GENE EXPRESSION ANALYSIS OF DUPUYTREN'S DISEASE: THE ROLE OF TGF-β2

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Dupuytren's disease is characterised by nodular fibroblastic proliferation of the palmar fascia leading to contracture of the hand. Transforming growth factor beta (TGF-β) is thought to play a role in its pathogenesis. We performed a cDNA microarray analysis of Dupuytren's diseased cord tissue with an emphasis on TGF-β isoforms. Normal-appearing transverse ligament of the palmar fascia from adjacent to the diseased cord and palmar fascia from patients undergoing carpal tunnel release were used as controls. TGF-β gene expression was confirmed by quantitative real-time polymerase chain reaction. Over 20 unique genes were found to be significantly up-regulated, including several previously reported genes. A dominant increase in TGF-β2 expression was seen in the cord tissue, whereas TGF-β1 and TGF-β3 were found not to be significantly up-regulated. Quantitative real-time polymerase chain reaction confirmed these findings. This gene expression profile allows for further experiments that may eventually lead to gene therapy to block the development and progression of Dupuytren's disease clinically.

Keywords: microarray analysis, Dupuytren's disease, TGF-β, gene expression

Dupuytren's disease is characterised by nodular fibroblastic proliferation of the palmar fascia, leading to contracture of the digits of the affected hand, most frequently affecting the ring and small fingers. In 1972, Gabbiani and Majno identified the myofibroblast as the contractile force behind the formation of the disease nodules. Other studies later identified fibronectin, α-smooth muscle actin and α5 β1 integrin as major components of the cellular apparatus through which intracellular force was generated and transmitted to the extracelluar matrix, leading to contracture formation (Magro et al., 1995; Tomasek et al., 1986, 1987). Some of these genes have been used as markers for myofibroblasts in later studies of Dupuytren's disease. Laminin and tenascin C have also been implicated in immunohistochemical studies by Berndt et al. (1994). Recently, other extracellular matrix proteins, such as matrix metalloproteinases, and tissue inhibitors of metalloproteinases have been identified as possible components of the disease process (Ulrich et al., 2003). Howard et al. (2004) found wound-healing-associated heat shock protein 47 (Hsp47) in Dupuytren's disease tissue through Western blot analysis (a method using antibody to detect specific protein from tissue extract using electrophoresis) and immunohistochemistry. Finally, Dupuytren's disease is also characterised by deranged collagen production (Bailey et al., 1977; Bazin et al., 1980; Margro et al., 1997; Menzel et al., 1979).

Recently, studies have focused on transforming growth factor beta (TGF-β) as a major factor in Dupuytren's disease pathogenesis. TGF-β regulates the transcription of a wide spectrum of matrix proteins including collagen, fibronectin, glycosaminoglycans, matrix-degrading proteases and their inhibitors, and integrin receptors (Roberts and Sporn, 1995). Many of these proteins have been found to be abnormal in Dupuytren's disease. TGF-β is released from many cell types that are involved in inflammation and fibrosis, including lymphocytes, macrophages, endothelial cells, smooth muscle cells, epithelial cells and fibroblasts. There are three mammalian isoforms of TGF-β viz. TGF-β1, TGF-β2 and TGF-β3. These TGF-β isoforms have been identified in the fibroblastic nodule and surrounding tissue of Dupuytren's disease palmar facsia. Using real-time polymerase chain reaction (RT-PCR) (a method to detect the expression of the messanger RNA for a protein), Baird et al. (1993) found increased TGF-β expression in Dupuytren's disease. TGF-β isoforms, however, were not distinguished in this study. In a later study, Berndt et al. (1995) identified all three TGF-β isoforms in Dupuytren's disease cord tissue and TGF-β1 and β3 were also found in the surrounding tissue using in situ hybridisation (a method to detect specific protein expression in tissue sections using probes designed for the protein). Badalamente et al. (1996) demonstrated TGF-β1 staining in fibroblasts, myofibroblasts and capillary endothelial cells in Dupuytren's disease samples. TGF-β2 was found only in myofibroblasts in that study.

