Identification of Biomarkers in Dupuytren's Disease by Comparative Analysis of Fibroblasts Versus Tissue Biopsies in Disease-Specific Phenotypes

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Purpose Biomarkers are molecular mediators that can serve as indicators of normal biological processes, pathologic processes, and therapeutic interventions. This study aims to identify potential biomarkers in Dupuytren's disease (DD), a fibroproliferative benign tumor with an unknown etiology and high recurrence after surgery.

Methods Bioinformatic analytical techniques were employed to identify candidate genes that may be differentially expressed in DD, which included gene expression analysis of microarray data and thorough literature searches in genetic linkage and other related biomolecular studies. All DD cases were males with advanced DD (n = 5, 66 years ± 14). RNA was extracted from biopsies and corresponding cultures of normal fascia (unaffected transverse palmar fascia), palmar nodule and cord from each patient. Real-time reverse transcription–polymerase chain reactions were performed to determine the gene expression levels for disease-related transcripts.

Results The bioinformatic analysis revealed 25 candidate genes, which were further short-listed to 6 genes via functional annotation. The 6 selected candidate genes included: A disintegrin and metalloproteinase domain (ADAM12), aldehyde dehydrogenase 1 family member (ALDH1) A1, Iroquois homeobox protein 6 (IRX6), proteoglycan 4 (PRG4), tenascin C (TNC), and periostin (POSTN). The culturing treatments were shown to have significant impact on the gene expression for ALDH1A1, PRG4, and TNC. In tissue biopsies, significant fold changes were observed for ADAM12, POSTN, and TNC in the cord and/or nodule when compared with that of normal fascia. ADAM12 and POSTN are associated with accelerated or abnormal cell growth, whereas TNC has been associated with fibrotic diseases and cell migration.

Conclusions This study demonstrated differential gene expression results in DD tissue biopsies compared with that of their corresponding cultures. ADAM12, POSTN, and TNC were identified from the cord and nodule biopsy samples as potential biomarkers in relation to DD development. *(J Hand Surg 2009;34A:124–136. © 2009 Published by Elsevier Inc. on behalf of the American Society for Surgery of the Hand.)*

Key words Biomarkers, Dupuytren's contracture, Dupuytren's disease, Dupuytren's tissue and fibroblasts culture, RT-qPCR.

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DUPUYTREN'S DISEASE IS a nodular fibroproliferative disorder that can cause permanent and irreversible flexion contracture of the digits.[1] It is often familial and highly prevalent among the Northern European Caucasian population.[2,3] Surgical intervention remains the mainstay of treatment for Dupuytren's disease (DD).[1] However, there is a high recurrence rate of DD after surgery.[4–6] Despite the recent advances in our understanding of the biochemical and cellular processes involved in the development of DD, the exact pathogenesis of DD remains unknown.[7]

Biomarkers can be cellular or molecular mediators or responders that can serve as an indicator of a normal biological process, a pathologic process, or a pharmacologic response to a treatment.[8] By identifying new biomarkers for DD, novel strategies for prognosis, diagnosis, and treatments tailored to diagnostic and prognostic indicators may be developed.

Dupuytren's disease has 2 structurally distinctive fibrotic structures: the nodule and the cord.[9] It is speculated whether the nodule develops into the cord as the disease progresses or the 2 structures represent different stages of the disease.[10,11] The nodule is thought to be involved in the most biologically active phase of the disease, as it is characterized by elevated vascularized soft tissue masses and contains a dense population of fibroblasts, which are largely myofibroblasts,[12,13] On the other hand, the cord is a relatively avascular, acellular, and collagen-rich structure that contains a smaller population of myofibroblasts.[10]

Despite accumulating evidence that *in vitro* conditions have an impact on gene expression patterns, there are limited studies that have investigated differential gene expression findings in both tissue culture and biopsies.[14–16] The aim of the study was to identify biomarkers by investigating gene expression levels of candidate genes differentially expressed in various DD tissue phenotypes as well as to determine whether the obtained results from tissue biopsies are comparable with those from cell cultures.

MATERIALS AND METHODS

Patients

All cases involved in the study were diagnosed to have primary advanced stage of DD, which was determined by the presence of nodule and cord causing contracture of the metacarpophalangeal joint and the proximal interphalangeal joint in the involved hand. The mean age of the patients participating was 66 years 14. All patients were Caucasian men who had not had any previous surgical or nonsurgical treatments.

Samples

Using magnifying loupes, we carefully dissected the cord, nodule, and transverse palmar fascia from each patient at the time of surgery (Fig. 1). Each biopsy was then bisected for either total RNA extraction or cell culture processing. Therefore, 2 sets of tissue biopsy samples were obtained from each patient. The biopsies used for establishing tissue cultures were thoroughly washed for 15 minutes in 1× Dulbecco's phosphate-buffered saline (Lonza, Verviers, Belgium) and 1% penicillin/streptomycin (Lonza) at room temperature. For RNA extraction, the biopsy specimens were stored in a solution (RNA*later*; Ambion, Austin, TX) and incubated at 4°C overnight, followed by storage at −20°C for short-term storage and −80°C for long-term storage.

