target\ gene} - CT_{reference\ gene})}$); an interplate calibrator was not required, as the quantitative polymerase chain reactions for the corresponding blood and nodule DNA were done on the same plate. In cases in which external controls are included in the comparison, multiple quantitative polymerase chain reaction plates were used, and Promega female reference DNA was used as an interplate calibrator. The relative copy numbers were then determined using the equation $\left(\frac{2^{-(CT_{target\ gene} - CT_{target\ caliberator})}}{2^{-(CT_{reference\ gene} - CT_{reference\ gene\ calibrator})}}\right)$. For gene expression analysis, the relative gene expression levels were calculated using the equation ($2^{-(CT_{target\ gene} - CT_{reference\ gene})}$). Statistic tests and n number used for each comparison are described in Table 2.

Table 2. Statistical Tests Used and *p* Value Obtained for Each Quantitative PCR Assay

Assay	Comparison	Statistical Test	*p*	Ratio (Test/Control)
Chr1A	DD ($n = 25$)/external control ($n = 30$)	Mann–Whitney U test	0.546	1.2
Chr1B	DD ($n = 25$)/external control ($n = 30$)	Mann–Whitney U test	0.670	1.2
Chr7A	DD nodule/internal control blood ($n = 10$)	Wilcoxon signed rank test	0.002*	1.4
Chr7B	DD nodule/internal control blood ($n = 10$)	Wilcoxon signed rank test	0.002*	1.4
Chr7C	DD nodule/internal control blood ($n = 10$)	Wilcoxon signed rank test	0.002*	1.6
Chr7C	DD nodule ($n = 20$)/external control ($n = 30$)	Mann–Whitney U test	0.000*	1.7
Chr14A	DD nodule/internal control blood ($n = 10$)	Wilcoxon signed rank test	0.002*	1.6
Chr14B	DD nodule/internal control blood ($n = 10$)	Wilcoxon signed rank test	0.002*	1.4
Chr14C	DD nodule/internal control blood ($n = 10$)	Wilcoxon signed rank test	0.002*	1.6
Chr14C	DD nodule ($n = 20$)/external control ($n = 30$)	Mann–Whitney U test	0.000*	1.6
Chr17A	DD ($n = 25$)/external control ($n = 30$)	Mann–Whitney U test	0.155	1.1
Chr17B	DD ($n = 25$)/external control ($n = 30$)	Mann–Whitney U test	0.533	1.0
Chr20A	DD ($n = 25$)/external control ($n = 30$)	Mann–Whitney U test	0.627	0.8
Chr20B	DD ($n = 25$)/external control ($n = 30$)	Mann–Whitney U test	0.616	0.9
DAD1	DD nodule/internal control fascia ($n = 7$)	Wilcoxon signed rank test	0.237	1.2
MMP14	DD nodule/internal control fascia ($n = 7$)	Wilcoxon signed rank test	0.018*	8.6
SFRP4	DD nodule/internal control fascia ($n = 7$)	Wilcoxon signed rank test	0.028*	1.9

DD, Dupuytren's disease; DAD1, defender against cell death-*1*; MMP14, matrix metalloproteinase-14; SFRP4, secreted frizzled-related protein 4.
*Statistically significant ($p < 0.05$).

RESULTS

Comparative Genomic Hybridization

When comparing nodules with blood of the same patients (internal controls), two copy number variation regions were found on the arrays: three of four nodules showed copy number gains on chromosomes 14q11.2 and 7p14.1. In addition, when comparing nodules with external controls, the copy number variation region on chromosome 7p14.1 was also present in 87.5 percent of the cases, and the copy number variation region at chromosome 14q11.2 was present in all Dupuytren's disease cases. This suggests that the copy number changes were only present at the disease site (nodule) but not in blood (Fig. 1). An additional three significant copy number variation regions were found in more than 87.5 percent of the Dupuytren's disease cases when comparing them with external control DNA. A summary of the array-based comparative genomic hybridization results can be found in Table 3 and Figure 2. All copy number variations have previously been reported (Database of Genomic Variants), although the ones identified on chromosomes 7p14.1 and 14q11.2 are relatively rare.

