Microarray Gene Analysis and Expression Profiles of Dupuytren's Contracture

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Dupuytren's disease, although not altogether uncommon, has eluded scientists who have sought to explain the cause of this palmar fibroproliferative disorder. It can lead to severe limitations of hand function if left untreated. This study is the first broad genetic survey using microarray technology to find gene products that are overexpressed or underexpressed in diseased tissues. The authors found 23 genes with levels that differ consistently from control levels. Nine were selected for further verification using reverse transcription–polymerase chain reaction. These genes hold potential promise in explaining some of the demographic trends seen with disease, such as correlation with alcoholism and the striking predisposition for the male gender.

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Dupuytren's disease is a chronic fibroproliferative process involving the palmar and digital fascia of unknown pathogenesis. It is named after Baron Guillaume Dupuytren, a French physician and surgeon who popularized the recognition of this hand disease through a series of lectures in 1831. If this disease is left untreated, nodules and cords develop that may lead to flexion deformities of the fingers, most commonly affecting the proximal interphalangeal and metacarpophalangeal joints. It occurs predominantly in whites, and men are 7 to 15 times more likely to be affected than women. It is observed less frequently among other ethnicities.[1] This disease is also linked to environmental factors such as alcohol intake, tobacco use, and antiseizure medication, as well as other metabolic diseases such as diabetes mellitus.[2] Other trends include increased incidence with advancing age and strong familial genetic patterns. However, the etiopathogenesis of this fibromatosis is still unknown.

Several hypotheses have been proposed, including theories involving pathological change in normal palmar fascia,[3] an overaggressive allergic response,[4] multifactorial effects on microvessels,[2] and overexpression of α smooth muscle actin.[5] Furthermore, there appears to be some role of genetic predisposition, because there are observed family clusters in northern Europe as well as a decreased incidence among Africans.[2] It has also been shown that the predominant collagen of palmar fascia is type I, and diseased fascia has an increased ratio of type III to type I collagen.[3]

To elucidate possible genetic causes of Dupuytren's disease, we hoped to identify genes that were either overexpressed or underexpressed in diseased tissues compared with normal palmar fascia. Until recently, the ability to survey cells for the presence and abundance of a large number of gene transcripts in a single experiment had been limited because of the small number of identified genes and the cumbersome nature of transcription analysis. Recent advances in the Human Genome Project and the development of microarray technology have helped to overcome these limitations. Microarray analysis is a hybridization-based process that uses messenger ribonucleic acid (RNA) harvested from cells and tissues, and reverse transcribes them to their complementary deoxyribonucleic acid (DNA). The complementary DNAs are labeled radioactively and then exposed to a membrane

Table 1. Primer Sequences for RT-PCR

Designation	Gene	Forward Primer	Reverse Primer
P1	Protein kinase PKX1	gcttaaagcaggagcaacac	cagagtagaagagccccgtg
P2	SEF2-1B	ggaggtgacatggatatggg	gtgcttgctgatggagcata
P3	ICAM-2	attcgaggtacacgtgaggc	cgtgtcatgggagatgtttg
P4	Tetranectin	catgaaatgctttctggcct	ctggcgcaggtactcataca
P5	Dihydrodiol dehydrogenase	gatcccatcgagaagaacca	acacctgcacgttctgtctg
P6	Aldehyde dehydrogenase	ctggaaacgtggttgtgatg	ccagctccaaggtcactctc
P7	Amyloid A4 precursor	gagacacctggggatgagaa	cttgacgttctgcctcttcc
P8	Archain	cagtatctggaggcagcaca	cttcagaagtacgcttgccc
P9	Lymphocyte-specific protein 1	gagcaccagaaatgtcagca	gagtttggtggtagggctca

ICAM = intercellular adhesion molecule 2.

for which sequences of known genes are secured. The complementary DNAs that match these affixed genes are then hybridized and the radioactive signal processed to determine the relative amounts of the messenger RNA within the subject tissue.

Our study used this microarray technique to survey broadly genes that may be preferentially over- or underexpressed in tissue affected by Dupuytren's disease. We were able to survey human genetic expression using an array of 1,176 genes to identify genes with expression levels that were different from control subjects.