Tissue culture

To establish the tissue cultures, the biopsy specimens were further dissected into small pieces, roughly 1 mm^3 in size, with sterile scalpels. The tissue pieces were incubated in 0.25% to 5% collagenase A solution (Roche Diagnostics GmbH, Mannheim, Germany) at 37°C for 2.5 to 3 hours. The collagenase activity was inhibited using fibroblast culturing media, which is Dulbecco's Modified Eagle's Medium 3 (Lonza) supplemented with 10% heat-inactivated fetal bovine serum (Sigma Aldrich, Irvine, UK), 1% penicillin/streptomycin (Lonza), and 1% nonessential amino acids (Lonza). The digested samples were centrifuged at 1,500 rpm (approximately 400 × *g*) for 5 minutes. Each pellet was resuspended in 5 mL fibroblast culturing media, seeded to a 25 cm^2 culturing flask (Corning, USA), and incubated at 37°C in 5% CO_2. The culturing media was replaced every 48 hours, and cell passages were carried out at approximately 80% confluency using trypsin–ethylene diamine tetraacetic acid (200 mg L-ethylene diamine tetraacetic acid, 500 mg L-trypsin; Lonza). The second passage of the cell cultures was used in this study, as several studies have demonstrated that more prolonged passaging of cell cultures shows altered gene expression patterns compared with those of early passages.[17,18]

RNA extraction

To extract RNA from a biopsy sample, we removed approximately 2 mm^3 of the tissue, finely diced, and placed it in a 2-mL round-bottom Eppendorf tube possessing a flame-sterilized steel ball-bearing. This procedure was performed in 4 replicates for each sample. One milliliter of TRIzol (Invitrogen, Carlsbad, CA) was added into each tube. The tissues were homogenized at

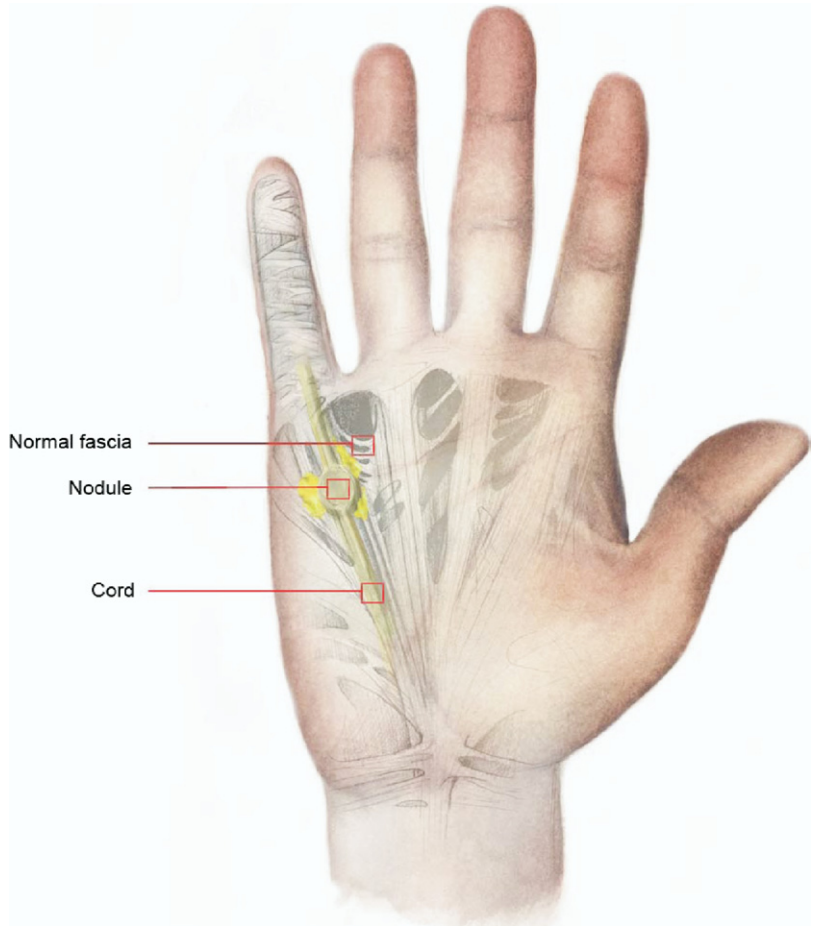


FIGURE 1: The DD-associated tissues that are subjected to analysis in this study. Three tissue biopsies are collected from each DD patient's affected hand: the normal fascia (unaffected transverse palmar fascia), palmar nodule, and cord.

30 oscillations per second for 12 minutes in tissue lysis machinery (Qiagen Tissue Lyser; Qiagen, Hilden, Germany). The solution was transferred to a sterile 1.5-mL round-bottom Eppendorf tube and centrifuged at 13,000 rpm (approximately 16,000 × *g*) for 10 minutes to remove cell debris. The supernatant was transferred to a new Eppendorf tube and mixed with 0.2 mL chloroform/1 mL TRIzol (Invitrogen). The solutions were mixed well and left at room temperature for 2 minutes, followed by centrifugation at 13,000 rpm (approximately 16,000 × *g*) for 15 minutes. The upper aqueous layer was pipetted into a fresh Eppendorf tube, and an equal volume of 70% ethanol was added and mixed well.

The resulting mixture was further processed using RNeasy kit (Qiagen) according to the manufacturer's instructions. The resulting extracted RNA was subjected to DNase treatment (DNAFree kit; Ambion, Austin, TX) according to the manufacturer's protocol. The concentration and the integrity of extracted RNA were measured using a spectrophotometer (NanoDrop ND-1000 UV-visible spectrophotometer; Labtech International, Montchanin, DE) and an analyzer (Agilent 2100 Bioanalyzer; Agilent Technologies, Gmbh, Waldbronn, Germany), respectively.

