HAND
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ORIGINAL PAPER

Differential Gene Expression Analysis of Subcutaneous Fat, Fascia, and Skin Overlying a Dupuytren's Disease Nodule in Comparison to Control Tissue

Barbara Shih · Jason J. Brown · Daniel J. Armstrong · Tommy Lindau · Ardeshir Bayat

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Abstract Dupuytren's disease (DD) is a benign fibroproliferative tumor with an unknown etiology and high recurrence postsurgery. Several observations suggest the possible involvement of skin overlying nodule (SON) and the subcutaneous fat in the pathogenesis of DD. This study aims to (1) compare the gene expression levels of SON and subcutaneous fat in DD and normal subjects and (2) to compare transverse palmar fascia (Skoog's fibers) from DD patients as internal control tissue, with palmar fascia (transverse carpal ligament) from patients undergoing carpal tunnel release as external control. Skin, fat, and fascia were obtained from five DD patients of Caucasian origin (age=66±14) and from five control subjects (age=57±19) undergoing carpal tunnel release. Total ribonucleic acids was extracted from each sample and used for complementary deoxyribonucleic acid synthesis. Real-time quantitative polymerase chain reaction was used to assess the gene expression levels of six candidate genes: A disintegrin and metalloproteinase domain (ADAM12), aldehyde dehydrogenase 1 family member A1 (ALDH1A1), iroquois homeoboxprotein 6 (IRX6), periostin, osteoblast specific factor, proteoglycan 4, and tenascin C. Using independent t test, ADAM12, ALDH1A1, and IRX6 expression levels in DD fats were significantly ($p<0.05$) higher than those in the controls. There is no significant difference in the gene expression levels of all six genes when comparing disease and control fascia and skin. Interestingly, ADAM12 up-regulation has also been observed in several other fibrotic and proliferative disorders. In conclusion, this study demonstrates potential roles for subcutaneous fat in DD pathogenesis as well as supports the use of transverse palmar fascia as appropriate control tissues in DD research.

Keywords Dupuytren's disease · Dupuytren's contracture · Subcutaneous fat · Skin overlying nodule · Transverse palmar fascia · Recurrence · Gene expression · Transcriptomics

Introduction

Dupuytren's disease (DD) is a common fibroproliferative disorder that affects palmar and digital fascia, leading to disability in affected individuals due to progressive and permanent flexion contracture of the digits [14].

Typically, a nodule appears first, followed by the development of cord, which progressively and irreversibly shortens and leads to the contracture of the digits [29]. It is believed that the contracture in DD is caused by the myofibroblast [10], a cell type believed to play an important role in contraction of connective tissues post injury and fibrosis [15]. However, the origin of the causative cells, the original fibroblast that myofibroblasts differentiate from, for DD formation and contracture remains unknown.

While surgery remains the mainstay of treatment for DD, the recurrence rate is high [36]. Several groups have reported lower postsurgery DD recurrence with dermofas-

B. Shih · J. J. Brown · A. Bayat (✉)
Plastic & Reconstructive Surgery Research,
Manchester Interdisciplinary Biocentre, University of Manchester,
Manchester, UK
e-mail: ardeshir.bayat@manchester.ac.uk

A. Bayat
Department of Plastic, Reconstructive and Hand Surgery,
South Manchester University Hospital Foundation Trust,
Manchester, UK

D. J. Armstrong · T. Lindau
The Pulvertaft Hand Centre, Derbyshire Royal Infirmary,
London Road,
Derby DE1 2QY, UK

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ciectomy, a procedure that involves replacing the overlying affected skin as well as subcutaneous fat adjacent to nodule with a skin graft [13, 40, 41]. Several other observations also suggest possible involvements of skin overlying nodule (SON) or subcutaneous fat in DD pathogenesis [2, 13, 27, 28, 40, 41, 46]. Despite this, there are no studies that have investigated the possible role of skin and fat in DD by transcriptomic analysis.

