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The Expression of Myoglobin and ROR2 Protein in Dupuytren's Disease[1]

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Background. **Dupuytren's disease (DD) is a hand disease inherited as an autosomal dominant trait with variable penetrance, especially among populations of northern European ancestry. The etiology and pathophysiology of DD are not clear. The purpose of this study was to examine the gene expression profiles of palmar fascia of DD and healthy patients using microarray analysis to highlight the genes that might contribute to the pathogenesis of DD.**

Materials and methods. **Dupuytren contracture samples were taken from excised mature cords of DD patients during aponeurectomies. Control samples were collected from healthy hand trauma patients. Microarray analysis was performed with the Affymetrix HGU133A genome array (Affymetrix, Santa Clara, CA). Expression changes of selected proteins were confirmed at the protein level with Western and dot blotting or by immunohistochemistry.**

Results. **At least an 8-fold change in gene expression was found with 127 genes, including a 90-fold down-regulation of myoglobin and a 14-fold up-regulation of tyrosine kinase-like orphan receptor 2 (= ROR2) from absent to present during the disease. The changes in myoglobin and ROR2 expression were confirmed at the protein level.**

Conclusions. **In this study, we showed for the first time the connection of ROR2 in Dupuytren's disease. ROR2 and myoglobin may play an important role in the pathophysiology of this disease.**

Key Words: **Dupuytren's disease; gene expression; microarray.**

INTRODUCTION

Dupuytren's contracture is a troublesome disease of palmar fascia, contracting fingers permanently and impairing normal hand function. It affects mainly populations of northern European ancestry [1, 2], is male dominant [3], and its prevalence increases with age [1]. Diabetes, smoking, and genetic susceptibility are generally accepted predisposing factors for Dupuytren's disease (DD) [4, 5]. Alcohol abuse, epilepsy and anticonvulsants, microtraumas caused by manual work, and HIV are somewhat controversial as predisposing factors [3, 6–8]. The possible connection between cancer and DD has been under surveillance [9–12]. Histologically, DD resembles fibrosarcoma at the early stages of the disease, while maturing it becomes more like scar tissue [13]. Myofibroblasts, a characteristic feature of DD, also participate in the normal wound healing process and are involved in different inflammatory conditions [14].

In our study, we wanted to examine the differences between the DD tissue and healthy palmar fascia at the mRNA level to identify any gene expression changes associated with the disease and, thus, find factors that could explain the etiology of the disease. For this purpose, we performed a microarray analysis. This allowed the simultaneous study of almost the whole genome in one experiment. To verify the microarray results at the protein level of myoglobin and ROR2, which we assume to be important in the development of DD, we performed Western blot or immunohistochemical staining.

The tyrosine kinase-like orphan receptor 2 (ROR2) belongs to the family of receptor tyrosine kinases. It has an important role in tissue development and morphogenesis through cell migration. Mutations in ROR2 cause brachydactyly and Robinow syndrome. In both

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diseases there is variability in phenotypic expression from complete absence to hypoplasia of limbs and spine [15].

MATERIALS AND METHODS

Twelve DD palmar fascia samples were recruited from April 2002 to August 2003 at the University Hospital by two hand surgeons. The Dupuytren patients were male, and the average age was 59 y (range 31 to 78). One took medication for coronary disease and the other had Type 1 diabetes mellitus. The patient with coronary disease had recurrent DD and positive hereditary diathesis, having two uncles with the disease. The patient with diabetes mellitus also had recurrent DD, but no diathesis. Nine of the patients had a recurrent disease, and eight of them had a positive diathesis for Dupuytren's disease. One of the patients, a smoker, had recurrent DD as well as plantar fibroproliferative lesions. In all of the patients the DD was considered to be mature and in the residual, advanced stage by the time of operation. The justification for the aponeurectomy was an extension limitation of 30 degrees of metacarpal joint or more, or an annoying midpalmar cord. Operations were performed under tourniquet and under general anesthesia. The samples were divided into two halves to be used for immunohistochemical staining and RNA and protein isolation. For the RNA and protein analysis, the DD samples were divided into two randomized pools (D1 and D2) to improve the reliability of the study. Both pools contained six patients.