Complementary DNA synthesis

The synthesis of complementary DNA (cDNA) was carried out using the SuperScript II Reverse Transcriptase kit (Invitrogen). For each reaction, 500 ng total RNA, 1 μL nucleotides mix (10 mmol/L for each nucleotide; Invitrogen), 375 ng oligo-dT (Invitrogen), 62.5 ng random primers (Invitrogen), and sterile nuclease-free water (Ambion) were added to an Eppendorf tube to make up a total volume of 12 μL. The mixture was first incubated at 65°C for 5 minutes, followed by rapid cooling on ice. Two microliters of 0.1 mol/L dithiothreitol, 1 μL of RNaseOut (Invitrogen), and 4 μL of First-Strand Buffer (250 mmol/L Tris-hydrochloride, pH 8.3 at room temperature; 375 mmol/L potassium chloride; 15 mmol/L magnesium chloride) was added to each reaction tube. After incubation at 42°C for 2 minutes, 1 μL SuperScript II Reverse Tran-

TABLE 1. The qPCR Assays Designed to Amplify the 6 Candidate Genes

Gene	Location	Transcript ID	Forward Primer	Reverse Primer
ADAM12	10q26.3	NM_003474.3	tggaagaaggagaggagtgtg	cattgcagcagcgattcata
ALDH1A1	9q21.13	NM_000689.3	ccaaagacattgataaagccataa	cacgccatagcaattcacc
IRX6	16q11.2-q13	NM_024335.2	ctcactgtatggggcactga	gccaggctggatgtaaaact
POSTN	13q13.3	NM_006475.1	atgggagacaaagtggcttc	ctgctcctcccataatagactca
PRG4	1q25-q31	NM_005807.2	tcgtgattcagcaagtttcatc	cagttgcaggtggcatctc
TNC	9q32-q34	NM_002160.1	ccttgctgtagaggtcgtca	ccaacctcagacacggcta

The National Center for Biotechnology Information transcript ID for each gene is given, showing the transcript sequence used for the assay designs.

scriptase (Invitrogen) was added to each reaction. The reaction tubes were incubated for 10 minutes at 25°C, followed by a further 50 minutes at 42°C. The reaction was inactivated by incubation for 15 minutes at 70°C.

Bioinformatic search for candidate genes

The selection of target genes was done through several bioinformatic analytical techniques, including gene expression analysis of our previous microarray work,[10] searching through scientific literature in the English language, analysis of established linkage using Ensembl (http://www.ensembl.org/), and use of bioinformatic databases to perform gene function clustering. The genes in the region between, microsatellite markers D16S419 and D16S3032 on chromosome 16q, were analyzed using Ensembl. The region was associated with DD through the linkage analysis by Hu and colleagues.[19] Another list of candidate genes was produced from microarray data by using the MADAT software (Micro Array Data Analysis Tool; http://www.bioinf.manchester.ac.uk/MADAT/index.html).[10] Affymetrix data were uploaded to MADAT and normalized by using Robust Multi-array Average.[10] Principal component analysis was carried out to test the quality of the chips. Then a *t*-test was conducted by using a multiple testing correction method. Probe sets expression with a fold change more than 2 and a p value less than .05 were filtered. Eighty genes were selected on the basis of statistical significance. Candidate genes were clustered according to the biological processes by using gene annotation tools provided by NetAffx Analysis Center (http://www.affymetrix.com/analysis/index.affx) and DAVID Bioinformatic Resources 2008 (http://david.abcc.ncifcrf.gov/). Batch query was carried out by selecting Human Genome U133A (HG-U133A) gene chip array and uploading the list of candidate genes from microarray analysis onto the NetAffx Analysis Center. Candidate genes listed from linkage analysis was also uploaded to DAVID Bioinformatics Resources 2008.

Selection of reference genes

The selection of suitable reference genes was carried out by screening 8 reference genes that have been previously used by other authors using GeNorm.[20] The reference genes included beta actin, succinate dehydrogenase complex subunit A, beta-2-microglobulin, ribosomal protein L13a, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), hydroxymethyl-bilane synthase, hypoxanthine phosphoribosyl-transferase I, and ribosomal protein L32 (RPL32).[20,21] The stability for the 8 reference genes across different tissues was determined in 4 sample sets for both tissue biopsy and culture samples.

Assay design for real-time reverse transcription–quantitative polymerase chain reaction

Assay designs for real-time reverse transcription–quantitative polymerase chain reaction (real-time RT-qPCR) were carried out using the Universal Probe Library Assay Design Centre (https://www.roche-applied-science.com/sis/rtpcr/upl/). The melting temperatures of all primers were between 58°C and 60°C. Nucleotide basic local alignment search tool was used to search for homology of the designed primer sequences within the human genome. The designed primers are listed in Table 1.

Quantitative polymerase chain reaction

Quantitative PCR was carried out using a real-time PCR system (LightCycler 480 platform; Roche Diagnostics GmbH) and corresponding software (LightCycler; Roche Diagnostics, Mannheim, Germany). The second derivative method was employed for calculating the threshold cycle (C_T). The PCRs were performed in a final volume of 10 μL and were placed in 384 multiwell plates (Roche Diagnostics GmbH). Three

replicates of each reaction were carried out. The reaction volume is composed of 4 μL 1:20 diluted template cDNA, 5 μL LightCycler 480 Probes Master (Roche Diagnostics GmbH), 0.2 μmol/L of each primer (Metabion International AG, Martinsried, Germany) (Table 1), 0.1 μL probe from Universal Probe Library (Roche Diagnostics GmbH), and nuclease-free water (Ambion). For no template control, nuclease-free water was substituted for cDNA. The conditions used for qPCR consisted of a single activation cycle at 95°C for 5 minutes to activate the Hot Start Taq polymerase, followed by 45 cycles of amplification. Each cycle in the amplification stage consisted of 10 seconds at 95°C for denaturation and 30 seconds at 60°C for the annealing and extension. The fluorescence intensity at each cycle was recorded at the 60°C stage. After amplification, a cooling step at 40°C was programmed.