Materials and Methods

Our study group consisted of 6 patients, 4 men and 2 women, ranging in age from 45 to 76 years. Our control group consisted of 2 patients, 1 man and 1 woman. The study was approved by the Human Investigation Committee, Yale University, protocol no. 10785.

Microarray Analysis

Diseased palmar fascia from patients and normal palmar fascia from control subjects were stored at −70°C. Expression array analysis was performed at Clontech Laboratories with their Atlas Microarray System (Palo Alto, CA). Briefly, the Atlas Pure Total RNA labeling system was used to extract RNA, and then radioactive probes were synthesized by reverse transcribing each RNA population using a complementary DNA synthesis protocol. Probes were then hybridized to a Human 1.2k Array II membrane containing 1,176 genes, and analyzed by autoradiography. The image data were then analyzed using specialized software including AtlasImage and AtlasNavigator Software (Clontech Laboratories, Palo Alto, CA). Results were then interpreted using the AtlasInfo database. Two control subjects were combined into one sample, and each experimental patient was run individually. Differences in gene expression were then analyzed for all patients in comparison with the combined control. Nine of 23 genes that were expressed at ratios consistently divergent from the control levels in both increased and decreased levels were then chosen for further analysis by reverse transcription–polymerase chain reaction (RT-PCR).

Reverse Transcription–Polymerase Chain Reaction

The Promega Access RT-PCR system (Madison, WI) was used according to the manufacturer's protocol. The RT-PCR mix included approximately 150 ng template messenger RNA and the standard concentrations of reaction buffer, dNTP mix, magnesium sulfate, upstream and downstream primers, *Tfl* DNA polymerase, and avian myeloblastosis virus reverse transcriptase. Each reaction was then brought up to a final volume of 50 μl with double distilled H_2O.

A negative control using no RNA template was performed and a positive control using glyceraldehyde phosphate dehydrogenase was run with the same volume of RNA template and primers. All 6 patients were run with primers specific for the following genes: protein kinase X1, SEF2-1B, intercellular adhesion molecule 2, tetranectin, dihydrodiol dehydrogenase, aldehyde dehydrogenase 2, amyloid A4 precursor, archain, and lymphocyte-specific protein 1. Primer sequences are listed in Table 1.

Table 2. Gene Expression Direction/Level

Gene	Expression Direction	Expression Level
Aldehyde dehydrogenase 2	Down	1.9–25.5
Alzheimer's disease amyloid A4 protein precursor	Up	1.7–2.4
Archain	Up	3.0–5.0
Brain-specific tubulin α1 subunit	Up	1.8–2.6
CD81 antigen	Down	2.0–4.1
Cytochrome B5	Down	D–3.0
Cytoplasmic glycerol-3-phosphate dehydrogenase	Down	D–3.0
Dematin	Down	D–5.0
Dihydrodiol dehydrogenase	Down	2.6–11.8
Hormone-sensitive lipase	Down	3.3–13.0
Intercellular adhesion molecule 2 precursor	Down	4.0
Laminin β3 subunit precursor	Down	D
Lung group 1B phospholipase	Down	D
Lymphocyte-specific protein 1	Down	2.5
Mitochondrial enoyl-CoA hydratase short subunit 1	Down	2.4–4.4
Monocyte differentiation CD14 antigen precursor	Down	2.5–4.8
Protein kinase PKX1	Up	1.8–3.4
SEF2-1B protein	Up	2.0–5.5
Symplekin	Down	D–2.0
Tartrate-resistant acid phosphatase type 5 precursor	Down	1.9–4.3
Tetranectin	Down	2.2–11.0
Tissue factor pathway inhibitor 2	Down	D–3.0
TWEAK	Down	2.0–4.0

These genes were found to be consistently overexpressed or underexpressed in all patient tissues compared with control tissues with microarray analysis. Underlined genes were chosen for further review by reverse transcription–polymerase chain reaction. D = decreased expression level but weakly quantified in some subjects; CoA = coenzyme A; TWEAK = TnF like weak inducer of apoptosis.

The RT-PCR cycle profile used was as follows: one cycle at 48°C for 45 minutes and one cycle at 94°C for 2 minutes. The following 40 amplification cycles were run at 94°C for 30 seconds, 55°C for 1 minute, and 68°C for 1 minute. The final cycle was held at 68°C for 7 minutes and then the reactions were held to cool at 4°C. All samples were then run on gel electrophoresis against a standard nucleotide marker to verify reaction products and gene length. Reactions that did not yield strong single bands on electrophoresis were then repeated using twice the magnesium sulfate concentration or increased template concentrations.