Copy Number Variation Validation by Quantitative Polymerase Chain Reaction

Significantly higher copy numbers of the chromosome 14q11.2 and 7p14.1 copy number variation regions were observed in Dupuytren's disease nodules in comparison with internal control blood in all quantitative polymerase chain reaction validation assays ($p < 0.005$; Table 2 and Figs. 3 and 4). This may suggest that certain copy number gains have more selective advantages in tumor growth. One patient who did not show copy number differences between blood and nodules for these two copy number variation regions on the microarrays (Fig. 1) did, in fact, show a change when tested in the quantitative polymerase chain reaction validation assays for these loci; this false-negative result may be due to quality problems of the array-based comparative genomic hybridization assay. There is also a significantly higher copy number of these copy number variations in nodules compared with external controls. Significantly different copy numbers for the two copy number variation regions also occur when comparing nodule and blood DNA from the same patient (only Dupuytren's disease nodules were used in the comparisons with external controls).

It has been previously demonstrated that copy number variations influence the expression of genes in their vicinity, even up to 450 kb away from the boundary of the copy number variation.[32] One gene (*SFRP4*) 450 kb upstream of the chromosome 7p14.1 copy number variation region (Fig. 5) and two genes (*DAD1* and *MMP14*) 450 kb upstream of the chromosome 14q11.2 copy num-

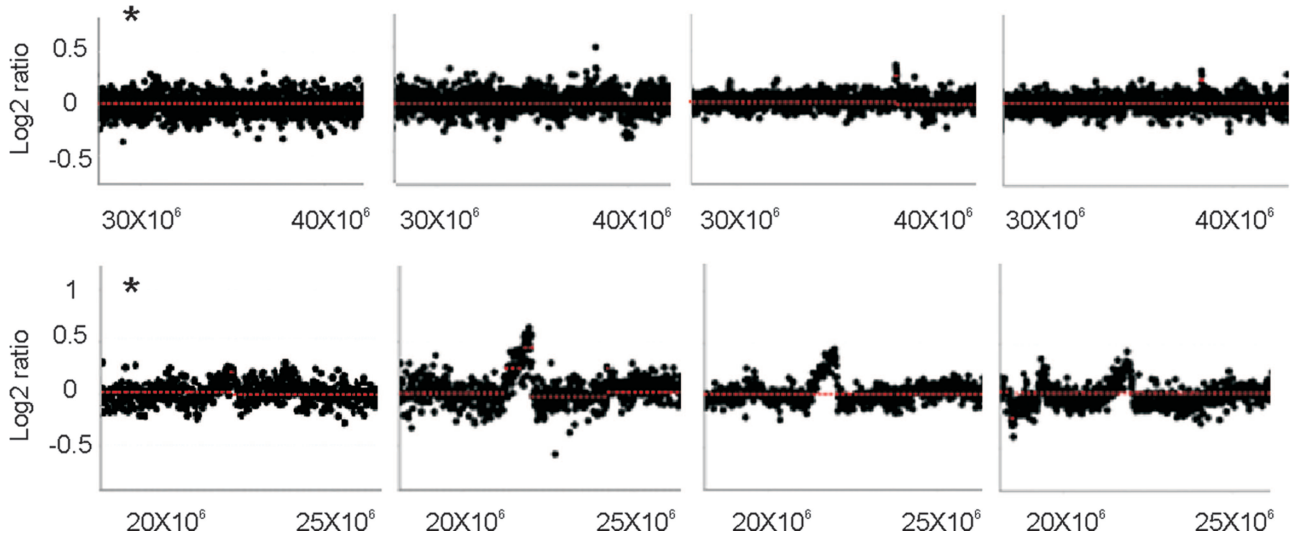

Fig. 1. Copy number changes detected by array-based comparative genomic hybridization. Comparisons between blood and nodular DNA in three Dupuytren's disease patients. Copy number alterations at (*above*) chromosome 7: 38,285,500 to 38,302,725 bp and (*below*) chromosome 14: 21,810,963 to 22,029,889 bp. If the data points were near 0.0 along the Log2 ratio axis, the control (internal blood DNA) and test (nodule DNA) had the same copy number. If the value for Log2 ratio is greater than 0, it indicated that there was a higher copy number of the genomic region in the nodule than in the blood. *One patient did not show significant difference in copy numbers on array-based comparative genomic hybridization.