Gene expression level analysis

The significance of the difference in gene expression levels between the control fascia, cords, and nodules was determined by using the relative threshold cycle (C_T) method.[22] First, internal control reference gene expression levels were measured. The C_T from the PCRs for the reference transcripts were averaged and deducted from the C_T for the candidate transcripts, resulting in the value ΔC_T. The ΔC_T of each gene in the 3 tissues were compared with each other using paired *t*-test to determine statistical significance in their differences, as suggested by Yuan and Stewart.[23] Repeated-measures analysis of variance was performed on the ΔC_T values to determine the effect of culturing treatments on gene expressions. Where there was no significant interaction between culturing treatments and different tissues, the ΔC_T for all tissues were averaged per patient. Paired *t*-test was then carried out to determine if there was significant difference in the average ΔC_T of the biopsy samples and tissue cultures. SPSS ver. 14.0 (SPSS Inc., Chicago, IL) was used for all the statistical tests.

As 2 copies of amplicons should be made during each PCR cycle, $2^{-\Delta CT}$ was used to represent the relative expression levels in natural numbers for the purpose of creating bar charts. The fold change was then calculated using the $2^{-\Delta\Delta CT}$ method. Genes were considered to be differentially expressed when there was a statistically significant difference in the average ΔC_T as well as an average fold change of higher than 2. A summary of steps taken to identify the potential biomarkers for DD are shown in Figure 2.

RESULTS

Candidate genes selected from the bioinformatic analysis

Two gene lists were the sources for the selection of potential candidate genes to be examined in this study: the previously established gene linkage and gene expression analysis of microarray data.[10]

The analysis of the 6cM region on chromosome 16 where a linkage for DD was established[19] revealed the following candidate genes: Iroquois homeobox protein 6 (IRX6), IRX5, IRX3, protein kinase B interacting protein (AKTIP), and retinoblastoma like 2/p130 (RBL32) (Table 2). In addition to the linkage analysis results, 20 more candidate genes in DD were chosen according to the gene expression analysis obtained from microarray data due to their high fold change,[10] including periostin (POSTN), collagen type I alpha I (COL1A1), tenascin C (TNC), cysteine- and glycine-rich protein 2 (CSRP2), leucine-rich repeat containing 17 (LRRC17), collagen type V alpha 1 (COL5A1), A disintegrin and metalloproteinase domain (ADAM12), collagen type V alpha 2 (COL5A2), lysyl-oxidase like 2 (LOXL2), aggrecan (ACAN), laminin, beta 1 (LAMB1), myristoylated alanine-rich protein kinase C substrate (MARCKS), collagen type IV alpha 2 (COL4A2), collagen type VI alpha 1 (COL6A1), matrix metallopeptidase 14 (membrane-inserted) (MMP14), aldehyde dehydrogenase 1 family, member A1 (ALDH1A1), and proteoglycan 4 (PRG4), as shown in Table 2. The 25 potential candidate genes were short-listed to 6 candidate genes (IRX6, POSTN, TNC, ALDH1A1, ADAM12, and IRX6) on the basis of higher fold change (upregulated and downregulated) from microarray data[10] and functional clustering of genes (Table 2 and Fig. 3). The criteria chosen were to select those genes that were associated with fibrotic conditions, cell growth, extracellular matrix, and cell adhesion. The 6 candidate genes included ADAM12, ALDH1A1, IRX6, POSTN, PRG4, and TNC.

As well as possessing involvement in liver fibrogenesis and keloid tumor formation, ADAM12 has been shown to contribute to the activation of transforming growth factor beta (TGFβ) signaling pathway, a pathway found to be associated with the pathogenesis of DD.[24–27] The roles of ADAMS in various cancers have been recognized, and ADAM12 has been proposed to be a potential biomarker for breast and bladder cancers.[28–30] ALDH1A1 was selected because of its role in hepatic fibrosis.[31] IRX6 was selected because it is located between the markers D16S419 and D16S3032 on chromosome 16, a single 6cM region where Hu et al. had established linkage.[19]

