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Genetic susceptibility in Dupuytren's disease

TGF-β1 POLYMORPHISMS AND DUPUYTREN'S DISEASE

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Dupuytren's disease is a benign fibroproliferative disease of unknown aetiology. It is often familial and commonly affects Northern European Caucasian men, but genetic studies have yet to identify the relevant genes.

Transforming growth factor beta one (TGF-β1) is a multifunctional cytokine which plays a central role in wound healing and fibrosis. It stimulates the proliferation of fibroblasts and the deposition of extracellular matrix. Previous studies have implicated TGF-β1 in Dupuytren's disease, suggesting that it may represent a candidate susceptibility gene for this condition.

We have investigated the association of four common single nucleotide polymorphisms in TGF-β1 with the risk of developing Dupuytren's disease. A polymerase chain reaction-restriction fragment length polymorphism method was used for genotyping TGF-β1 polymorphisms. DNA samples from 135 patients with Dupuytren's disease and 200 control subjects were examined.

There was no statistically significant difference in TGF-β1 genotype or allele frequency distributions between the patients and controls for the codons 10, 25, -509 and -800 polymorphisms.

Our observations suggest that common TGF-β1 polymorphisms are not associated with a risk of developing Dupuytren's disease. These data should be interpreted with caution since the lack of association was shown in only one series of patients with only known, common polymorphisms of TGF-β1. To our knowledge, this is the first report of a case-control association study in Dupuytren's disease using single nucleotide polymorphisms in TGF-β1.

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Dupuytren's disease (DD) is a nodular palmar fibromatosis causing progressive and permanent contracture of the digits. It is often familial and is common in individuals of Northern European extraction.[1] More than 25% of men of Celtic origins over 60 years of age have evidence of DD,[2] and it is one of the most common inheritable disorders of connective tissue in Caucasians.[3]

Autosomal dominance with variable penetrance has been proposed as the likely mode of inheritance,[4] although no single gene has so far been identified. It is, however, unclear whether DD is a complex oligogenic condition or a simple monogenic Mendelian disorder. The identification of susceptible genetic loci would provide an ideal approach to unravelling the hereditary component of this common disease.

The myofibroblast has been shown to be a key cell responsible for the tissue contraction in DD.[5,6] Iwasaki et al[7] studied the histopathological changes in 43 patients and concluded that growth factors may induce proliferation of genetically abnormal myofibroblasts.

Transforming growth factor beta one (TGF-β1) is a multifunctional cytokine which has been implicated in the pathogenesis of DD.[6,8-13] It modulates cellular growth and differentiation in a wide variety of cell types including fibroblasts. It also stimulates the proliferation and migration of fibroblasts and deposition of extracellular matrix (ECM) and inhibits degradation of the latter.[14,15] The precursor to TGF-β1 is a latent protein composed of 390 amino acids,[16] while the active form consists of two identical linked peptide chains of 112 amino acids, which are highly conserved between species.

Variability in the TGF-β1 gene resulting in the induction of different levels of protein expression of ECM is a possible cause of DD,[17] which would result in different

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levels of deposition and cellular growth, proliferation and differentiation of ECM.[18]

Recently, pathological dysregulation of the TGF-β pathway has been implicated in the development of fibrotic disease.[19] The TGF-β1 gene is polymorphic and is associated with increased production of TGF-β1 in fibrotic conditions. Several polymorphisms of the TGF-β1 gene have been reported.[18,20] One of these at codon position 25 has been associated with increased production of TGF-β1 and fibrosis.[20,21]

TGF-β1 promotes the development of a myofibroblast phenotype in normal fibroblasts.[22] Several experiments have suggested that it could be involved in the pathogenesis of DD.[8-11] For example, TGF-β1 is widespread in fibroblasts in all stages (proliferative, involutional and residual) of the disease,[10] and significantly stimulates proliferation of myofibroblasts in DD.[13] By contrast, normal palmar fascia contains only an occasional cell staining positively for TGF-β1. We have tested the hypothesis that there is an association between four known common TGF-β1 polymorphisms and the development of DD.

TGF-β1 genotyping was undertaken in Caucasian individuals with DD and compared with a control Caucasian population.