Molecular analysis has become an essential component in many areas of modern medical research [34]; however, there have been few molecular studies to date carried out in molecular dissection of DD. Limited number of previous microarray and linkage studies have demonstrated lists of genes that may potentially be involved in DD pathogenesis; however, these potential findings require further validation with more sensitive experimental approaches [18, 35]. Quantitative polymerase chain reaction (qPCR) is a sensitive, rapid, and accurate method of quantifying the transcript levels of genes of interest in a study differentiating between disease and healthy tissue [43].

Therefore, in order to further investigate the possible involvement of fat and skin in DD pathology, transcriptomic or differential gene expression analysis of various candidate genes was performed in skin, fat, and fascia from DD cases and control subjects.

Materials and Methods

The steps taken in the study were summarized in Fig. 1. In brief, ribonucleic acids (RNA) were extracted from biopsies of skin, fat, and fascia (Fig. 2) from five DD and five control subjects. Using the extracted RNA, complementary DNA (cDNA) was synthesized and used in quantitative polymerase chain reactions. The results from qPCR were analyzed using independent t test.

Patients

Five DD cases and five control subjects were included in the study. All recruited DD cases were diagnosed with advanced stage of DD, which was determined clinically by an experienced hand surgeon. All patients presented flexion contracture of the metacarpophalangeal joint and proximal interphalangeal joint as well as presence of nodules. All participating DD patients were men, of Caucasian origin, with a mean age of 66±14 years who had not previously received treatments for DD. Four of the five control subjects included in the study were Caucasians, with the remaining one being Asian Indian. Three of the control subjects were male, and two were female. The average age of the control subjects was 57± 19 years. The study was approved by the institutional review board for human subject research.

Sample Collection

Three tissue biopsies, including the SON, subcutaneous fat superficial to nodule, and unaffected transverse palmar fascia (Skoog's fibers), were carefully dissected from each DD patient at the time of surgery (Fig. 2a). Three tissue biopsies, including the skin, subcutaneous fat, and palmar fascia (transverse carpal ligament), were obtained from individuals undergoing carpal tunnel release (Fig. 2b). The harvested biopsy samples were kept in RNA*later* (Ambion, UK) at 4°C overnight and stored at −80 until use.

RNA Extraction

For each biopsy sample, approximately 8 mm^3 of tissue was finely diced and placed in four separate 2-mL round-bottom Eppendorf tubes, each containing a flame-sterilized steel ball bearing and 1 mL Trizol (Invitrogen, UK). Qiagen Tissue Lyser (Qiagen, UK) was used to homogenize the tissues, at 30 oscillations per second for 12 min. The homogenized tissue suspension in each tube was transferred to a 1.5-mL Eppendorf tube and centrifuged at 13,000 rpm for 10 min. The resulting supernatant was transferred to a new Eppendorf tube, mixed well with 0.2 mL chlorophorm, and left at room temperature for 2 min. The mixture was then spun at 13,000 rpm for 15 min. The upper aqueous layer was collected and mixed with an equal volume of 70% ethanol, which was then further processed with RNeasy kit (Qiagen) according the manufacturer's instructions. DNase treatment was then carried out using DNAfree kit (Ambion) according to the manufacturer's protocol. NanoDrop ND-1000 UV-visible spectrophotometer (Labtech International, UK) was used to estimate the total RNA concentration.

Complementary DNA Synthesis

SuperScript II ™ Reverse Transcriptase kit (Invitrogen) was used for complementary DNA synthesis. One microliter of nucleotide mix (10 mM for each nucleotide; Invitrogen), 375 ng oligo-dT (Invitrogen), 62.5 ng random primers (Invitrogen), total RNA, and nuclease-free water (Ambion) were used to make up 12 μL reaction volume. Total RNA (0.5–1 μg) was used in each reaction, and the gene expression levels were normalized with internal reference gene using relative gene expression level method at the stage of quantitative polymerase chain reaction.