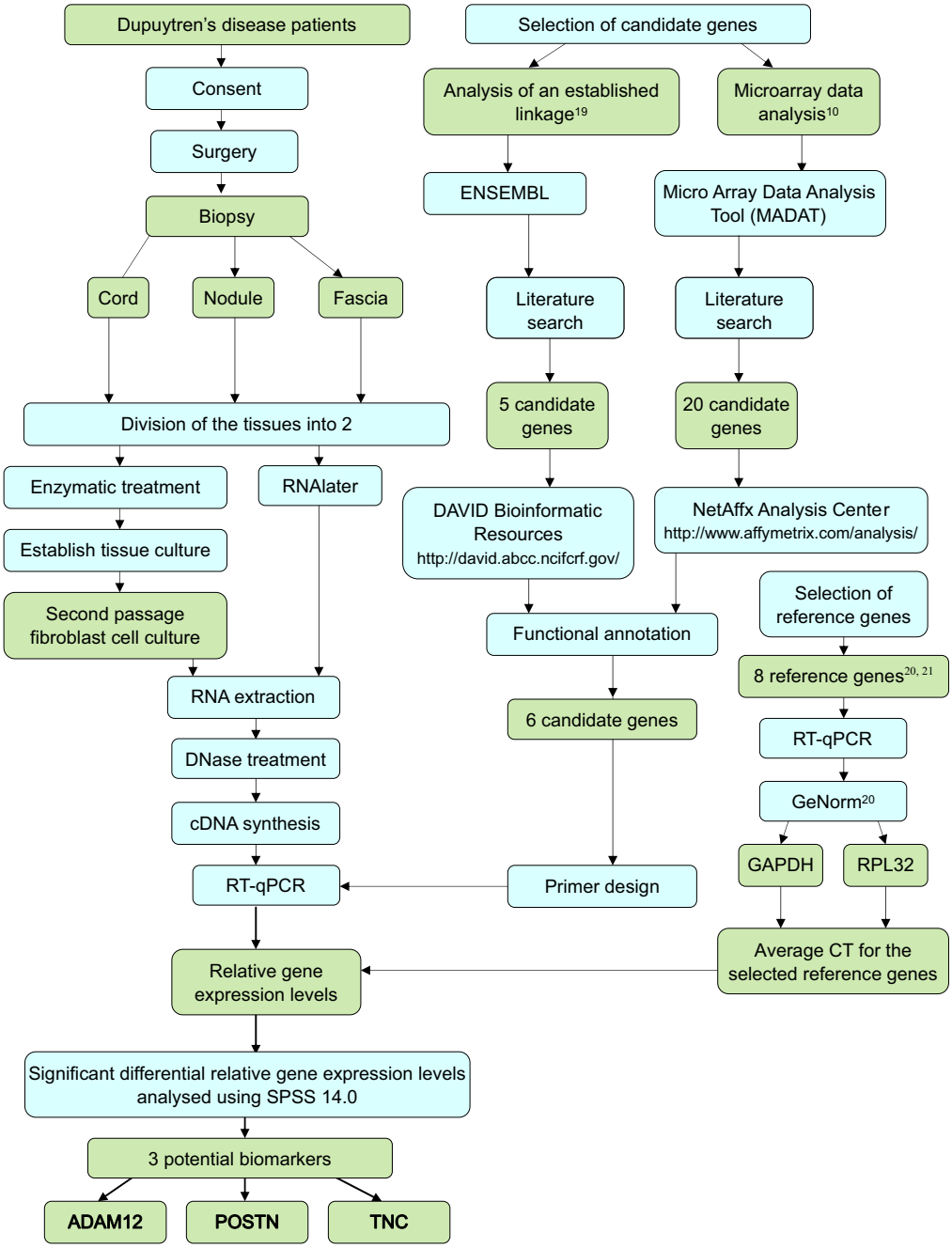


FIGURE 2: Summary of steps taken to determine potential biomarkers for DD. The flowchart presents a summary of the steps taken and the results obtained in this study. The green-shaded boxes show the obtained material or results; the blue-shaded boxes show the methods used.

PRG4 was a candidate gene because DD tissues were reported to have altered proteoglycan profile and was found to be dysregulated in DD cultures.[32,33] POSTN was also shown to be upregulated in DD and Peyronie's disease and had been related to abnormal cell growth and found to be overexpressed in several types of cancers, such as breast, oral, and colon cancers.[34–42] TNC was selected for several reasons. First, the myofibroblastic phenotype in DD was found to be associated with tenascin matrix using immunohistochemistry.[43] In contrast with ADAM12, TGFβ levels were shown to regulate TNC expression.[44,45] TNC was also thought to be involved in fibroblast and myofibroblast migration, wound healing, and fibrosis in various animal models.[46–49] The sequence details of the primers for the qPCR analysis of the selected 6 transcript are listed in Table 1.

TABLE 2. Potential Candidate Genes

Symbol	Gene	Location	Information	Reference
IRX6	Iroquois homeobox protein 6	16q11.2-q13	As a treatment in hyperproliferative disorders, inhibition of the Iroquois family of nucleic acids or proteins, such as IRX1, IRX2, IRX3, IRX4, IRX5, or IRX6, is carried out.	57
IRX5	Iroquois homeobox protein 5	16q11.2-q13	Same as above.	57
IRX3	Iroquois homeobox protein 3	16q12.2	Same as above.	57
AKTIP	Protein kinase B (Akt) interacting protein	16q12.2	Fused toes mice had decreased (AKTIP) FT1 expression. This disease is caused by impaired regulation of programmed cell death.	58
RBL2	Retinoblastoma like 2/p130	16q12.2	Dysregulation of the Cdk/Rb/E2F axis reconstructed for mammary epithelial cells to initiate a paracrine loop with tumor-associated fibroblasts involving TGFβ and hepatocyte growth factor resulting in desmoplasia.	59
POSTN	Periostin	13q13.3	POSTN is upregulated in nodules from patients with DD. The gene is also upregulated in various cancers.	34–37, 39–40
COL1A1	Collagen type I alpha I	17q21.33	Collagen type I is upregulated in DD and idiopathic pulmonary fibrosis.	60
TNC	Tenascin C (hexabrachion)	9q33	Immunohistochemical studies showed that TNC expression is associated with the proliferative nodules in DD. TNC is also involved in fibrosis and the migration of fibroblast and myofibroblasts upon wounding.	44–49
CSRP2	Cysteine- and glycine-rich protein 2	12q21.1	CSPR2 is involved in liver fibrosis.	61
LRRC17	Leucine-rich repeat containing 17	7q22.1	LRRC17 is upregulated in DD.	62
COL5A1	Collagen type V alpha 1	9q34.2-q34.3	COL5A1 is upregulated in DD.	62
ADAM12	A disintegrin and metalloproteinase domain	10q26.3	ADAM12 is upregulated in liver fibrogenesis, keloids, and various cancers.	24–25, 27–31
COL5A2	Collagen type V alpha 2	2q14-q32	COL5A2 is upregulated in DD.	62
LOXL2	Lysyl-oxidase like 2	8p21.3-p21.2	LOXL2 is involved in Wilson's disease.	63
ACAN	Aggrecan	15q26.1	Upregulation of ACAN is observed during the period of acute inflammatory response to toxic liver damage in rats. Cell shape changes have been associated with upregulation of ACAN in animal cell cultures.	64, 65
LAMB1	Laminin, beta 1	7q22	The myofibroblastic phenotype in DD is associated with laminin.	43
MARCKS	Myristoylated alanine-rich protein kinase C substrate	6q22.2	Inhibitors of MARCKS were proposed as anti-hypersecretory drugs for the treatment of airway mucus hypersecretion in cystic fibrosis.	66
COL4A2	Collagen type IV alpha 2	13q34	COL4A2 is upregulated in DD.	45
COL6A1	Collagen type VI alpha 1	21q22.3	COL6A1 induces cardiac myofibroblast differentiation.	67