cDNA microarray has become a powerful tool for studying human disease. Microarray technology allows

the researcher to look at gene expression of the entire genome of an organism. This technology allows comparison of tissue gene expression patterns both quantitatively and qualitatively across an entire genome. All the genes that are expressed at a higher level (ie up-regulated) in a disease state at one time can be seen and quantified. This had led to an explosion of discovery of new genes involved in many disease processes. We applied cDNA microarray analysis to the study of the gene expression patterns of the Dupuytren's disease cords, normal-appearing transverse ligament of the palmar fascia from adjacent to the diseased cord and control palmar fascia from patients with carpal tunnel disease. The purpose of our research is to use this technology to (1) validate previous findings, (2) to further characterise and quantify TGF-β involvement and (3) to screen for new genes that might be involved in Dupuytren's disease.

MATERIALS AND METHODS

Tissue procurement and processing

Diseased cords and normal-appearing transverse ligament of the palmar fascia from adjacent to the diseased cord were obtained from patients who had undergone palmar fasciectomy for the surgical release of Dupuytren's contractures. Control palmar fascia samples were also obtained from patients who had undergone carpal tunnel release. Four diseased cords, four corresponding normal-appearing transverse ligaments of the palmar fascia from adjacent to the diseased cord and three control palmar fascia samples were examined. Collection of tissue and use for this study were approved according to standard guidelines set by the institutional review boards of Stanford University and the Veterans Affairs Palo Alto Health Care System. Informed consent was obtained prior to enrolment of each patient.

Patient's age, gender, ethnicity, history of alcohol use, smoking and diabetes, and the presence of bilateral disease were determined and are summarised in Table 1. After excision, each tissue sample was divided. The larger portion was flash-frozen in liquid nitrogen and stored at $-80\,°C$ prior to RNA isolation. Frozen sections of the smaller portion were used for immunohistochemical analysis and haematoxylin and eosin staining.

Table 1—Demographics of patients with Dupuytren's disease in this study

Case	Age[1]	European descent	Smoking	Alcohol use	Diabetes	Bilateral disease
1	67	No	No	No	No	Yes
2	62	Yes	No	No	No	Yes
3	52	Yes	Yes	No	No	Yes
4	74	Yes	Yes	Yes	No	Yes

[1]The average age of the control patients was 53.

Gene profiling using microarrays

Total RNA from tissue samples were extracted with Trizol reagent (Life Technologies Inc., Gaithersburg, MD, USA), and further purified with an RNeasy mini kit (Qiagen, Germantown, MD, USA) according to the kit manufacturer's protocols. To obtain sufficient amounts of RNA for microarray experiments, total RNA isolated from all tissue samples were amplified using the MessageAmp aRNA kit (Ambion Inc., Austin, TX, USA). RNA quality and quantity were measured by a spectrophotometer. Each sample used in the microarray experiments achieved an absorbance ratio (A260/A280) in the range of 1.7 to 2.3. Amplified RNA was reverse-transcribed into cDNA using Superscript II reverse transcriptase (Life Technologies Inc., Gaithersburg, MD, USA) and random hexamer (Amersham Biosciences, Buckinghamshire, UK). Microarray analysis was performed on cDNA chips manufactured by the Stanford Functional Genomics Facility (www.microarray.org). Each array chip contains approximately 42,000 human cDNA elements, representing over 30,000 unique genes. A type II experimental design was applied in which a universal human reference RNA (Stratagene, La Jolla, CA, USA) served as the common reference probe in all hybridisations. The universal human RNA was amplified and used to make cDNA probes labelled with Cy3 (Amersham Biosciences, Buckinghamshire, UK). Amplified RNA from tissue samples was used to make cDNA probes labelled with Cy5. Probe hybridisation was performed by adding $20\,\mu g$ of yeast tRNA, $20\,\mu g$ of polydeoxyadenylic acid and $20\,\mu g$ of human CoT1 DNA (Invitrogen, Carlsbad, CA, USA) to a solution containing $3\times$ standard saline citrate, 0.3% sodium dodecyl sulphate and labelled probes. This mixture was aliquoted onto the microarray chip and incubated for 18 hours at $65\,°C$. Washes and scans were performed as described previously (DeRisi et al., 1996, 1997). After gridding, array data were uploaded to the Stanford Microarray Database (http://genome-www5.stanford.edu/MicroArray/SMD) and analysed.