The mixture was incubated at 65°C for 5 min. Following rapid cooling on ice, 2 μL of 0.1 M dithiothreitol, 1 μL of RNaseOut (Invitrogen), and 4 μL of First-Strand Buffer (250 mM Tris–hydrochloride, pH 8.3 at room temperature; 375 mM potassium chloride; 15 mM magnesium chloride) were then added to each reaction tube. The mixtures were then incubated 42°C. After 2 min, 1 μL SuperScript ™ II

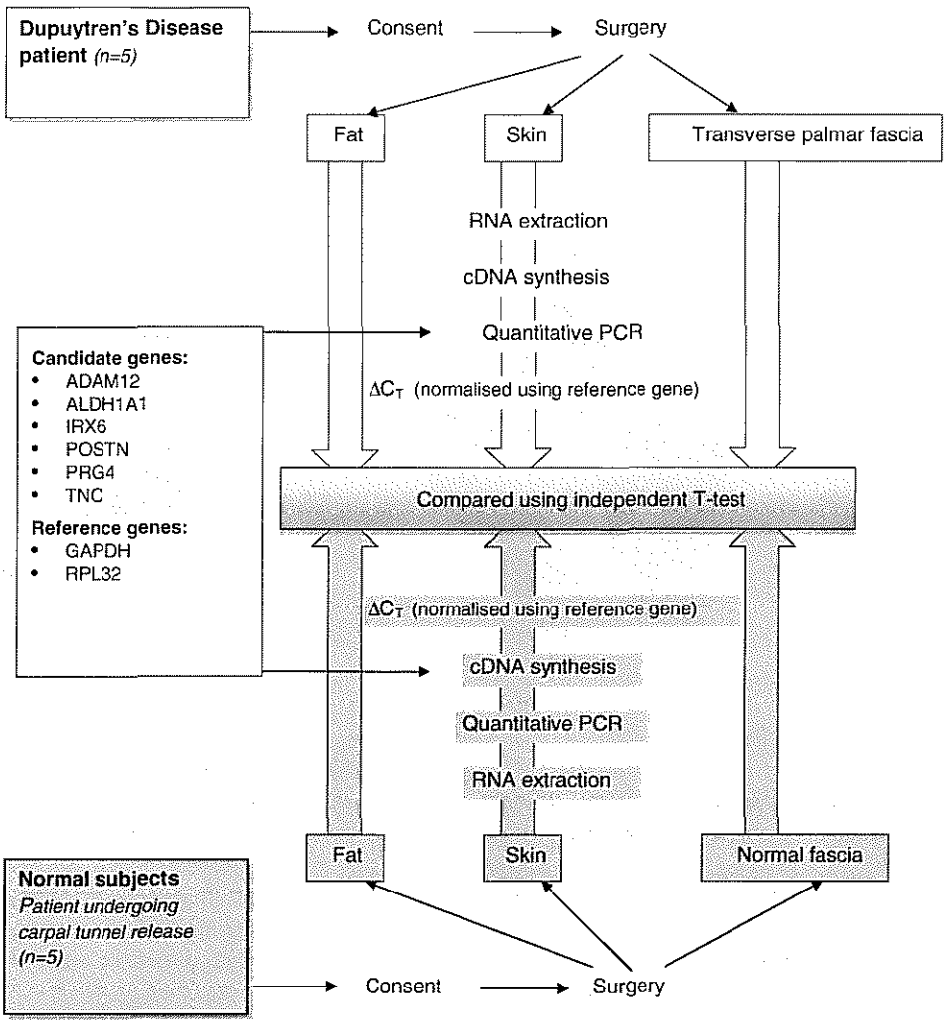

Figure 1 Summary flowchart of the steps taken in the study. Summary of the steps taken in the study to determine whether there is differential gene expression levels for genes in DD and control skin, fat, and fascia [39]. Following the consent of all subjects selected for the study, relevant surgical procedures (dermofasciectomy for cases and carpal tunnel release for controls) were carried out, and biopsies of skin, fat and palmar fascia were obtained. The biopsy samples were subjected to RNA extraction, following which cDNA synthesis and quantitative PCR were carried out. Relative gene expression levels were determined using the $2^{-\Delta CT}$. ΔC_T was determined by subtracting the threshold cycle of reference gene from those of target genes. Independent *t* tests were then carried out on relative gene expression levels to determine genes that are significantly ($p<0.05$) differentially expressed. *ADAM12* A disintegrin and metalloproteinase domain; *ALDH1A1* aldehyde dehydrogenase 1 family member; *IRX6* iroquois homeoboxprotein 6; *PRG4* proteoglycan 4; *TNC* tenascin C; *POSTN* periostin, osteoblast specific factor; *RPL32* ribosomal protein L32; *GAPDH* glyceraldehyde-3-phosphate dehydrogenase; ΔC_T delta threshold cycle; *PCR* polymerase chain reactions; *RNA* ribonucleic acid; *cDNA* complementary deoxyribonucleic acid.

Reverse Transcriptase (Invitrogen) was added to each reaction tube, which was incubated for 10 min at 25°C and then 50 min at 42°C. The reaction was inactivated by incubating at 70°C for 15 min.

Gene Selection

Six candidate genes and two reference genes were taken from Shih et al. [39]. In short, using bioinformatic approaches, including functional clustering with DAVID Bioinformatic Resources, existing microarray data, and linkage analysis, six candidate genes were short-listed on the basis of value of fold changes observed in microarray and functions [18, 35, 39]. The candidate genes were: A disintegrin and metalloproteinase domain (ADAM12), aldehyde dehydrogenase 1 family member (ALDH1) A1, iroquois homeoboxprotein 6 (IRX6), proteoglycan 4 (PRG4), tenascin C (TNC), and periostin, osteoblast specific factor (POSTN). The reference genes were ribosomal protein L32 (RPL32) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [39].