(*continued*)

TABLE 2. Potential Candidate Genes (*Continued*)

Symbol	Gene	Location	Information	Reference
MMP14	Matrix metallopeptidase 14 (membrane-inserted)	14q11-q12	MMP14 is upregulated in DD.	52
ALDH1A1	Aldehyde dehydrogenase 1 family, member A1	9q21.13	Hepatic fibrosis inactive aldehyde dehydrogenase (ALDH2) alleles may promote hepatic fibrosis.	68, 69
PRG4	Proteoglycan 4	1q25-q31	Proteoglycan alterations observed in DD fascia (some of the chain molecular masses were increased); an increased content of iduronate disaccharide clusters were observed; oversulfation of disaccharide repeats.	32, 70
			Proteoglycan is involved in idiopathic pulmonary fibrosis (helps in early repair process in the lung).	
ADH1B	Alcohol dehydrogenase IB (class I), beta polypeptide	4q21-q23	ADH1B is involved in hepatic fibrosis (alleles were associated).	32
CLU	Clusterin	8p21-p12	CLU is upregulated in tubulointerstitial fibrosis.	71
PCOLCE2	Procollagen C-endopeptidase enhancer 2	3q21-q24	PCOLCE2 is upregulated in liver fibrogenesis in rats.	72

Using the list of differentially expressed genes in a study by Rehman et al.[10] and established linkage by Hu et al.,[22] a list of 25 potential genes was generated. The literature reviews of each gene are summarized in the table.

Selected reference genes

RPL32 and GAPDH were selected for their suitability to be used as stable internal control genes for the normalization in the real-time RT-qPCR.

Quantitative polymerase chain reaction

The gene expression levels of all tissues were compared with each other. The fold changes were considered to be significant when (1) there was a probability of less than .05 for the ΔC_T of a gene being the same and (2) the average fold change was greater than 2.

Four genes were significantly differentially regulated ($p < .05$) between nodules and control fascia (Fig. 4 and Table 3). ADAM12 upregulation was found in nodules when compared with fascia. A fold change of 3 for IRX6 expression level was observed between fascia and nodules. ADAM12, POSTN, and TNC were found to be highly differentially expressed between nodules and control fascia (Fig. 4 and Table 3).

Repeated-measures analysis of variance was used to determine whether culturing treatments had a statistically significant impact on gene expression. Culturing conditions significantly affected the gene expression levels of ALDH1A1, IRX6, and PRG4 ($p < .005$) (Table 4). For ADAM12, POSTN, and TNC, significant effect was not observed ($p < .05$) for either culturing conditions or the interaction between the 2 variables: culturing conditions and different tissues (Table 4).

DISCUSSION

This study has identified statistically significant differences in gene expression for ADAM12, POSTN, and TNC ($p < .05$), all candidate genes generated from bioinformatic analyses found in the biopsy tissues. In addition, gene expression patterns and levels for most transcripts were observed to be significantly different in the cell cultures compared with that in biopsy samples derived from the same tissue ($p < .05$).

Significantly different gene expression levels ($p < .05$) were observed for 4 of the 6 selected candidate genes when comparing nodules with control fascia. However, no statistically significant difference was observed ($p < .05$) in the gene expression levels between the cords and nodules or between cords and fascia. This may be a result of the higher biological variations in the cord tissues (Fig. 4). Although not statistically significant ($p < .05$), it is evident that the gene expression patterns of cords and nodules are similar (Fig. 4). This observation may support the hypothesis that cords are developed from changes in phenotypes in nodule cells.[10,11] A possible explanation for this observation