The normal palmar fascia samples (control samples) were obtained from three healthy, non-smoking male hand trauma patients, aged 18, 19, and 31 y. The study protocol was approved by the Ethical Committee of the Faculty of Medicine, University Hospital, and all of the patients gave their written consent for the study.

Isolation of Total RNA and Microarray Analysis

All fascia samples were cut into 15 μm cryosections and pulverized. Total RNA was isolated using the Trizol protocol (GIBCO-BRL, Gaithersburg, MD) and further purified with the Qiagen RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. For the microarray analysis the RNA was pooled from three healthy (H) samples as well as from the six diseased samples of both the D1 and D2 pools.

Experimental procedures for microarray (Affymetrix, Santa Clara, CA) were performed according to the Affymetrix GeneChip Expression Analysis Technical Manual. Affymetrix HGU133A array containing over 22,000 probe sets for over 14,500 genes was used. The samples were hybridized once.

Analysis of Protein Expression with Western Blot and Dot Blot

Proteins were isolated from the same samples as total RNA according to the Trizol reagent protocol. The pooled samples (pooling as above) were run on a 10% sodium dodecyl sulfate-polyacrylamide gel and transferred to an Immobilon P filter (Millipore Corp., Bedford, MA). Non-specific binding was blocked by incubation with phosphate-buffered saline supplemented with 5% nonfat dry milk (Valio, Helsinki, Finland) for 60 min. The filter was first incubated overnight at room temperature with primary antibodies against myoglobin (1:3000 dilution) (Novocastra Laboratoires Ltd., Newcastle upon Tyne, United Kingdom). Then the filter was treated with peroxidase-conjugated secondary antibody (DAKO A/S, Glostrup, Denmark) (1:500), followed by incubation with ABComplex solution (DAKO), including avidin and biotinylated horseradish peroxidase. For detection, ECL Western blotting reagents (Amersham Pharmacia Biotech, Buckinghmashire, United Kingdom) were used as described in the product protocol.

For myoglobin protein expression analysis from individual samples, dot blot was performed using two healthy and ten DD samples. One of the healthy samples used for Western blot and one DD sample in both the D1 and D2 pools were excluded from the dot blot analysis because of their low protein content. Proteins (18 μg) were transferred onto Protran filters (Schleicher and Schuell GmbH, Dassel, Germany) with suction, and the membranes were handled similar to the Western filters above. Intensities of the dots were measured using Scion Image software (Scion Corporation, Frederick, MA).

Immunohistochemical Analysis

Immunohistochemical staining was performed on 3 μm-thick formalin-fixed paraffin sections. The samples were prepared by deparaffination and rehydration. The sections were then microwaved for 15 min (300 W) in 10 mM Tris/EDTA (pH 9) for epitope retrieval. Endogenous peroxidase activity was inhibited by incubation with peroxidase blocking solution (ChemMate; DakoCytomation, Glostrup, Denmark) for 5 min. The primary antibodies ROR2 (1:100) were incubated for 30 min at room temperature. For detection, the ChemMate Dako EnVision Detection Kit (ChemMate) and Labvision Autostainer (Labvision Corp., Fremont, CA) were used, and the sections were counterstained with Mayer's hematoxylin. A section incubated with a non-immune primary antibody was used as a negative control.

RESULTS

Microarray

Microarray analysis was performed to screen for gene expression changes between healthy palmar fascia and DD samples on a large scale. The analysis revealed 127 genes whose expression was altered at least 8-fold, when comparing the healthy samples and one of the pooled diseased (D1 and D2) samples, and at least 4-fold, when comparing the healthy sample with other diseased sample (D1 or D2). These genes can be divided into the following categories: immune response, angiogenesis, apoptosis, carbohydrate metabolism, cell adhesion and cell-matrix adhesion, cell cycle and proliferation, cell differentiation, transcription, development, signaling and signal transduction, protein synthesis and folding, oxygen transport, muscle-specific genes, and other genes.

Of the genes displaying varying expression levels, 27 of the total 127 were present in both healthy and DD samples. Four belonged to the immune response category, two from each of the cell adhesion, others, and development categories (e.g., ROR2), three from each of the signaling and oxygen transport categories, and eleven from the muscle category.

The genes, which were absent from diseased samples, belonged mostly to the muscle category (e.g., myoglobin belonged to this category, although it was present in healthy and diseased samples), the immune response category, and the others category. Genes involved in carbohydrate metabolism exhibited the greatest changes in expression levels.