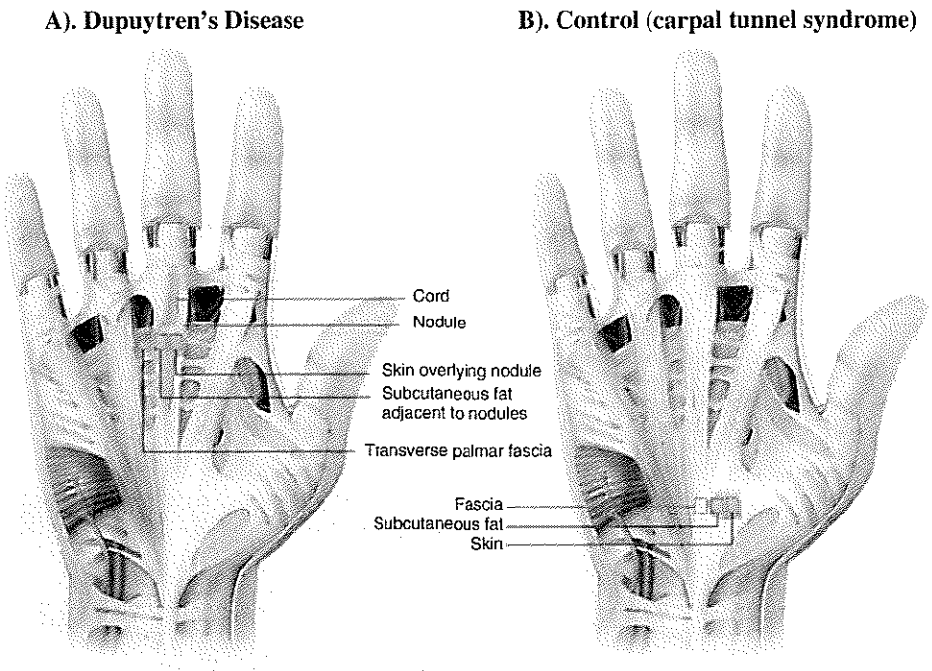

Figure 2 Tissues subjected to analysis in this study. **a** This figure demonstrates the palm of the hand of an individual affected with Dupuytren's disease, where the overlying skin has been removed to demonstrate the position of the palmar fascia in relation to the disease and harvested samples as indicated. Skin overlying palmar nodule, subcutaneous fat adjacent to palmar nodule, and transverse palmar fascia (Skoog's fibers) were obtained from Dupuytren's disease patients. **b** This figure demonstrates the palm of the hand of a control subject, where the overlying skin has been removed to demonstrate the position of the palmar fascia harvested. Skin, palmar fascia (transverse carpal ligament), and fat were obtained from control subjects, individuals undergoing carpal tunnel release.

Quantitative Polymerase Chain Reaction

Quantitative polymerase chain reactions were done in real-time using the LightCycler®480 platform (Roche Diagnostics GmBh, Germany) and corresponding LightCycler® (Roche Diagnoistics, UK) 480 software release 1.5.0 (version 1.5.0.39, Roche Diagnostics).

Each qPCR reaction was carried out in a final volume of 10 μL, consisting of 4 μL diluted template cDNA (approximately 5 ng cDNA), 5 μL LightCycler 480 Probes Master (Roche Diagnostics GmBh, Germany), 0.2 μM of forward primer (Metabion International AG, Martinsried, Germany), 0.2 μM of reverse primer (Metabion International AG, Martinsried, Germany), 0.1 μL probe from Universal Probe Library (Roche Diagnostics GmBh, Germany), and 0.7 μL nuclease-free water (Ambion; see Table 1 for the primer and probe details). In no template controls, water was used instead of cDNA. Each reaction was done in triplicates. Three hundred eighty-four multi-well plates (Roche Diagnostics GmBh, Germany) were used.

The qPCR reactions were initiated at 95°C for 5 min to activate the Hot Start Taq polymerase. Each of the 45 amplification cycles consisted of a 10-s denaturation step at 95°C and a 30-s annealing and elongation step at 60°C. The fluorescence intensity was recorded at the end of the 30-s annealing and elongation step in each cycle. After the 45 cycles of amplification, a cooling step at 40°C was carried out.

Data Analysis

In order to determine the significant difference between the gene expression levels of DD and control fat, skin, and fascia, the relative threshold cycle (C_T) method was used [25]. C_T values were obtained from qPCR. The C_T values for DD fascia (n=4) were previously published in Shih et al. [39]. ΔC_T was calculated by deducting C_T of the

Table 1 Primer and probe details.

Gene	Transcript ID	Forward primer	Reverse primer	Probe
ADAM12	NM_003474.3 NM_021641.3	tggaagaaggagaggagtgtg	cattgcagcagcgattcata	ttcctctg
ALDH1A1	NM_000689.3	ccaaagacattgataaagccataa	cacgccatagcaattcacc	ctcctctg
IRX6	NM_024335.2	ctcactgtatggggcactga	gccaggctggatgtaaaact	ggaggctg
POSTN	NM_006475.1	atgggagacaaagtggcttc	ctgctcctcccataatagactca	tccagtgt
PRG4	NM_005807.2	tcgtgattcagcaagttcatc	cagttgcaggtggcatctc	tggggaag
TNC	NM_002160.1	ccttgctgtagaggtcgtca	ccaacctcagacacggcta	ctgggaga

ADAM12 A disintegrin and metalloproteinase domain, *ALDH1A1* aldehyde dehydrogenase 1 family member, *IRX6* iroquois homeoboxprotein 6, *PRG4* proteoglycan 4, *TNC* tenascin C, *POSTN* periostin, osteoblast specific factor

internal controls, which was the averaged C_T of the reference genes, from the C_T of the target genes. Levels of target gene expression normalized using reference genes or relative gene expression levels were represented by $2^{-\Delta CT}$ [37].