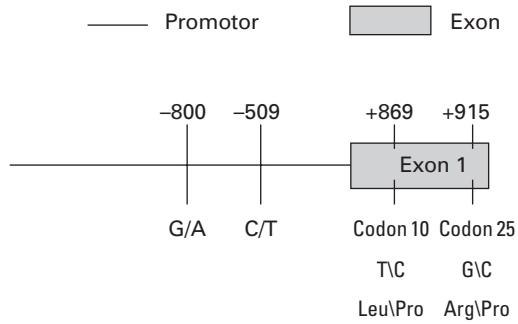

Fig. 1

Positions of common polymorphisms in the TGF-β1 gene.

Patients and Methods

There were 135 patients, 117 men and 18 women, in the study with a mean age of 56.9 years (37 to 90). All were unrelated Caucasians from the Northwest region of England and were identified from the operative records at the South Manchester University Hospital Trust and Wrightington Hospital. All were seen by the first author (AB) who took a full medical history using a proforma and examined both hands. They all had a confirmed diagnosis of DD with characteristic nodules in the palm of the hand and/or digits with or without joint contractures. The 200 control subjects, 100 men and 100 women, were ethnically-matched healthy Caucasians with a mean age of 45.8 years (24 to 76), who were selected from the register of general practices. The age and gender ratios differed between the patients and the controls, but since the gene polymorphisms examined are non-gender-determined and lie on chromosome 19 q13.1, the gender ratio was unlikely to affect the outcome of the study. We have compared the incidence of disease-causing alleles between our patients and a normal population control group in view of the distribution of age and gender. The local and hospital Ethical Committees gave approval for the study and written consent was obtained from all individuals.

DNA extraction. Blood samples were collected using a standard venesection technique; 5 ml of venous blood were collected from each subject. DNA was extracted from peripheral blood cells using a commercially available DNA extraction kit (Qiagen, UK) and concentrations of DNA were measured and diluted in buffer to 100 ng/μl using sterile, Qiagen buffer.

Genotyping. Four common polymorphisms of TGF-β1 were typed using the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method.[23] These were selected using previously confirmed and published single nucleotide polymorphisms (SNPs) found in the TGF-β1 gene in association with different fibrotic

Table I. Details of the position, primer sequences and reaction conditions used for the detection of the four TGF-β1 gene polymorphisms

Position	Primer sequence (5'-3')	Size (bp)	Annealing temperature (°C)
-800	F: 5'- ACA GTT GGC ACG GGC TTT CG -3' R: 5'- TCA ACA CCC TGC GAC CCC AT -3'	388	62
-509	F: 5'- CAG TAA ATG TAT GGG GTC GCA G -3' R: 5'- GGT GTC AGT GGG AGG AGG G -3'	153	59
Codon 10	F: 5'- CTC CGG GCT GCG GCT GCG GC -3' R: 5'- CGG CAC CTC CCC CTG GCT CG -3'	199	65
Codon 25	F: 5'- GCT ACC GCT GCT GTG GCT ACT -3' R: 5'- ACG CGG GTG ACC TCC TTG-3'	297	64

Table II. TGF-β1 codon 10, codon 25, -509 and -800 allele and genotype frequencies by number and *percentage*

	DD	Control	p value
Codon 10			
Number of subjects	131	199	
Allele frequency[1]			[1] 0.293 (OR = 1.19, 95% CI 0.86 to 1.65)
(T)	177 (*68*)	253 (*64*)	
(C)	85 (*32*)	145 (*36*)	
Genotype frequency[2]			[2] 0.455
(T/T)	59 (*45*)	83 (*42*)	
(T/C)	59 (*45*)	87 (*44*)	
(C/C)	13 (*10*)	29 (*14*)	
Codon 25			
Number of subjects	134	197	
Allele frequency[1]			[1] 0.687 (OR = 0.89, 95% CI = 0.53 to 1.51)
(G)	241 (*90*)	358 (*91*)	
(C)	27 (*10*)	36 (*9*)	
Genotype frequency[2]			[2] 0.597
(G/G)	108 (*81*)	164 (*83*)	
(C/G)	25 (*18*)	30 (*15*)	
(C/C)	1 (*1*)	3 (*2*)	
-509			
Number of subjects	131	200	
Allele frequency[1]			[1] 0.357 (OR = 1.17, 95% CI 0.84 to 1.65)
(C)	187 (*71*)	272 (*68*)	
(T)	75 (*29*)	128 (*32*)	
Genotype frequency[2]			[2] 0.573
(C/C)	66 (*50*)	94 (*47*)	
(C/T)	55 (*42*)	84 (*42*)	
(T/T)	10 (*8*)	22 (*11*)	
-800			
Number of subjects	135	197	
Allele frequency[1]			[1]0.281 (OR = 0.74, 95% CI 0.43 to 1.27)
(G)	243 (*90*)	364 (*92*)	
(A)	27 (*10*)	30 (*8*)	
Genotype frequency[2]			[2] 0.498
(G/G)	110 (*81*)	168 (*85*)	
(G/A)	23 (*17*)	28 (*14*)	
(A/A)	2 (*2*)	1 (*1*)	