Table 3. Details of Copy Number Variation Regions Detected by Array-Based Comparative Genomic Hybridization

CNVR Loci (hg18)	Size of CNVR	Genes within the Region	Genes 450 kb Upstream of the Region	Genes 450 kb Downstream of the Region	No. of Samples Showing Copy Number Gain/Loss
Chr1: 72,539,425–72,584,227	44,802	N/A	NEGR1	N/A	7/8 gain, 1/8 loss (case versus external control) 4/4 no change (nodule vs. internal control blood)
Chr7: 38,285,500–38,302,725	17,225	TRGC2	STARD3NL	TRGV5	7/8 gain (case versus external control)
		TRGV9	EPDR1	TRGV3	3/4 gain, 1/4 no change (nodule versus internal control blood)
		TCRG	TARP TCRg SFRP4	AMPH FAM183B VPS41	
Chr14: 21,810,963–22,029,889	218,926	TRAC	N/A	OXA1L	8/8 gain (case versus external control)
		ABHD4		MMP14	3/4 gain, 1/4 no change (nodule versus internal control blood)
		TCRA		LRP10 REM2 RBM23 PRMT5 JUB C14orf93 C14orf94 DAD1 MRPL52 SLC7A7	
Chr17: 31,462,055–31,491,755	29,700	N/A	CCL3L1	C17orf66	8/8 loss (case versus external control)
			CCL3L3	CCL5	4/4 no change (nodule versus internal control blood)
			CCL4L CCL4L1 CCL4L2	RDM1 LYZL6 CCL16 SCYA16 CCL14 CCL15 CCL23 CCL18 CCL3 CCL4	
Chr20: 1,514,240–1,527,293	13,053	N/A	FKBP1A	SIRPG	6/8 loss, 2/8 gain (case versus external control)
			NSFL1C		1/4 loss, 3/4 no change (nodule versus internal control blood)
			SIRPD		

CNVR, copy number variation region; N/A, not applicable.

ber variation were selected for gene expression analysis in seven Dupuytren's disease cases (nodules compared with internal control transverse palmar fascia). Five of the seven cases were also used in copy number variation region validation by quantitative polymerase chain reaction , and all have higher copy numbers in nodules. *MMP14* and *SFRP4* show significant up-regulation in Dupuytren's disease nodules when compared with internal control fascia (Table 2 and Fig. 6).

No significant associations were found in the validation assays for the three remaining copy number variation regions detected on chromosomes 17q12, 1p31.1, and 20p13 in case–control comparisons. The statistical tests and significance values for each quantitative polymerase chain reaction assay are summarized in Table 2. The findings of this study are summarized in Figure 7.

DISCUSSION

In this study, we investigated the possible involvement of copy number changes in Dupuytren's disease from two different perspectives,