Independent t tests on the $2^{-\Delta CT}$ of DD and control fat, skin, and fascia were carried out using SPSS 15.0 (SPSS, USA), as suggested by Schmittgen and Livak [37]. Fold change of the genes between the normal and DD tissues were calculated using the $2^{-\Delta\Delta CT}$ method [25].

Results

As described in Table 2 and Fig. 3, using independent t test, ADAM12, ALDH1A1, and IRX6 expression were significantly ($p<0.05$) higher in DD fat than in control fat (Table 2). The average up-regulation for ADAM12, ALDH1A1, and IRX6 in DD fat was 3.9-, 3.1-, and 2.5-fold, respectively (Table 2; Fig. 3). However, there was no significant difference in the relative gene expression levels of the six investigated genes between DD and control skin or fascia (Table 2; Fig. 3).

Discussion

This study has demonstrated differential gene expression levels in subcutaneous fat, superficial and adjacent to nodules, compared to normal nonaffected tissue. ADAM12, IRX6, and ALDH1A1 were found to be up-regulated in DD fat when compared to normal fat. However, of the six selected genes, there were no statistically significant differences in their expression levels between DD and control skin and fascia.

ADAM12 belongs to the group of disintegrin and metalloproteases (ADAMs). There has been an increasing interest in ADAM12, which has been suggested to be