Microarray data analysis

Using on-line software from Stanford Microarray Database, data points that met the following spot-quality criteria were selected for analysis: spot regression correlation >0.4 and "spot flag" and "failed" filters equal 0. These user-selectable criteria are intended to exclude spots with non-uniform, dim or otherwise unreliable signals. Relative changes in gene expression were evaluated by fold change as determined from the $\log_2$ of red/green normalised ratio reported by Stanford Microarray Database, as previously described (DeRisi et al., 1997). For a given gene, a four-fold change from the mean in at least one array was used as a cut-off in our experiments (non-biased analysis). Only genes and

arrays with greater than 80% good data were included in the results. The gene expression profile, in terms of fold change from the mean, was compared using the Student's t-test. Results with $P<0.05$ were considered significant. Genes and arrays were also clustered according to their expression patterns using Stanford Microarray Database on-line software (Eisen et al., 1998). The data discussed in this publication have been deposited in NCBIs Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) and are accessible through GEO Series accession number GSE2688.

Quantitative real-time polymerase chain reaction

For confirmation of TGF-β expression, we performed quantitative real-time polymerase chain reaction (QRT-PCR) on total RNA isolated from each sample. cDNA were obtained by reverse transcription of 1 µg of DNAse-treated total RNA using oligo dT-priming in 50 µl reactions according to the manufacturer's recommendations (Taqman® Reverse Transcription Reagent Kit, Applied Biosystems, Foster City, CA, USA). QRT-PCR was performed using the Applied Biosystems Prism® 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA). A Taqman-based assay was used in which each cDNA sample was evaluated at least in triplicate. The 20 µl reactions were used for all target transcripts. Expression values were normalised to the expression levels of glyceraldehyde 3-phosphate dehydrogenase. QRT-PCR primers and probes are summarised in Table 2. Human glyceraldehyde 3-phosphate dehydrogenase primers and probes were purchased from Applied Biosystems (Applied Biosystems, Foster City, CA, USA). All others were designed using Primer Express version 2.0.0 (Applied Biosystems, Foster City, CA, USA) and were tested to confirm appropriate product size and optimal concentrations.

RESULTS

Expression profiles of genes known to be involved in Dupuytren's disease

Cord tissue samples from Dupuytren's disease ($n = 4$) were compared to normal-appearing transverse ligament of the palmar fascia from adjacent to the diseased cord in the same patient ($n = 4$) and control palmar fascia from patients who underwent carpal tunnel release ($n = 3$). Many genes that have been previously described in Dupuytren's disease were found to be up-regulated, in other word these genes are expressed at much higher levels when compared to the controls. These included α-smooth muscle actin, fibronectin, β1 integrin, laminin, tanascin C, Hsp47, TGF-β2 and collagen I. These genes were clustered together as having similar expression profiles across tissue type. In other words, the expression levels of these genes are uniformly high in the diseased cord tissue and uniformly low in the controls. Furthermore, the diseased cords had similar gene expression patterns, while controls – both normal-appearing transverse ligament of the palmar fascia from adjacent to the diseased cord and palmar fascia from carpal tunnel release patients – had similar expression patterns (Fig 1). Levels of expression, in terms of fold changes, are summarised in Table 3. Because both control fascias had very similar gene expression patterns and because more data from normal-appearing transverse ligaments of the palmar fascia from adjacent to the diseased cord survived the data-filtering protocols, statistical analyses were made between diseased cord and normal-appearing transverse ligament of the palmar fascia from the adjacent to the diseased cord. A trend of increased expression of β1 integrin was seen in diseased cord tissue. A trend of increased expression of α-smooth muscle actin was seen in both the diseased cord and normal-appearing transverse ligaments of the palmar fascia from the adjacent to the diseased cord in some samples. All other genes were significantly up-regulated exclusively in the diseased cord tissue.