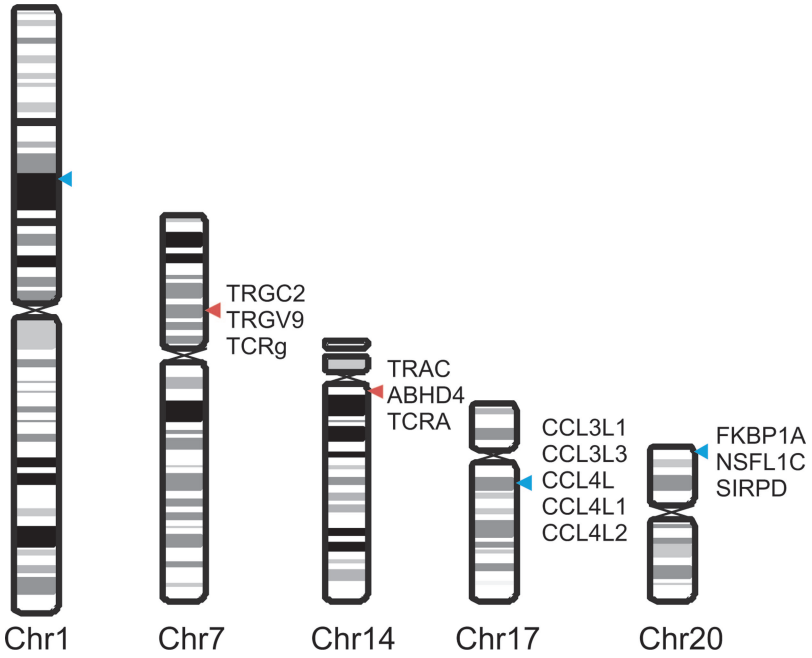

Fig. 2. Copy number variation regions selected for validation using quantitative polymerase chain reaction. The *arrows* indicate the genes within the copy number variation regions investigated by quantitative polymerase chain reaction. The *blue arrows* indicate the regions with significantly different copy numbers between Dupuytren's disease DNA and external control DNA. The *red arrows* indicate the regions with significantly different copy numbers between DNA from nodules and blood of the same patient. TRGC2, T-cell receptor gamma constant 2; TRGV9, T-cell receptor gamma variable 9; TCRG, T-cell receptor gamma locus; TRAC, T-cell receptor alpha constant; ABHD4, abhydrolase domain containing 4; TCRA, T-cell receptor alpha locus; CCL3L, chemokine (C-C motif) ligand 3-like; CCL4L, chemokine (C-C motif) ligand 4-like; FKBP1A, FK506 binding protein 1A; NSFL1C, NSFL1 (p97) cofactor (p47); SIRPD, signal-regulatory protein delta.

comparing copy number variations detected in Dupuytren's disease subjects with control subjects and determining copy number alterations that may have arisen sporadically within the Dupuytren's disease disease site (at the nodules).

Two regions with significant ($p < 0.001$) copy number changes were detected when comparing DNA from nodules and blood from the same individual on chromosomes 7p14.1 and 14q11.2. In addition, at least a 1.6-fold copy number increase was observed at these loci when comparing Dupuytren's disease nodules with the control group ($p < 0.001$). The copy number variation region on 7p14.1 is adjacent (within 450 kb) to a single nucleotide polymorphism (rs16879765) recently reported to be significantly associated with Dupuytren's disease.[9]

The copy number variation region on chromosome 7p14.1 (chr7: 38,285,500 to 38,302,725 bp) has previously been reported to show copy number gains in two of 1190 control samples in one study,[33] and one of 50 control samples in a different study.[34] The chromosome 14q11.2 (chr14: 21,810,963 to 22,029,889 bp) copy number gain is reported to be even more common in the normal population; 125 of 1190 control samples[33] and one of 70 control samples.[35] The 7p14.1 copy number variation region contains the genes T-cell receptor gamma constant 2 (*TRGC2*), T-cell receptor gamma variable 9 (*TRGV9*), and T-cell receptor gamma (*TCRG*); and at chromosome 14q11.2, the genes T-cell receptor alpha locus (*TCRA*), T-cell receptor alpha constant (*TRAC*), and abhydrolase domain–containing 4 (*ABHD4*) are present in the copy number variation region. Interestingly, reduced numbers of *TCRG* have been associated with childhood allergic asthma,[36] and higher copy numbers of *TCRG* and *TCRA* have been associated with hepatocellular carcinoma.[37] Because T-cell maturation in-