disorders.[18,20] Two of the SNPs are in the promoter region and two in exon 1. The location, relative position and nucleotide substitution of the SNPs in the TGF-β1 gene are shown in Figure 1. The known polymorphisms chosen for the study were named according to their relative position on the TGF-β1 gene; Genbank accession number X05839.

PCRs were carried out in 96-well plates. Each PCR consisted of 1 μl of DNA (100 ng/μl), 2.5 μl of x 1 NH_4 buffer (Bioline Ltd, UK), 2.5 μl of each 200 mM d'NTP, 0.1 μl of 0.5 unit Taq polymerase (Bioline), 1 μl of $MgCl_2$ at either 2 mM for codon 10 and -509 or 1.75 μl at 3.5 mM for codon 25 and -800 and 0.1 μl each of 50 μmol forward and reverse primer, and made up to 25 μl reaction mix with autoclaved, distilled water. The sequences of the primers used are given in Table I. PCR was carried out under the following conditions: denaturation for two minutes at 95ºC, followed by 35 cycles of further denaturation of 45 seconds at 95ºC and then of one minute at annealing temperature followed by extension for 45 seconds at 72ºC. A final elongation step of five minutes at 72ºC was included.

For PCR-RFLP conditions amplified DNA (5 μl) was incubated overnight in a Hybaid Omnigene thermal cycler with the appropriate enzyme and buffer and made up to a 10 μl reaction mixture. The enzymes used were Tai I for -800, Bsu 361 for -509, Not I for codon 10 and Bgl I for codon 25. All enzymes were purchased from New England Biolabs, USA. The products were fractionated in 2% polyacrylamide gels and visualised by ethidium bromide and ultraviolet light.

Statistical analysis. For each SNP we compared the distribution of its allele frequencies between patients and control subjects using a single global Pearson's chi-squared test. The STATA 7.0 (STATA Corporation, Texas) statistical analysis program was used to calculate p values and odds ratios.

Results

Four known SNPs, two in the promoter region and two in exon 1, were genotyped using the PCR-RFLP method. The minor differences between the numbers of patients and control subjects for different SNPs were due to technical problems in genotyping some of the samples. The genotype distributions in both groups were in Hardy-Weinberg equilibrium for all SNPs examined. Allele and genotype frequencies of all four SNPs were compared using chi-squared analysis (Table II). The frequency of the genotypes

($p = 0.293$) and alleles ($p = 0.455$) for codon 10 polymorphism was similar for patients and controls as were the frequency of the genotypes ($p = 0.281$) and alleles ($p = 0.687$) for codon 25 polymorphism. The genotype frequency ($p = 0.498$) and allele frequency ($p = 0.597$) for -800 polymorphism and genotype frequency ($p = 0.573$) and allele frequency ($p = 0.357$) for -509 polymorphism were both similar for patients and controls.

Codon 10 and -509 SNPs in TGF-β1 gene show a similar allele frequency of approximately 65% and 35% for both patients and controls. By comparison, frequency of alleles of approximately 90% and 10% were observed in TGF-β1 gene SNPs at codon 25 and -800 for both patients and controls. These ratios are of interest in determining the appropriate sample sizes for such studies. The genotype and allele frequencies of all TGF-β1 gene SNPs examined were not significantly different ($p > 0.05$ for both genotype and allele frequency) between patients and controls.