Expression profiles of TGF-β isoforms

The gene expression profile of TGF-β isoforms was further characterised. An array list was generated that included all three isoforms. Raw data of the TGF-β expression obtained from the microarray experiment were filtered as described in the Materials and methods. In this case, data were not filtered by fold changes in order to include genes that had not achieved a four-fold change in at least one array. The isoforms and arrays were centred by mean and clustered. Again, gene expression patterns were compared between diseased cord and normal-appearing transverse ligament of the palmar fascia from adjacent to the diseased cord (Fig 2). There were no statistically significant differences in the

Table 2—Sequences of primers and probes used in this study

| Gene	Forward primer	Reverse primer	Probe
GAPDH	GAAGGTGAAGGTCGGAGTC	GAAGATGGTGATGGGATTTC	CAAGCTTCCCGTTCTCAGCC
TGFβ1	GCGCATCCTAGACCCTTTCTC	CAGAAGGTGGGGTGGTCTTGAA	TCTCCGACCTGCCACAGATCCCCT
TGFβ2	CGAGAGGAGCGACGAAGAGTA	CACTGAGCCAGAGGGTGTTGT	TCCCCTCCGAAACTGTCTGCC
TGFβ3	CGGAATGAGCAGAGGATCGA	GCCACCGATATAGCGCTGTT	TCGGCCAGATGAGCACATTGCC
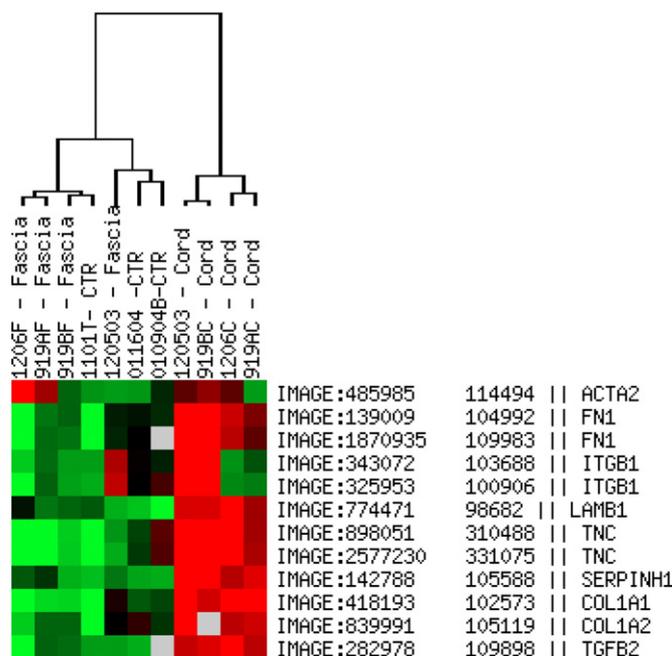
Fig 1 Hierarchical clustering analysis of genes known to be involved in the pathogenesis of Dupuytren's Disease. Rows represent individual genes including clone ID and gene symbol; columns represent individual samples. Cord = Dupuytren's disease cords, Fascia = normal-appearing palmar fascia from adjacent to the diseased cord, CTR = control fascia from carpal tunnel patients. Genes that are up-regulated appear in red, those that are down-regulated appear in green; black indicates approximately the same gene expression as the mean for that gene across all samples. Colour saturation is proportional to the magnitude of the difference from the mean. This list was clustered by an on-line software from SMD.