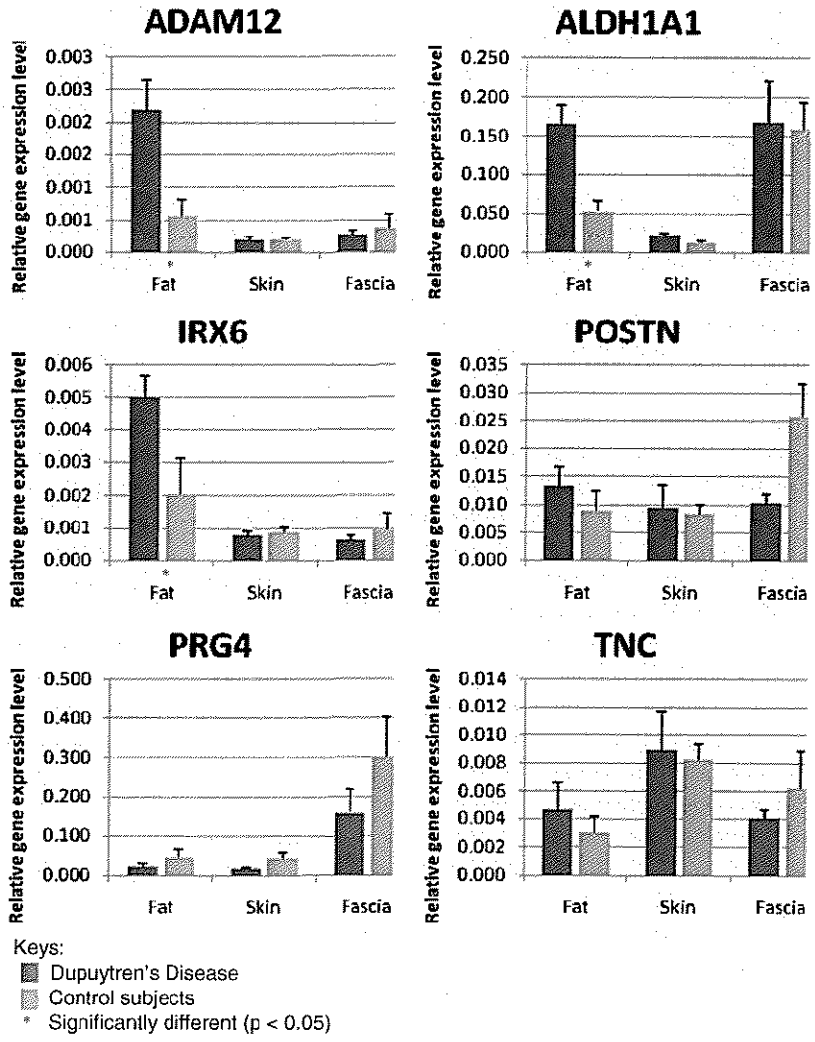

Figure 3 Relative gene expression in each sample. The relative gene expression levels, obtained by normalizing candidate gene expression levels to reference genes, were obtained from quantitative polymerase chain reactions. The *dark-colored bars* represent the data for Dupuytren's disease samples, and the *light colored bars* represent the data for control samples. Statistically significant ($p<0.05$) differential gene expression for ADAM12, ALDH1A1, and IRX6 were observed in DD fat, which are marked with *asterisk* in the graphs. *ADAM12* A disintegrin and metalloproteinase domain; *ALDH1A1* aldehyde dehydrogenase 1 family member; *IRX6* iroquois homeoboxprotein 6; *PRG4* proteoglycan 4; *TNC* tenascin C; *POSTN* periostin, osteoblast specific factor. The C_T values for DD fascia ($n=4$) were previously published in Shih et al. [39].

Table 2 Statistical comparison of the gene expression levels in Dupuytren's disease and control fat, skin, and fascia.

Gene symbol	Gene name	*p* value			Average fold change		
		Fat	Skin	Fascia	Fat	Skin	Fascia
ADAM12	A disintegrin and metalloproteinase domain 12	0.012*	0.937	0.648	3.9*	1.0	0.7
ALDH1A1	Aldehyde dehydrogenase family 1 member A1	0.004*	0.067	0.892	3.1*	1.7	1.1
IRX6	Iroquois homeobox protein 6	0.044*	0.608	0.607	2.5*	0.9	0.7
POSTN	Periostin, osteoblast specific factor	0.377	0.820	0.056	1.5	1.1	0.4
PRG4	Proteoglycan 4	0.309	0.147	0.299	0.5	0.4	0.5
TNC	Tenascin C	0.493	0.824	0.801	1.5	1.1	0.7