Discussion

Since its description by Dupuytren in 1833,[24] the exact pathogenesis of DD has remained an enigma. Various risk factors such as age, gender, smoking, diabetes, anticonvulsant medication, alcohol abuse, cirrhosis of the liver and manual labour have been implicated in its pathology.[25] The relevance of some of these factors has been questioned and none so far has been proven to be of significant value in understanding the pathology.[26]

There are, however, two elements in the aetiology of DD which stand out. One is its common occurrence in Caucasians and the other is its familial nature.[1] No genetic study has been carried out to identify a gene or genes involved in the pathogenesis. The identification of candidate loci in the development of DD is now possible using polymorphism association.

Of the growth factors which have been studied for a possible role in the development of DD, TGF-β appears to be the most likely candidate.[27] In view of the pathogenic role of TGF-β1 in the formation of Dupuytren's tissue which has been shown in numerous experiments,[6] the TGF-β1 gene was selected for the purpose of identifying the genetic regulation of this condition.

Our results show that there is no statistically significant association between the development of DD in Caucasian patients for known polymorphisms of TGF-β1. In our investigation we only undertook genotypic analysis in patients with the disease. Patients suffering from other fibrotic conditions of the skin, such as scleroderma and keloid scarring were excluded.

There are a number of possible explanations for the observed lack of association between DD and the polymorphisms investigated in our study. A difference may have been present, but undetectable because of the sample size. Larger numbers may have to be studied. For an SNP with allele frequencies of approximately 65%:35% in the population, to detect an odds ratio of 2.0 as being significant at the 5% level with 80% power, would require a sample size of approximately 142 individuals. These approximate percentages do not represent averages of allele frequencies derived in our study. These values serve only as guides for calculating sample sizes. Codon 10 and -509 SNPs of TGF-β1 gene have an approximately similar allele frequency in both patients and controls. Therefore, it would seem that our sample size is adequate to detect an association of that strength. By contrast, allele frequencies of approximately 90%:10% for any SNP in the general population would require a sample size of 283 to detect an odds ratio of 2.0 with an 80% power and p value of 0.05. Codon 25 and -800 SNPs in the TGF-β1 gene have an approximately similar frequency of alleles. It is therefore possible that our present sample size would need to be increased to demonstrate any significant association.

Another possibility is that an association does not exist with the SNPs examined in this study, but is present in other as yet unidentified SNPs in the TGF-β1 gene. It may be necessary to identify new SNPs within the TGF-β1 gene. The common SNPs used in our experiments have been identified as lying within a specific region of the gene, between position -1321 and +966 relative to the first major transcription start site.[20] It is possible that unknown TGF-β1 polymorphisms located outside this region may be associated with DD. The technique used by previous groups[18,20] for the detection and characterisation of mutations (SNPs) within the TFG-β1 gene has been the single-stranded conformational polymorphism method. This technique is not 100% sensitive. Better techniques for detecting mutations are emerging, such as denaturing high-performance liquid chromatography using transgenomic wave nucleic acid fragment analysis. Furthermore, the association of TGF-β1 in the pathogenesis of DD described by a number of previous authors may result from polymorphisms in other members of the TGF-β regulatory system such as other superfamily members.

Presently, we are increasing our sample size and looking for new polymorphisms in the TGF-β1 gene and other members of the TGF-β signalling system. The detailed genetic basis of DD is essential to provide prognostic and diagnostic advice to patients and to develop new regimes of treatment.

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References

1. **Burge P.** Genetics of Dupuytren's disease. *Hand Clin* 1999;15: 63-72.
2. **Hueston JT.** Dupuytren's contracture. In: Jupiter JB, ed. *Flynn's hand surgery,* 4th Edn. Williams & Wilkins, Baltimore, 1991:864-89.
3. **Hunter JAA, Ogden C, Norris MG.** Dupuytren's contracture: I--Chemical pathology. *Br J Plast Surg* 1975;28:10-8.
4. **Ling RSM.** The genetic factors in Dupuytren's disease. *J Bone Joint Surg [Br]* 1963;45-B:709-18.