Table 3—Known up-regulated genes involved in disease pathogenesis in terms of fold changes

Description	Average fold change in carpal tunnel control fascia	Average fold change in adjacent normal fascia	Average fold change in diseased cord[1]
Actin, alpha 2, smooth muscle	0.4	1.8	1.4
Fibronectin 1	0.6	0.6	**3.5**
Integrin, beta 1 (fibronectin receptor)	0.9	1.0	2.9
Laminin, beta 1	0.5	0.6	**2.9**
Tenascin C (hexabrachion)	0.8	0.3	**5.2**
Hsp 47	0.4	0.6	**3.4**
Collagen, type I, alpha 1	0.7	0.6	**4.4**
Collagen, type I, alpha 2	0.7	0.6	**3.2**

[1]Numbers shown in bold reached statistical significance of $P < 0.05$ when compared to adjacent normal fascia using a Student's t-test.

TGF-β1 expression between diseased cord, normal-appearing transverse ligaments of the palmar fascia from the adjacent to the diseased cord and control fascia from carpal tunnel patients. A trend of decreased TGF-β1 expression was seen in the diseased cord tissue. TGF-β2 was significantly up-regulated in the diseased cord tissue and down-regulated in the normal-appearing transverse ligament of the palmar fascia from adjacent to the diseased cord and control fascia from carpal tunnel patients. TGF-β3 showed a trend of increased expression in the diseased cord tissue. However, this increase is not statistically significant.

To validate microarray results, total RNA from two cord samples and corresponding cord adjacent fascia was tested by QRT-PCR (Fig 3). Expression levels were normalised to that of glyceraldehyde 3-phosphate dehydrogenase. The QRT-PCR expression profiles of TGF-β1 and 2 corresponded well with array data. One sample showed a 20-fold increase in TGF-β2 expression in the diseased cord tissue. TGF-β3 showed a trend of decreased expression in the diseased cord and increased expression in the normal-appearing transverse ligament of the palmar fascia from adjacent to the diseased cord. Statistically, this change was not significant.

Expression profile of new genes

Of the over 20 significantly up-regulated genes, many had not previously been associated with the pathogenesis of Dupuytren's disease (Fig 4). These included fibroblast activation protein (FAP), osteonectin (SPARC), thrombospondin 2 (THBS2), v-maf musculoaponeurotic fibrosarcoma oncogene homologue B (MAFB), RAB31-member RAS oncogene family
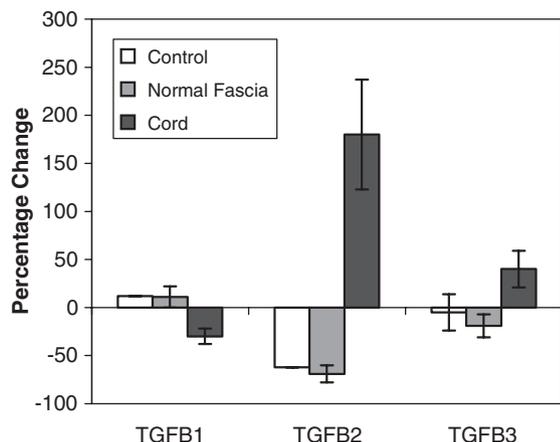
Fig 2 TGF-β gene expression profile. Compared with normal-appearing palmar fascia from adjacent to the diseased cord, TGF-β1 and 3 were not significantly up-regulated in the diseased cord tissue although a trend of increase was seen with TGF-β3 in the diseased cord tissue. TGF-β2 was significantly up-regulated in the diseased cord tissue compared with the normal-appearing palmar fascia from the adjacent to the diseased cord ($P < 0.02$).