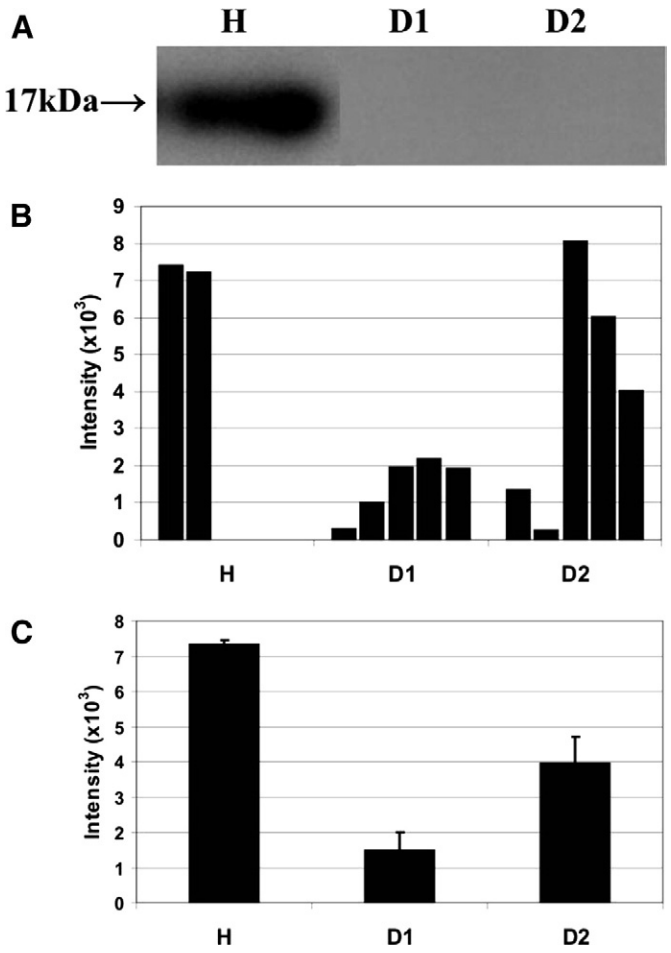

FIG. 1. (A) Myoglobin Western blot showing expected 17 kDa band in healthy (H) but not in either of the pooled diseased samples (D1 and D2). (B) Dot blot analysis was performed with each of the individual samples. (C) Mean values and standard deviations of H, D1, and D2.

Confirmation of Microarray Results at the Protein Level

To confirm the microarray results at the protein level, Western blot and immunohistochemistry were used. Using the Western blot technique, myoglobin protein was detected from the pooled healthy sample, but not in either of the pooled diseased samples (Fig. 1A). To examine the individual samples, dot blot analysis of myoglobin expression was performed using two healthy samples, five of the D1 samples, and five of the D2 samples. The strongest expression level was observed in healthy samples, whereas diseased samples showed varying but lower expression (Fig. 1B and C). The dot blot result confirmed the findings of the Western blot.

Since ROR2 is linked to other hand deformities [16], it was selected for further study at the protein level. Immunohistochemical staining revealed ROR2 expression in diseased but not in healthy samples, thus confirming the microarray results (Fig. 2).

DISCUSSION

Although much of the pathogenesis and nature of DD has been revealed, its etiology is still unclear. In our study, the expression of over 20,000 transcripts was analyzed with microarray. A microarray technique is a powerful tool for studying the gene expression patterns of tissues. The technique has been used previously to compare palmar fascia of healthy controls and DD patients [17], as well as those with Peyronie's disease and Dupuytren's contracture [18]. In our study, the expression of more than 2000 genes was altered between the samples, and at least an 8-fold expression difference was detected in 127 genes. The relationship between the quantity of mRNA and its protein are not directly proportional, so these results must be interpreted with caution. A few select genes considered to be of interest in Dupuytren's disease were selected for analysis also at the protein level to verify changes in expression detected at the mRNA level. Because of the vulnerability of Western blot, immunohistochemical staining was also used to detect the protein and to confirm the results.