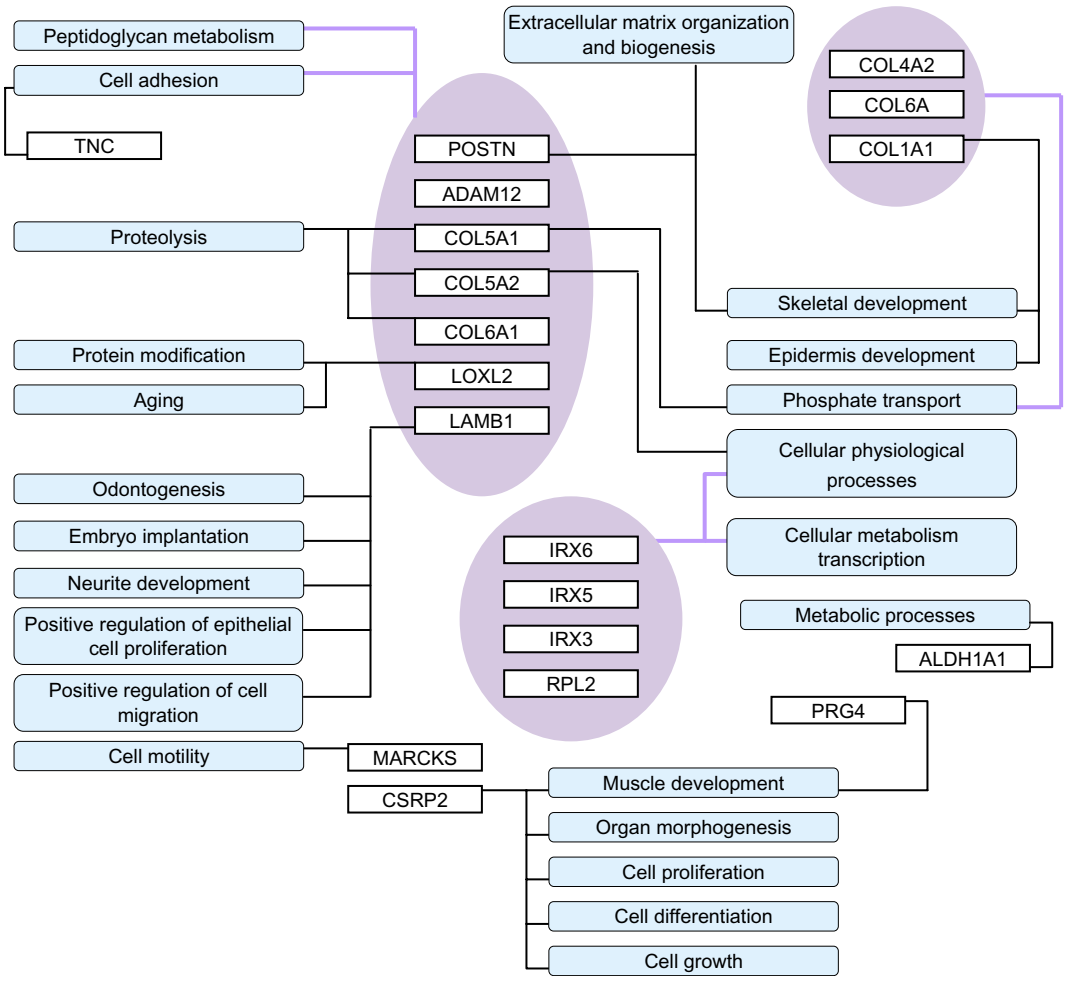


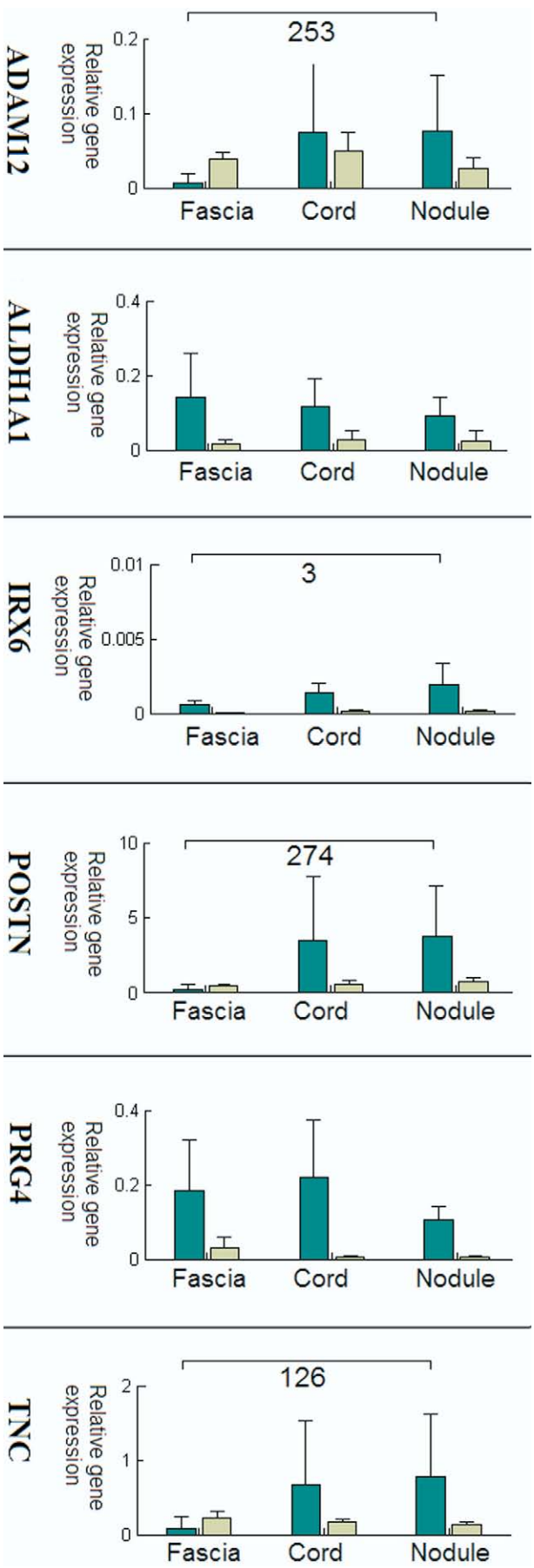
FIGURE 3: Functional annotation of the candidate genes. Using gene annotation programs, NetAffx Analysis Centre and DAVID Bioinformatic Resources, 18 genes are clustered and the pathways each gene is involved in are indicated in the figure. Genes that are involved in similar functions are circled together See Table 2 for abbreviations.

may be that as the cords develop from the active cells in nodules, which is a feature considered to be the most biologically active part of the disease,[12,13] the high gene expression levels gradually decrease over time, which may in turn result in a larger variation in gene expression levels between affected individuals.

ADAM12, POSTN, and TNC were all significantly upregulated in the nodules when compared with control fascia ($p < .05$; Table 3 and Fig. 4). ADAM12, POSTN, and TNC have been previously associated with other abnormal cell growth disorders, as well as molecular pathways associated with DD pathology. Overexpression of POSTN and ADAM12 was associated with other fibrotic disorders or abnormal cell growths.[27–30,41,42]

POSTN upregulation has been associated with various cancers, including melanoma, pancreatic cancer, and oral cancers.[50,51] The upregulation of POSTN, however, may be an effect rather than the cause of DD development. Shimazaki and Kudo demonstrated that POSTN may negatively regulate tumor growth by promoting capsule formation.[38] It is possible that an abnormal cell growth in nodules may lead to the upregulation of POSTN and lead to the development of cords with subsequent contracture.