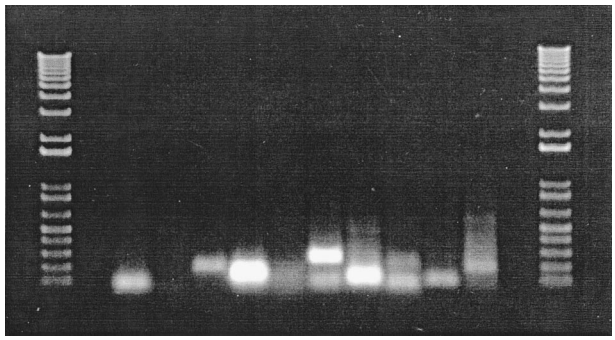

Fig 1. Representative reverse transcription–polymerase chain reaction gel. Primer designations are listed in Table 1. Any products not obtained were repeated with doubling of the magnesium or template concentration. Control was conducted using glyceraldehyde phosphate dehydrogenase.

Results

Microarray analysis revealed 23 genes, 5 of which were consistently overexpressed and 18 underexpressed when compared with control tissue gene expression levels. Nine genes were then chosen for further analysis using RT-PCR based on expression levels differing at least approximately twice that of control levels and with gene products that were not widely expressed proteins. These genes are listed in Table 2, and the nine chosen are underlined within. A representative gel illustrating the RT-PCR products is shown in Figure 1.

Discussion

The genes in Table 2 are the genes with variations from control-level expression. These are notable

in that the upward or downward trends of these genes were the same across all patients compared with control subjects, and at the varying ratios shown. The numbers represent the range of expression as multiples of the control tissue levels.

It is widely accepted that alcohol consumption is linked with Dupuytren's disease, and two of these genes are involved in alcohol metabolism: dihydrodiol dehydrogenase and aldehyde dehydrogenase. The first is a member of the aldo-keto reductase family that catalyzes the reduction of aldehyde or ketone to a corresponding alcohol. The latter is an enzyme in the mitochondrial oxidative degradation of ethanol as well as the metabolism of the neurotransmitter serotonin.[6]

The second intriguing finding lies in protein kinase X1. This is a regulatory enzyme in the phosphorylation of proteins and maps to an area of the sex chromosome with some evidence of Xp and Yp recombination, which may provide a clue as to why it is seen predominantly in men and yet sporadically in women.[7]

The other genes have other interesting associations: Tetranectin is a protein that binds plasminogen in the fibrinolytic pathway.[8] Amyloid A4 precursor was found initially in the brain tissue of Alzheimer's patients and has been found recently to be a membrane-spanning glycoprotein in adult human muscle, cultured human myoblasts, and myotubes.[9] Lymphocyte-specific protein is involved in lymphocyte signal transduction and may be involved in the binding of actin to the plasma membrane.[10] These last two substances seem to play roles governing cellular contractile properties and may have a role in this disease. SEF2-1B and archain are two poorly defined gene products with roles that are still being elucidated. The former is a transcription factor and the latter is a modulator of protein trafficking.[11,12]

It should be noted that there are disadvantages to microarray data, in that the numbers generated are only expression ratios. This provides some problems for data analysis because no information is given on the absolute gene expression levels. Therefore, only trends can be deduced from this data. The true power of microarray data, however, comes from the analysis of not just one subject but of several subjects, and identifying common patterns of gene expression.[13] In this way, our results with 6 patients, all with similar expression patterns of these nine genes appears to be etiologically significant.

Furthermore, microarray data present a challenge because of the sheer number of tests and a small number of replicates, and may therefore be subject to false-positive results. To avoid this, many have advocated an unrelated second method of evaluating gene expression. This may include in situ hybridization, Northern blots, or RT-PCR.[14] We chose RT-PCR as an independent method to verify that these gene products were, in fact, present in diseased tissues. However, this too is not a quantitative method and can only validate the presence of these gene transcripts and not their actual expression levels.