Myoglobin, which belongs to the muscle gene category, demonstrated a 52.0-fold to 90.5-fold reduction in DD samples. Muscle-related genes were the largest category with the greatest changes among differentially expressed genes, and myoglobin expression was detected both in healthy and DD samples. The differential protein expression was confirmed using Western blot, which showed expression in healthy tissue but did not show any expression in diseased samples. Myoglobin is a hemoprotein that transports, stores, and releases oxygen to muscle tissues during hypoxia or anoxia [19]. The marked decrease in expression is probably due to the late stage of DD, which is hypocellular, and therefore the main cells, myofibroblasts, decrease in number and are substituted by non-muscle-consisting fibroblasts. Local tissue ischemia is supposed to be one of the underlying factors of DD [5]. Usually, myoglobin is considered to exist only in striated muscle, yet according to some studies it has also been found in smooth muscle tissue [20]. Myofibroblasts are known to have features of both smooth muscle tissue and fibroblasts [21].

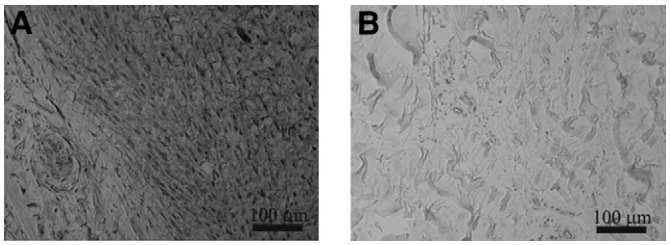

FIG. 2. Immunohistochemical analysis of ROR2 protein expression showed expression in diseased tissue (A) but not in healthy control tissue (B). 300 × magnification was used.

Dot blot analysis of myoglobin expression in individual samples confirmed higher expression in healthy tissue, but also showed varying expression in diseased tissue. The result supports the ischemia theory. The tissue in Dupuytren's disease is probably avascular because the expression of genes from the oxygen transport group is also decreased in DD tissue. In addition, there were differences in myoglobin protein expression between D1 and D2 pools in dot blot. Interestingly, the pool containing samples from patients who had recurrent disease seemed to have a stronger expression.

ROR2 protein expression was confirmed immunohistochemically. This gene was considered to be of interest due to its role in development and association with certain hand deformities [16]. ROR2 is a member of the ROR subfamily. Microarray analysis revealed a surprising up-regulation of this gene, from an absence of expression in healthy samples to its expression in diseased tissue. ROR2 is responsible for chondrocyte and cartilage development, amino acid phosphorylation, and signal transduction between cells. This protein affects the process of differentiation and has a significant role in cell apoptosis, which is a typical feature of DD at the residual stage [22]. The protein has not been studied before in DD, although it is involved in some hand disorders. Brachydactyly, an autosomal dominant hand deficiency, has been recently shown to be caused by mutations in ROR2 [16]. Drosophila tyrosine kinase from the ROR-family plays an important role in neural development [23]. Tyrosine kinase like-proteins might have an important role in the etiology of DD, affecting either the developmental or differentiation stage of cells. Furthermore, ROR2 also controls skeletal development. Chondrocytes can display contractile behavior [24], and DD is characterized as a contractile disease. Peyronie's disease is considered to simulate DD, and cartilage and bone tissue have been found in diseased samples [25]. The protein has never before been linked to DD and was therefore a new discovery.

The main interest in DD research has been on genes and proteins with a known role in the tissue development and differentiation. One of the proteins (MafB) of this group was recently found in DD tissue samples in a study of Lee *et al.* [26]. However, only 50% of the samples were positively stained in immunohistochemistry. The reason for this may be the maturation stage of the DD. This may also explain the variability of myoglobin expression in our study between groups D1 and D2, and in group D2 (Fig. 1B).

We found a number of genes with striking changes in their mRNA expression levels between controls and DD samples. The expression of ROR2 and myoglobin was also detected at the protein level. The most interesting of the genes with altered expression was ROR2 that may have an important role in the development of DD and which has not been reported earlier to be connected with DD. Since the sample material used in this study consisted only of residual cases, the possibility remains that the gene expression profiles are different during the proliferative and involutional disease stages.