ADAM12 is a matrix metalloproteinase that has been proposed to be a potential biomarker for bladder and breast cancers.[28,29,31] Another matrix metalloproteinase, A disintegrin and metalloproteinase with thrombospondin motifs 14 (ADAMTS14), was demonstrated to be upregulated in DD.[52] Whereas the expression of ADAM12 was almost undetectable in normal liver and benign tumors, there was a 6-fold upregulation in hepatocellular carcinomas and an up-to 60-fold upregulation in liver metastasis from colonic carcinomas.[30] As previously demonstrated, ADAM12 and TNC have both been shown to be involved in the TGFβ pathways, a pathway previously suggested to be in-



volved in DD pathology.[50,53] ADAM12 is involved in the activation of the TGFβ pathways, whereas TNC is regulated by TGFβ expression.[24-25,44,45] The upregulation of TNC may be important in the development of DD through other mechanisms; TNC was also shown to be involved in the migration of fibroblasts and myofibroblasts upon wounding.[48,49] The 2 cell types densely populated in the DD cords and nodules are fibroblast and myofibroblasts.[7,51]

Even though a statistically significant difference in gene expression levels for IRX6 was observed between cords and nodule when compared with fascia ($p < .05$), IRX6 may not be an ideal biomarker due to the following reasons. When compared with normal fascia, the fold changes of IRX6 in nodules or cords were only 3-fold. However, as IRX6 was found to have an important role in morphogenesis and embryo development,[54,55] DD pathology may be caused by an abnormal gradient of IRX6 expression. The function of the protein would need to be further characterized to make further conclusion.

Downregulation of PRG4 has also been observed in DD cord–derived fibroblasts as shown by Satish et al.[33] Although not statistically significant ($p < .05$), the PRG4 expression in our control fascia is approximately 10 times higher than that in the cord, yet this differential gene expression was not found in biopsy samples, indicating the discrepancy in use of tissue culture compared with tissue biopsy to identify biomarkers.

The culturing conditions had significant impact ($p < .05$) on gene expression levels of ALDH1A1, IRX6, and PRG4 (Table 4). ADAM12, POSTN, and TNC gene expression were not significantly different ($p < .05$) between tissue cultures and biopsies, possible due to the high variation caused by biological difference and the upregulation in nodules and cords (Fig. 4 and Table 4).

FIGURE 4: Relative gene expression levels of each gene in all samples. The relative gene expression levels of each gene, normalized to the expression of reference gene, are given in the graph. Where the relative expression levels are significantly different ($p < .05$) between the different tissues, for groups that have significantly different relative gene expression levels, average fold changes are indicated on the graphs. The average fold change is the average of the fold change observed in the patients, obtained using the $2^{-\Delta\Delta CT}$ method ($n = 5$ for tissue biopsies, $n = 4$ for tissue cultures). The dark-green bars represent the results for biopsy and the yellow bars represent the results for the tissue cultures.

TABLE 3. Significant Fold Changes Observed

Gene	Tissue	p Value*	Average Fold Change†	Biopsy or Culture
Compared with Normal Fascia				
ADAM12	Nodule	.046	+253	Biopsy
IRX6	Nodule	.034	+3	Biopsy
POSTN	Nodule	.046	+274	Biopsy
TNC	Nodule	.047	+126	Biopsy

*Derived from the qPCR results, the significantly different relative gene expression levels ($p < .05$) between the internal controls, fascia, and test tissues are listed ($n = 5$ for tissue biopsies; $n = 4$ for tissue cultures). The data were analyzed using paired T-test.

†The fold changes of those that are significant at 5% confidence level when compared with normal fascia.

TABLE 4. Significance of the Difference in the Relative Gene Expression Levels Between Tissue Biopsy and Culture Samples

Gene	Tissue × Culturing Treatment	Culturing Treatment
ADAM12	.211	.111
ALDH1A1	.946	.003
IRX6	.858	.001
POSTN	.389	.289
PRG4	.081	.001
TNC	.282	.275

Repeated-measures analysis of variance was performed on the relative C_T (ΔC_T) values to determine the effect of culturing treatments on gene expression. p values are indicated in the table. If there were statistically significant interactions ($p < .05$) between use of different tissues and tissue culturing treatment (tissue × culturing treatment), the ΔC_T of various tissues cannot be pooled for each patient. Where there was no significant interaction between use of different tissues and culturing treatments, the ΔC_T for all tissues were averaged per patient. Paired *t*-test was then carried out to determine if there was significant difference in the average ΔC_T of the biopsy samples and tissue cultures.

In support of our findings, culturing conditions were demonstrated to have a profound impact on gene expression of bladder cancers.[15] In contrast, Bignotti et al., using microarray and real-time RT-qPCR of some selected genes, proposed that short-term ovarian serous carcinoma cultures may not significantly affect the ovarian tumor gene expression ($p < .05$).[56] The difference observed in these studies may be due to (1) use of real-time RT-qPCR with a greater sensitivity than that of microarray analysis; (2) culturing treatments having a greater impact on the genes selected in that study; (3) the effect of culturing treatments on DD tissue phenotypes; (4) the differences in processing and the maintenance of the cultures; (5) the exact number of passages (variation between early and late passages). Several studies have demonstrated the effect of long-term passaging on gene expression in cell cultures.[17,18]

Pathogenesis of DD remains unresolved, and surgery continues to be the mainstay of treatment despite a high rate of recurrence after excision. It is feasible that the identification of biomarkers may help in determining the biological pathways involved in the development of DD, possibly leading to better diagnosis, prognosis, and treatment tailored to diagnostic and prognostic indicators.

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