(RAB31), lung type-I cell membrane-associated glycoprotein (T1A-2), brain abundant, membrane attached signal protein 1 (BASP1), SRY (sex determining region Y)-box 4 (Sox4), Down syndrome critical region protein 1 (DSCR1), adlican, neural cell adhesion molecule 1 (NCAM1), orthologue of rat vacuole membrane protein 1 (VMP1), protein regulator of cytokinesis 1 (PRC1), X-ray repair complementing defective repair in Chinese hamster cells 1 (XRCC1), regulator of G-protein signalling 3 (RGS3), leucine-rich repeat containing 17 (LRRC17), ADP-ribosylation factor-like 7 (ARL7), cellular retinoic acid binding protein 2 (CRABP2), collagen, type V, α2 (COL5A2), collagen, type VIII and α1 (COL8A1).

DISCUSSION

Dupuytren's disease is an intriguing disease because of its strong genetic components. Microarray technology has improved our ability to study the molecular genetics of human disease. It allows quantitative comparison of gene expression between multiple samples on a genomic scale. We applied this technology to the study of Dupuytren's disease by comparing the gene expression patterns of four Dupuytren's disease cord tissue samples, four corresponding to normal-appearing transverse ligaments of the palmar fascia from the adjacent to the diseased cord and three control palmar fascia samples from carpal tunnel patients.

Previous studies have established several genes in association with Dupuytren's disease. Specifically, the presence of myofibroblast and its associated extracel-
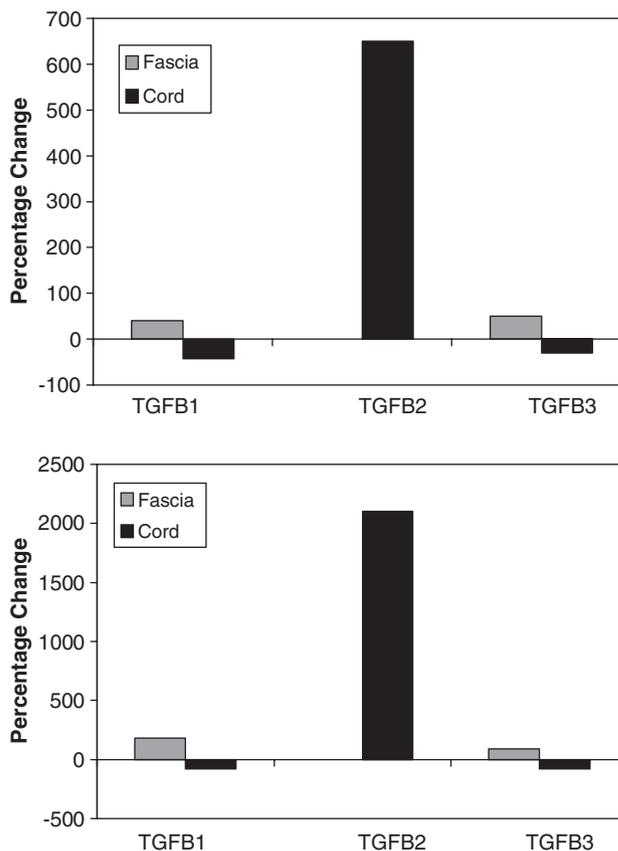

Fig 3 Quantitative real-time polymerase chain reaction (QRT-PCR) analyses of two patients' samples using glyceraldehyde 3-phosphate dehydrogenase expression level as the control. Absolute values on the y-axis are not consistently equal for array and QRT-PCR assay as different efficiencies of amplification in the LightCycler were encountered for these genes.