5. **Gabbiani G, Majno G.** Dupuytren's contracture fibroblast contraction? *Am J Pathol* 1972;66:131-46.
6. **Tomasek JJ, Vaughan MB, Haaksma CJ.** Cellular structure and biology of Dupuytren's disease. *Hand Clin* 1999;15:21-34.
7. **Iwasaki H, Muller H, Stutte HJ, Brennscheidt U.** Palmar fibromatosis (Dupuytren's contracture): ultrastructural and enzyme histochemical. *Virchows Arch A Pathol Anat Histopathol* 1984;405:41-53.
8. **Baird KS, Crossan F, Ralston SH.** Abnormal growth factor and cytokine expression in Dupuytren's contracture. *J Clin Pathol* 1993;46:425-8.
9. **Alioto RJ, Rosier RN, Burton RI, Puzas JE.** Comparative effects of growth factors on fibroblasts of Dupuytren's tissue and normal palmar fascia. *J Hand Surg [Am]* 1994;19-A:442-52.
10. **Badalamente MA, Sampson SP, Hurst LC, Dowd A, Miyasaka K.** The role of transforming growth factor beta in Dupuytren's disease. *J Hand Surg [Am]* 1996;21:210-5.
11. **Berndt A, Kosmeh H, Mandel U, et al.** TGF beta and bFGF synthesis and localization in Dupuytren's disease (nodular palmar fibromatosis) relative to cellular activity, myofibroblast phenotype and oncofetal variants of fibronectin. *Histochem J* 1995;27:1014-20.
12. **Meek RM, McLellan S, Crossan JF.** Dupuytren's disease: a model for the mechanism of fibrosis and its modulation by steroids. *J Bone Joint Surg [Br]* 1999;81-B:732-8.
13. **Kloen P, Jennings CL, Gebhardt MC, Springfield DS, Mankin HJ.** Transforming growth factor-beta: possible roles in Dupuytren's contracture. *J Hand Surg [Am]* 1995;20-A:101-8.
14. **Roberts AB, Sporn MB.** Transforming growth factor beta. *Adv Cancer Res* 1988;51:107-45.
15. **Border WA, Noble NA.** Transforming growth factor beta in tissue fibrosis. *N Engl J Med* 1994;331:1286-92.
16. **Derynck R, Jarrett JA, Chen EY, et al.** Human transforming growth factor-beta complementary DNA sequence and expression in normal and transformed cells. *Nature* 1985;316:701-5.
17. **Li B, Khanna A, Sharma V, et al.** TGF-beta 1 DNA polymorphisms, protein levels, and blood pressure. *Hypertension* 1999;33:271-5.
18. **Cambien F, Ricard S, Troesch A, et al.** Polymorphisms of the transforming growth factor-beta 1 gene in relation to myocardial infarction and blood pressure: The Etude Cas-Temoin de l'Infarctus due Myocarde (ECTIM) Study. *Hypertension* 1996;28:881-7.
19. **Anscher MS, Peters WP, Reisenbichler H, Petros WP, Jirtle RL.** Transforming growth factor beta as a predictor of liver and lung fibrosis after autologous bone marrow transplantation for advanced breast cancer. *N Engl J Med* 1993;328:1592-8.
20. **Awad MR, El-Gamel A, Hasleton P, et al.** Genotypic variation in the transforming growth factor-beta1 gene: association with transforming growth factor-beta1 production, fibrotic lung disease, and graft fibrosis after lung transplantation. *Transplantation* 1998;66:1014-20.
21. **El-Gamel A, Awad M, Sim E, et al.** Transforming growth factor beta 1 and lung allograft fibrosis. *Eur J Cardiothorac Surg* 1998;13:424-30.
22. **Desmouliere A, Geinoz A, Gabbiani F, Gabbiani G.** Transforming growth factor-beta 1 induces alpha-smooth muscle actin expression in granulation tissue myofibroblasts and in quiescent and growing cultured fibroblasts. *J Cell Biol* 1993;122:103-11.
23. **Wood NA, Thomson SC, Smith RM, Bidwell JL.** Identification of human TGF-beta 1 signal (leader) sequence polymorphisms by PCR-RFLP. *J Immunol Methods* 2000;234:117-22.
24. **Dupuytren G.** Permanent retraction of the fingers, produced by an affection of the palmar fascia. *Lancet* 1833;4:ii:222-5.
25. **Legge JW.** Dupuytren's disease. *Surg Annu* 1985;17:355-68.
26. **Yi IS, Johnson G, Moneim MS.** Etiology of Dupuytren's disease. *Hand Clin* 1999;15:43-52.
27. **Kloen P.** New insights in the development of Dupuytren's contracture: a review. *Br J Plast Surg* 1999;52:629-35.