In conclusion, this project outlines the first steps in a broad genetic survey of Dupuytren's disease in an attempt to elicit its possible genetic underpinnings. Of more than 1,000 genes, we found 23 that are expressed at consistently different levels from normal palmar fascia, and we confirmed this in a cohort of nine genes. Because these gene products have now been verified with RT-PCR, further refinements in investigation may now begin and our focus narrowed to these leading candidate genes. Further directions may involve immunohistochemical staining for cellular localization of gene expression as well as using quantitative PCR to obtain more concrete levels of gene transcript products. Hopefully, with this beginning, we will be able to answer more of the central questions regarding this disabling hand disorder.

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References

1 Ross DC. Epidemiology of Dupuytren's disease. *Hand Clin*. 1999;15:53–62.
2 Saar JD, Grothaus PC. Dupuytren's disease: an overview. *Plast Reconstr Surg*. 2000;106:125–134.

3 McFarlane RM. Patterns of diseased fascia in the fingers in Dupuytren's contracture: displacement of the neurovascular bundle. *Plast Reconstr Surg.* 1974;54:31–44.
4 Murrell GA, Hueston JT. Aetiology of Dupuytren's contracture. *Aust N Z J Surg.* 1990;60:247–252.
5 Dave SA, Banducci DR, Graham WP, et al. Differences in α smooth muscle actin expression between fibroblasts derived from Dupuytren's nodules or cords. *Exp Mol Pathol.* 2000;71:147–155.
6 Helander A, Walzer C, Beck O, et al. Influence of genetic variation in alcohol and aldehyde dehydrogenase on serotonin metabolism. *Life Sci.* 1994;55:359–366.
7 Klink A, Schiebel K, Winkelmann M, et al. The human protein kinase gene PKX1 on Xp22.3 displays Xp/Yp homology and is a site of chromosomal instability. *Hum Mol Genet.* 1995;4:869–878.
8 Holtet TL, Graversen JH, Clemmensen I, et al. Tetranectin, a trimeric plasminogen-binding C-type lectin. *Protein Sci.* 1997;6:1511–1515.
9 Calhoun ME, Burgermeister P, Phinney AL, et al. Neuronal overexpression of mutant amyloid precursor protein results in prominent deposition of cerebrovascular amyloid. *Proc Natl Acad Sci U S A.* 1999;96:14088–14093.
10 Pulford K, Jones M, Banham AH, et al. Lymphocyte-specific protein 1: a specific marker of human leucocytes. *Immunology (England).* 1999;96:262–271.
11 Kato T. Molecular genetics of bipolar disorder. *Neurosci Res (Ireland).* 2001;40:105–113.
12 Radice P, Pensotti V, Jones C, et al. The human archain gene, ARCN1, has highly conserved homologs in rice and *Drosophila*. *Genomics.* 1995;26:101–106.
13 Quackenbush J. Computational analysis of microarray data. *Nat Rev Genet.* 2001;2:418–427.
14 Geschwind DH. Sharing gene expression data: an array of options. *Nat Rev Neurosci.* 2001;2:435–438.

Open Discussion

Deborah Pan, MD

Robert X. Murphy, MD (Allentown, PA): Dr Pan, you implied in at least one of your markers that there is potential differential sexual expression. Were all of these subjects Caucasian? And if not, would you anticipate any racial differential expression?

Dr Pan: They were all Caucasian. Four were men; two were women. We wanted to make sure that we took out all sex differences. We were unable to find any other races for our study, but we are still looking for more subjects. As far as race is concerned, since this was kind of a shotgun approach and a fishing expedition for any genes that might be correlated, we were not expecting or looking for any racial differences at this time.

Amitabha Mitra, MD (Philadelphia, PA): If you did the same kind of gene analysis for hypertrophic scar and keloid, what would be your thought process, and how would you go about finding any difference. Because your control does not have any disease. I am making a guess. Am I right? Your control group of the patients have normal fascia that you are taking out?

Dr Pan: That's correct.

Dr Mitra: Now, if you did the same thing with hypertrophic scar or keloid, what would you suppose it would be?

Dr Pan: The correct way to approach that would be to take keloid tissue and to subject that to microarray analysis as well and to see if any of the same genes were brought out in comparison with the Dupuytren's disease. Then go from there and select out any similarities or differences and try to elucidate backward what genes may be differentially expressed and what processes may be going on vs. keloids vs. Dupuytren's disease.