lular fibronexus has been attributed to disease formation. Genes such as fibronectin, α-smooth muscle actin, α5 β1 integrin, laminin and tenascin C are thought to be major components of a cellular apparatus through which intracellular force is generated and transmitted to the extracellular matrix, thus leading to contracture formation. Some of these genes have been used as markers for Dupuytren's disease. These same genes were found to be up-regulated in our non-biased analysis. Fibronectin, laminin and tenascin C were consistently and significantly increased in the diseased cord tissue. α5 β1 integrin showed a trend of increased expression in the cord tissue. α-smooth muscle actin expression increased in both the cord and normal-appearing transverse ligaments of the palmar fascia from the adjacent to the diseased cord. Dupuytren's disease is also marked by increased extracellular matrix activity. This observation was further characterised by our findings of significantly increased expression of Col 1, MMP2 and Hsp47 in the fibrotic cord tissue. Confirmation of these previously described genes validates microarrays as useful tools in
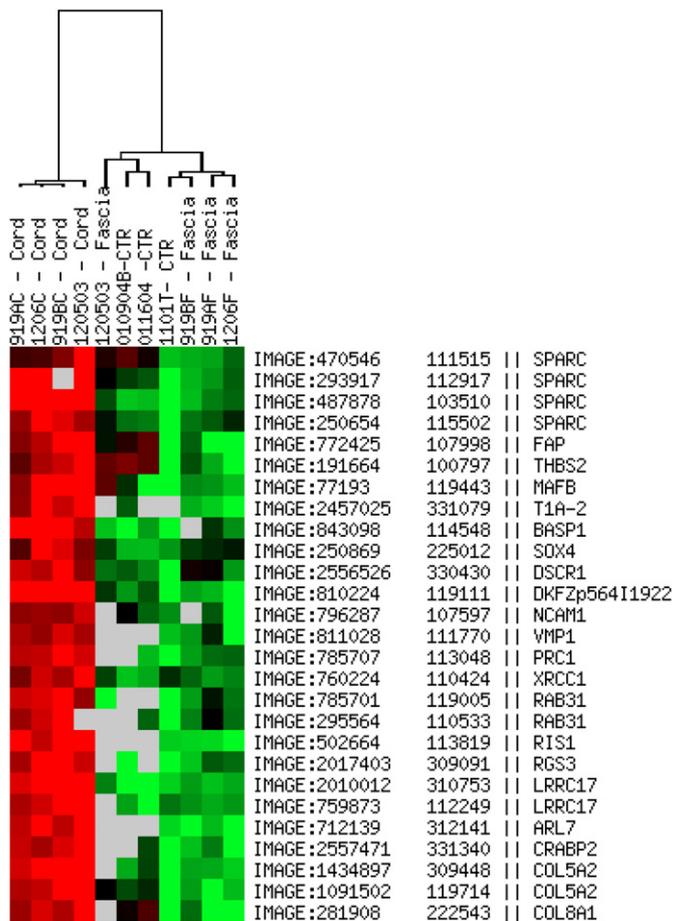
Fig 4 Novel genes that have significantly increased expressions in Dupuytren's disease. Rows represent individual genes including clone ID and gene symbol; columns represent individual samples. Cord = Dupuytren's disease cords, Fascia = normal-appearing palmar fascia from adjacent to the diseased cord-adjacent fascia, CTR = control fascia from carpal tunnel patients. Genes that are up-regulated appear in red, those that are down-regulated appear in green; black indicates approximately the same gene expression as the mean for that gene across all samples. Colour saturation is proportional to the magnitude of the difference from the mean. This list was clustered by an on-line software from Stanford Microarray Database.

the study of Dupuytren's disease. The microarray study also allowed quantification of gene expression levels for these genes.