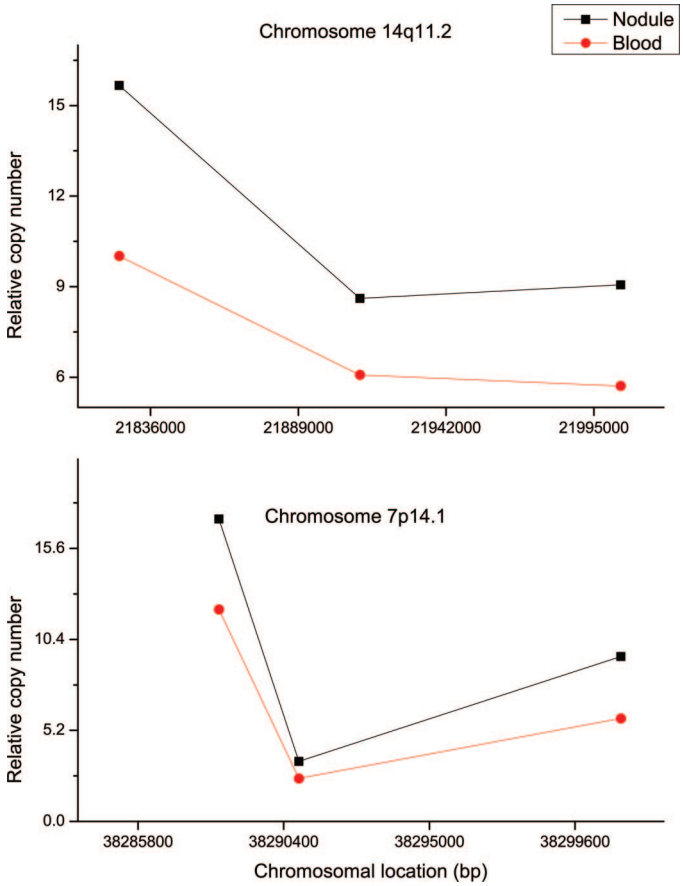

Fig. 3. Relative copy numbers of chromosome 7p14.1 and 14q11.2 copy number variation regions between blood and nodules from Dupuytren's disease patients. Three assays were designed for each loci. The figures demonstrate the average relative copy numbers for each assay. DNA from the nodules displayed a significantly higher number of copies for all assays at both loci.

volves T-cell receptor (TCR) gene rearrangements, it is unclear whether the significant copy number variations observed in this study are directly involved in the pathogenesis of Dupuytren's disease or occur as a result of a difference in T-cell populations between blood and nodule tissue. Schwienbacher et al.[38] have reported that copy number variations of the *TCR* genes may be influenced by the DNA source; blood contains T lymphocytes, which would have undergone chromosome rearrangement. The differences in *TCR* copy numbers that we observed may, in fact, reflect a high lymphocyte count in blood. Also, significantly lower copy number of the alpha and gamma, but not delta, *TCR*s in the blood of Dupuytren's disease patients may suggest a higher frequency of *TCR* rearrangement events that exclude the constant alpha region. Possible involvement of T cells in Dupuytren's disease has been suggested by several other observations, including higher levels of CD3-positive T lymphocytes and major histocompatibility complex class II molecules,[39] and higher levels of activated T cells and memory T cells in the blood from Dupuytren's disease patients and those with more severe forms of Dupuytren's disease.[40]

In addition, we have shown misregulation of expression of selected genes near the copy number variation region loci. It has been demonstrated that copy number variations can influence the expression of nearby genes, with effects up to 450 kb both upstream and downstream.[32] *SFRP4*, located within 450 kb upstream of the 7p14.1 copy number variation region (Fig. 5), and *MMP14 and DAD1*, which locate 450 Kb downstream from the 14q11.2 copy number variation region, were investigated due to their potential relevance to Dupuytren's disease. *SFRP4* is involved in the *Wnt*

pathway, and the *Wnt* signaling pathway has been suggested to be involved in Dupuytren's disease pathogenesis in several studies.[9,41] In addition, a single nucleotide polymorphism (rs16879765) adjacent to *SFRP4* has recently been associated with Dupuytren's disease[9] (Fig. 5). *SFRP4* is significantly up-regulated ($p < 0.05$) in Dupuytren's disease nodules when compared with an internal control (transverse palmar fascia). Dysregulated expression of both *MMP14* and *DAD1* has been previously reported to be associated with Dupuytren's disease,[42–44] and these genes are directly downstream of the 14q11.2 copy number variation region. *MMP14* expression was significantly higher in nodules, and higher levels of *MMP14* have been previously associated with recurrence of nodules following surgical intervention in Du-