The table indicates the fold changes and statistical probabilities (*p* values) of each gene being differentially expressed in tissue from Dupuytren's disease when compared to those from control subject. If the fold change value is 1, there is no difference in average gene expression levels between disease and control tissues. If the value is larger than 1, that indicates the gene is up-regulated in the tissues from DD. If the value is lower than 1, that indicates the gene is down-regulated. The fold changes that are statistically significant ($p<0.05$) are indicated with an asterisk. The C_T values for DD fascia ($n=4$) were previously published in Shih et al. [39]
*Statistically significant ($p<0.05$)

involved in several fibrotic and neoplastic disorders. Up-regulation of ADAM12 has been observed in nodules of DD [39], and in addition, ADAM12 dysregulation or mutation has also been reported in various fibrotic conditions or abnormal cell growth, including keloids [38], liver fibrosis, carcinoma [19] and cancer of the breast [7, 22], liver [23], stomach [4], bladder [9], colon [19], glioblastoma [21], and prostate [31]. ADAM12 is a multifunctional protein that is involved in various cellular process and pathways, including TGF-beta and epidermal growth factor signaling pathway [22].

ALDH1A1 is an aldehyde dehydrogenase, which is responsible for the oxidation of acetaldehyde, the metabolic product of ethanol [20]. Other substrates of ALDH1A1 include acetaldehyde, benzaldehyde, 4-hydroxynonenal, malondialdehyde, and retinaldehyde [5]. Although alcoholism has been reported to be associated to DD, there is discrepancy in this behavioral association [3, 6, 16]. Furthermore, our previous study showed no significant difference in ALDH1A1 expression in nodule or cord [39]. Whether the observed overexpression of ALDH1A1 in DD fat may be associated with DD pathology requires further characterization.