TGF-β has recently been implicated in governing growth, differentiation and function of the myofibroblasts of Dupuytren's disease. TGF-β1 was found to increase DNA synthesis in cultured DD cord cells (Kloen et al., 1995). TGF-β1 was also shown to increase collagen production in cultured Dupuytren's disease cord cells (Alioto et al., 1994). In a later study, TGF-β1 and TGF-β2 showed significant effects on myofibroblast proliferation, with TGF-β2 having the strongest effect (Badalamente et al., 1996). TGF-β isoforms have also been localised to Dupuytren's disease tissue through non-quantitative methods. TGF-β2 was found to reside only in myofibroblasts of diseased cord tissue. TGF-β1 and 3 were found both in the cord and in the surrounding tissue (Badalamente et al., 1996; Berndt

et al., 1995). In our non-biased analysis, TGF-β2 expression was found to be increased by 2.8-fold in the diseased cord tissue as compared to the level in normal-appearing transverse ligaments of the palmar fascia from adjacent to the diseased cord. When looking at TGF-β isoforms through gene-list analysis, TGF-β1 and 3 expression was found to be not significantly different across tissue types. These findings showing up-regulation of only TGF-β2 gene expression were confirmed by QRT-PCR. Selective up-regulation of TGF-β2 gene expression suggests that TGF-β2 may be the dominant factor of the TGF-β family in the pathogenesis of Dupuytren's disease. Other recent studies seem to support this finding. Kuhn et al. (2001) demonstrated that the addition of exogenous TGF-β2 to explanted Dupuytren's disease cord would increase collagen, DNA and protein production. This effect was blocked by TGF-β2 neutralising antibody. In a separate study by

the same authors, tamoxifen, an inhibitor of TGF-β activity, down-regulated TGF-β2 protein in cultured diseased cord fibroblasts and decreased their contractile function (Kuhn et al., 2002). Through ELISA assay, a 2.5-fold increase in TGF-β2 protein in Dupuytren's disease cords was seen in their study when compared to normal fascia obtained from carpal tunnel release.

Isolated TGF-β2 up-regulation has also been seen in other systems. In response to hypoxia, human umbilical vein endothelial cells expressed up to 25-fold more TGF-β2 mRNA and 12 times more protein at 48 hours (Akman et al., 2001). TGF-β is typically synthesised as a latent peptide complex that is stored in the extracellular matrix, cell surface and/or cytoplasm. Whether the marked increase in TGF-β2 expression seen in our microarray anaysis translates into higher TGF-β2 activity remains to be seen. Factors that govern TGF-β2 gene expression, such as hypoxia, will also be the subject of subsequent studies.

Of the over 20 genes with significantly up-regulated expression, many had not previously been associated with the pathogenesis of Dupuytren's disease. The function of some of the up-regulated genes, revealed by studies in other systems, suggests that they may also play important roles in Dupuytren's disease. FAP is a member of the serine protease family. Increased expression of FAP is a highly consistent trait of tumour stromal fibroblasts in most epithelial cancers. FAP, along with collagen I and tenascin C, have also been found to be induced by TGF-β1 in the reactive stromal myofibroblasts of prostate cancer (Tuxhorn et al., 2002). ADAM12, a disintegrin and metalloprotease, was found to induce actin cytoskeleton and extracellular matrix reorganisation during early adipocyte differentiation by regulating β1-integrin function (Kawaguchi et al., 2003). SPARC is a matricellular protein that has been found to have increased expression in dilating vascular aneurysm endothelium (Peters et al., 2001). TSP2 is another matricellular glycoprotein of the same family that is best known for its anti-angiogenic properties and its ability to modulate cell–matrix interactions (Bornstein et al., 2004). Further studies of these and other genes not previously identified in association with Dupuytren's disease are currently under way.

Gene expression analysis alone cannot provide an overall integrative molecular understanding of the genesis of progression of Dupuytren's disease. However, in these DNA microarray experiments, several previously described genes were confirmed and, in conjunction with QRT-PCR, the up-regulation of TGF-β2 was documented. Furthermore, many new genes have been identified. These genes that are found up-regulated cannot be implied to be the cause of disease but increased expression does suggest that they play a role in the disease process. Further studies to elucidate the specific roles of these genes are needed. It is hoped that the discovery of these genes and the work that follows may eventually lead to gene therapy to block the development and progression of Dupuytren's disease clinically.

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