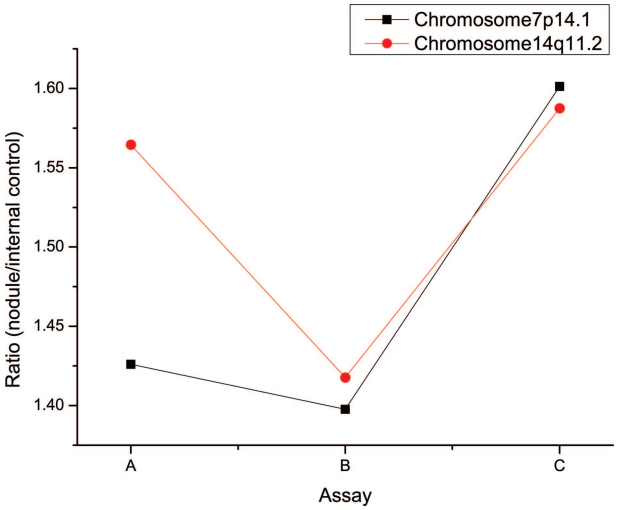

Fig. 4. Copy number ratios between blood and nodules from three Dupuytren's disease patients at two copy number variation regions. The figure demonstrates the average copy number changes when comparing DNA copy numbers in nodules with blood for each of the three assays. On average, there were 1.4 times as many copies of the two loci in nodule samples compared with blood; *$p < 0.05$ when comparing between nodule and internal control.

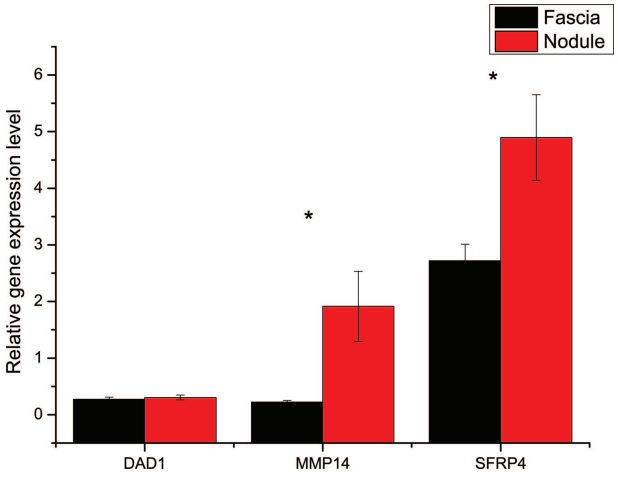

Fig. 6. Relative gene expression levels for *DAD1, MMP14*, and *SFRP4* in nodules and fascia. A significantly ($p < 0.05$) higher level of *MMP14* and *SFRP4* mRNA expression was found in the Dupuytren's disease nodules compared with internal controls (transverse palmar fascia; $n = 7$). The average change was approximately 8.6 and 1.9 times higher in nodules for *MMP14* and *SFRP4*, respectively. *DAD1* was not significantly differentially expressed in Dupuytren's disease nodules. Wilcoxon signed rank test was used for the analysis as the data are paired (internal control), and a skewed trend was observed in the box plot.