Up-regulation in of IRX6, which is a gene that locates within the linkage identified by Hu et al. [18], has been observed in fat in this study and in nodule by Shih et al. [39]. The function of IRX6 is currently not well-characterized in humans. The gene family of Iroquois homeobox gene (IRX) are involved in embryonic patterning, morphogenesis, growth, and differentiation [17, 30, 44]. By using overexpression and knockdown of IRX6 in prostate cancer cell lines, Myrthue et al. [30] demonstrated IRX5 may be involved in the regulation of cell cycle and apoptosis in prostate cancer cells.

There are three possible mechanisms through which subcutaneous fat may potentially be involved in DD pathogenesis. Firstly, as suggested by Flint [8], subcutaneous fat may provide shock-absorbing effect, and reduced fat in the palm may result in higher risk of repeated trauma damaging the palmar fascia. The following observations support this hypothesis. Subcutaneous fat tissues thickness is negatively correlated to age [32], which coincides with the higher prevalence of DD in older populations [1]. Bergenudd et al. [2] have reported that, within a 55-year-old population, lower skinfold index, a measurement for subcutaneous fat thickness, was observed in DD patients.

Another possible mechanism for the involvement of subcutaneous fat is through abnormal cellular activities in the tissue. Rabinowitz et al. [33] observed differential lipid composition in DD fat, which was richer in free fatty acids and showed a significantly higher content of octanoate and other short-chain fatty acids than control. In this study, ADAM12 and IRX6 have been shown to be significantly up-regulated in subcutaneous fat, which coincide with their up-regulation previously observed in nodules [39]. Also, several reports of DD fat histology described an infiltration of fibrous tissues or displacement by collagen fibers [27, 28, 46]. The use of dermofasciectomy, a procedure that replaces the skin and subcutaneous fat overlying the DD site, results in a lower recurrence rate [13, 40, 41]. In short, subcutaneous fat may be pathogenic because it has a differential lipid composition, fibrotic infiltrate, and similarity in gene expression pattern with nodules. The removal of subcutaneous fat during dermofasciectomy may therefore account for the lower recurrence rate.

A third possible mechanism is the presence of an abnormal population of progenitor cells in the subcutaneous fat, which then in turn may act as a source of cells that cause DD pathology. Mesenchymal stem cells and multipotent progenitor cells have been characterized in subcutaneous fat [26, 47]. It has been speculated that stem cells or progenitor cells may be the origins of cells which cause fibrogenesis in

peyronie's disease [11], liver fibrosis following mesenchymal stem cell transplantation [42], and fibrotic disorder of the skeletal muscles [24]. Stem cells are also involved in wound healing [12]. Involvement of stem-like cell in abnormal cell growth, such as cancers, has also been increasingly well characterized [45].

Of the six candidate genes studied, there were no significantly different gene expressions between the external fascia from samples obtained from patient undergoing carpal tunnel release in subjects suffering from carpal tunnel syndrome (CTS) and the internal fascia from DD cases. Using whole genome microarray analysis, Rehman et al. [35] demonstrated some differential gene expression between external and internal control fascia. However, only seven genes, out of approximately 14,500 genes, were reported to show significantly different gene expression [35]. Further studies may need to be carried out to investigate the use of transverse-palmar fascia as internal control.

While no differential gene expression was observed for the six selected candidate genes between skin or normal fascia from DD and control subjects, differential gene expression for three of the six genes was shown in DD subcutaneous fat. As well as suggesting a possibility of using internal nonaffected fascia from DD patients as internal control, these results also support the hypothesis that fat may be involved in DD. Further work involving DD subcutaneous fat should be encouraged to elucidate and confirm the role of fat in DD pathology or recurrence.

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