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REFERENCES

1. Ross DC. Epidemiology of Dupuytren's disease. Hand Clin 1999;15:53.
2. Finsen V, Dalen H, Nesheim J. The Prevalence of Dupuyten's disease among different ethnic groups in northern Norway. J Hand Surg 2002;27A:115.
3. Geoghegan JM, Forbes J, Clark DI, et al. Dupuytren's disease risk factors. J Hand Surge Br 2004;29B:423.
4. Gudmundsson KG, Arngrimsson R, Jónsson T. Dupuytren's disease, alcohol consumption, and alcoholism. Scand J Prim Health Care 2001;19:186.
5. Hueston JT, Murrell GA. Cell-controlling factors in Dupuytren's contracture. Ann Chir Main Memb Super 1990;9:135.
6. Abe Y, Rokkaku T, Ofuchi S, et al. An objective method to evaluate the risk of recurrence and extension of Dupuytren's disease. J Hand Surg Br 2004;29B:427.
7. Mc Farlane RM. Dupuytren's disease: Relation to work and injury. J Hand Surg Am 1991;16:775.
8. Khan AA, Rider OJ, Jayadev U, et al. The role of manual occupation in the aetiology of Dupuytren's disease in men in England and Wales. J Hand Surg Br 2004;29:12.
9. Mikkelsen OA, Hoyeraal HM, Sandvik L. Increased mortality in Dupuytren's disease. J Hand Surg Br 1999;24:515.
10. Gudmundsson KG, Arngrimsson R, Sigfússon N, et al. Increased total mortality and cancer mortality in men with Dupuytren's disease: A 15-year follow-up study. J Clin Epidemiol 2002;55:5.
11. Wilbrand S, Ekbom A, Gerdin B. Cancer incidence in patients treated surgically for Dupuytren's contracture. J Hand Surg Br 2000;25:283.
12. Wilbrand S, Ekbom A, Gerdin B. Dupuytren's contracture and sarcoma. J Hand Surg 2002;27:50.
13. Fitzgerald AMP, Kirkpatrick JJ, Naylor JL. Dupuytren's disease. The way forward? J Hand Surge Br 1999;24B:395.
14. Moulin V, Castilloux G, Auger FA, et al. Modulated response to cytokines of human wound healing myofibroblasts compared to dermal fibroblasts. Exp Cell Res 1998;238:283.
15. Afzal AR, Jeffery S. One gene, two phenotypes: ROR2 mutations in autosomal recessive Robinov Syndrome and autosomal dominant brachydactyly type B. Hum Mutat 2003;22:1.
16. Schwabe GC, Tinschert S, Buschow D, et al. Distinct mutations in the receptor tyrosine kinase gene ROR2 cause brachydactyly Type B. Am J Hum Genet 2000;67:822.
17. Pan D, Watson HK, Swigart C, et al. Microarray gene analysis and expression profiles of Dupuytren's contracture. Ann of Plastic Surg 2003;50:618.

18. Qian A, Meals RA, Rajfer J, et al. Comparison of gene expression profiles between Peyronie's disease and Dupuytren's contracture. Urology 2004;64:399.
19. Ordway GA, Garry DJ. Myoglobin: An essential hemoprotein in striated muscle. J Exp Biol 2004;207(Pt 20):3441.
20. Qiu Y, Sutton L, Riggs AF. Identification of myoglobin in human smooth muscle. J Biol Chem 1998;273:23426.
21. Tomasek JJ, Vaughan MB, Haaksma CJ. Cellular structure and biology of Dupuytren's disease. Hand Clin 1999;15:21.
22. Malemud CJ. Protein kinases in chondrocyte signaling and osteoarthritis. Clin Orthop Relat Res 2004;427:145.
23. Oishi J, Sugiyama S, Liu ZJ, et al. A novel Drosophila receptor tyrosine kinase expressed specifically in the nervous system. J Biol Chem 1997;272:11916.
24. Spector M. Musculoskeletal connective tissue cells with muscle: Expression of muscle actin in and contraction of fibroblasts, chondrocytes, and osteoblasts. Wound Repair Regen 2001;9:11.
25. Kapuller LL, Derevianko VP, Novak G, et al. Morphological characteristics of Peyronie's disease. Arkh Patol 1982;44:49.
26. Lee LC, Zhang AY, Alphonsus K, et al. Expression of a novel gene, MafB, in Dupuytren's disease. J Hand Surg 2006;31A: 211.