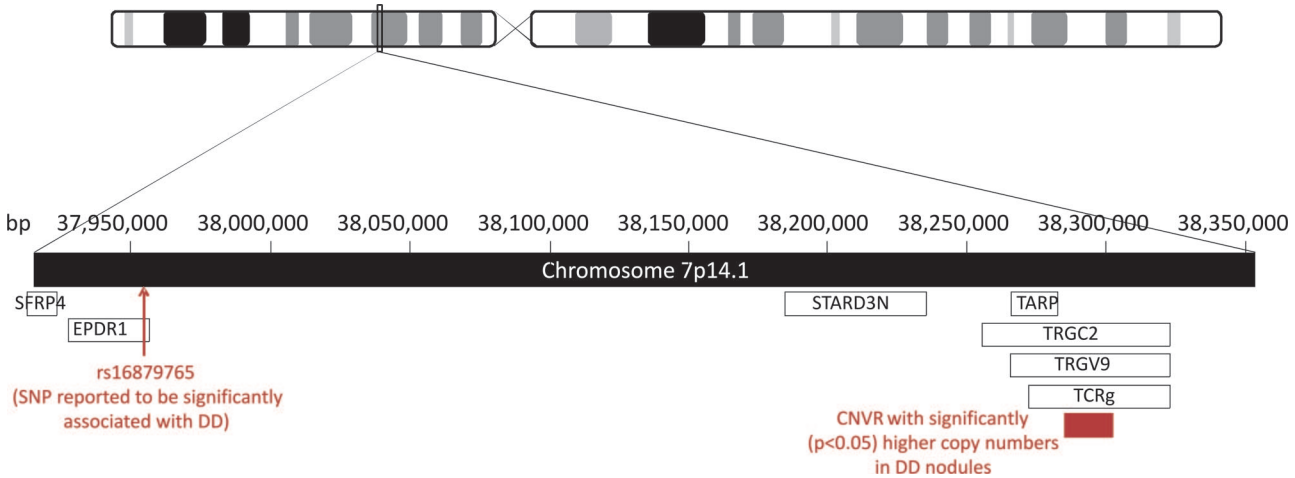

Fig. 5. Proximity of the chromosome 7p14.1 copy number variation region to single nucleotide polymorphism (rs16879765) associated with Dupuytren's disease. The *shaded red box* indicates the copy number variation region, and the *red arrow* indicates the relative location of the single nucleotide polymorphism (rs16879765) that has been reported to be significantly associated with Dupuytren's disease.[9]

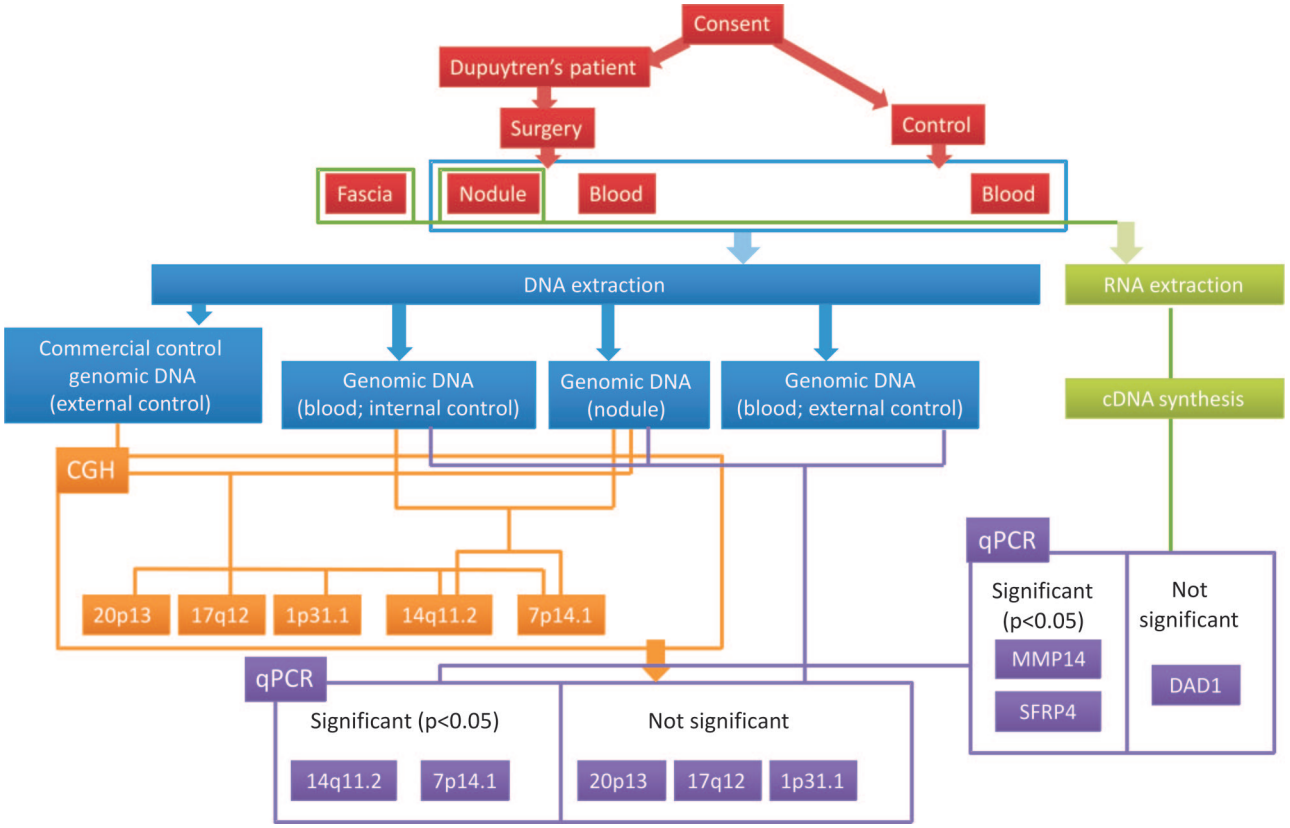

Fig. 7. Flowchart of the experimental procedures and results in this study.

puytren's disease cases.[44] *DAD1* has been reported to show an average four-fold up-regulation in Dupuytren's disease patients in a previous microarray study.[43] We confirmed dysregulation of *MMP14* and *SFRP4* by reverse transcriptase quantitative polymerase chain reaction in this study; however, the aberrant expression of *DAD1* mRNA found in the microarray results was not confirmed by reverse transcriptase quantitative polymerase chain reaction, indicating it may not be real.[43] Another interesting feature of the14q11.2 copy number variation region is a reported increased incidence of copy number variations on chromosome 14q12 in tumor samples, and the region may be associated with the onset or progression of neoplasia.[22]

The remaining three copy number variation regions on chromosomes 17q12, 1p31.1, and 20p13 were not found to be statistically significant when comparing 25 Dupuytren's disease patients with 30 controls. Although we did not find statistical significance with the 17q12 copy number variation region, Ojwang et al.[16] have reported an association between Dupuytren's disease and a single nucleotide polymorphism (rs1978136) on chromosome 17 (chr17:29,401,811bp), which is within the same cytoband as our copy number variation region at chromosome 17q12 (approximately 2 Mb away from the single nucleotide polymorphism). It is thus possible that an unknown genetic variation near the region is involved in Dupuytren's disease pathogenesis.

CONCLUSIONS

This study has identified and examined genetic variations that may contribute to Dupuytren's disease susceptibility. We have detected two genetic loci on chromosomes 14q11.2 and 7p14.1 that show copy number alterations between DNA from blood and palmar nodules from the same Dupuytren's disease patient and have higher copy numbers in nodules of Dupuytren's disease patients compared with external controls. The copy number variation region at chromosome 7p14.1 is in close proximity (within 450 kb) to a single nucleotide polymorphism (rs16879765) that has been positively associated with Dupuytren's disease.[9] A selection of genes near these copy number variation regions have also shown differential gene expression in Dupuytren's disease patients, including *MMP14* and *SFRP4*, and may therefore be involved in the disease etiology. These copy number variation regions also contain several genes involved in T-cell

receptor formation, which may support the hypothesis that T cells are involved in the development of Dupuytren's disease. These findings will help in better understanding the pathogenesis of Dupuytren's disease with potential diagnostic and therapeutic implications in the future.

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