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Genetic susceptibility to Dupuytren's disease: transforming growth factor beta receptor (TGFβR) gene polymorphisms and Dupuytren's disease☆

A. Bayat[a,c,d,*], J.K. Stanley[b], J.S. Watson[a], M.W.J. Ferguson[c], W.E.R. Ollier[d]

[a]*Hand Surgery Units, Wythenshawe Hospital, South Moor Road, Wythenshawe, Manchester M23 9LT, UK*
[b]*Hand Surgery Units, Wrightington Hospital, Wigan, Lancashire WN6 9EP, UK*
[c]*Division of Cells, Immunology and Development, School of Biological Sciences, University of Manchester, Manchester M13 9PT, UK*
[d]*Centre for Integrated Genomic Medical Research, University of Manchester, Stopford Building, Oxford Road, Manchester M13 9PT, UK*

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Summary Dupuytren's disease (DD) is a benign fibroproliferative disease of unknown cause. It is a familial condition that commonly affects Caucasians. Genetic studies have yet to identify the genes involved in DD. Transforming growth factor beta (TGFβ) family members are multifunctional; some play a central role in wound healing and fibrosis. Previous studies have implicated TGFβ cytokines and receptors in DD. In the light of this evidence, TGFβ receptors represent candidate susceptibility genes for this condition. In this study, we investigated the association of single nucleotide polymorphisms (SNPs) in TGFβ receptors one, two and three (TGFβRI, RII and RIII) with the risk of DD formation. A polymerase chain reaction-restriction fragment length polymorphism method was used for genotyping novel and known TGFβ receptor polymorphisms. DNA samples from 183 DD patients and 181 controls were examined. There was a statistically significant difference ($p < 0.05$) in genotype frequency distributions between cases and controls for TGFβRI polymorphisms in the recessive model. However, there were no significant difference in genotype or allele frequency distributions between cases and controls for the TGFβRII and TGFβRIII SNPs.

Dupuytren's disease (DD) is a nodular palmar fibromatosis causing progressive and irreversible contracture of the digits. DD is often familial and extremely common in Caucasians of Northern European ancestry.[1] A number of inheritance patterns including autosomal recessive have been proposed as the likely mode of inheritance.[1]

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*Corresponding author. Address: Centre for Integrated Genomic Medical Research, University of Manchester, Stopford Building, Oxford Road, Manchester M13 9PT, UK. Tel.: +44-794-009-0176; fax: +44-161-275-5043.
E-mail address: ardeshir.bayat@man.ac.uk

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Although no single gene has so far been identified, DD may have a complex oligogenic (conditions arising from a combination of environmental and multiple genetic factors) aetiology. Oligogenic disorders result from the combined action of alleles of more than one gene such as diabetes, and some cancers. The inheritance pattern of such disorders is usually complex when compared to monogenic disorders and depends on the simultaneous presence of multiple alleles.

The myofibroblast has been demonstrated in many studies as a key cell responsible for the tissue contraction in DD.[2,3] Transforming growth factor beta ($TGF\beta_1$) plays an important role in myofibroblast differentiation during wound healing and in fibrocontractive diseases including DD.[3] Iwasaki and colleagues[4] studied the histopathological changes in Dupuytren's tissue and concluded that growth factors induce proliferation of genetically abnormal myofibroblasts. TGFβ family members such as TGFβ1 and TGFβ2 have been implicated as growth factors involved in the pathogenesis of DD.[5–9]

TGFβ family cytokines regulate cell proliferation, migration, differentiation and play a key role in development, tissue turnover and repair.[10] There are three mammalian TGFβ isoforms (TGFβ1, TGFβ2 and TGFβ3) which have structural and functional similarities and mediate their effect by acting on a group of serine/threonine kinase membrane receptors.[10] TGFβ receptors are termed TGFβRI, TGFβRII and TGFβRIII and located on different chromosomes (9q22, 3p22 and 1p32, respectively). TGFβRI primarily functions as an intracellular signal transducer, whereas TGFβRII is required for ligand binding.[11] The functional receptor is a heterodimer consisting of two units of TGFβRI and two units of TGFβRII. TGFβRIII is a glycoprotein that binds TGFβ and serves both to present the ligand to the receptor and as a receptor accessory molecule.[12] All three TGFβ receptors are present in Dupuytren's tissue.[9] TGFβ receptors are expressed in varying levels in DD tissue.[9] Furthermore, myofibroblasts found in granulation tissue show strong expression of TGFβRI and RII compared to normal skin.[13] RI- and RII-overexpressing fibroblasts were found in high densities in post-excisional wounding,[14] and in hypertrophic scars up to 20 months after burn injury.[13] Fibrosis may be associated with a failure to eliminate TGFβ receptor-overexpressing fibroblasts during repair, leading to a persistent autocrine positive feedback loop that results in over-production of matrix proteins and subsequent fibrosis.[13]

In the light of the above findings, TGFβ receptors represent candidate susceptibility genes for this condition. Different mutations of all three TGFβ receptors have been demonstrated in a number of conditions, such as TGFβRI and RII in neoplastic conditions such as gastric and colon cancer and RIII in hereditary haemorrhagic telangiectasia.[15] TGFβRI gene polymorphism has also been shown in other fibrotic disorders such as systemic sclerosis.[16] In the present study, we tested the hypothesis that there is an association between TGFβ receptor polymorphisms and DD. We examined TGFβ receptor polymorphisms in a cohort of UK Caucasian DD patients.

Patients and methods

Dupuytren's patients ($n = 184$) were entered into the study. One hundred and fifty-one men with an age range of 35–85 years and a mean age of 63.4 years (SD = 10.5) and 33 women with an age range of 46–90 years and a mean age of 63.8 years (SD = 10.4) were entered into the study. Cases were all Caucasians from the Northwest region of England, UK. Successive Dupuytren's cases were identified through operative record clinical codes from the South Manchester University Hospital and Wrightington Hospital in the Northwest region. All cases had a confirmed diagnosis of advanced DD pre-operatively with the presence of characteristic Dupuytren's nodules in the palm of the hand and/or digits with contracture of either the metacarpophalangeal joint (MCPJ) or the proximal inter phalangeal joint (PIPJ). Early stage DD with nodules only and atypical cases of DD post injury were excluded from the study.

Controls ($n = 181$) were ethnically matched, healthy Caucasian men and women were selected from general practice registers. All controls were seen by the first author who took a full medical history using a proforma and examined both hands and feet of each individual to exclude the presence of DD in the control population. There were 150 men with a mean age of 57.8 years (SD = 12.9) and an age range of 31–76 years. Thirty-one women were recruited with a mean age of 56.5 years (SD = 11.0), and an age range of 34–75 years. The local and hospital ethical committees gave approval for the study protocol and proformas. Written consent was obtained from all individuals.

DNA extraction

Venous blood (15 ml) were collected from every subject. DNA was extracted from peripheral blood cells using a DNA extraction kit (Qiagen, UK). DNA

concentrations were measured and diluted to 100 ng/μl using sterile buffer.

Genotyping

In order to identify relevant single nucleotide polymorphisms (SNPs) for our candidate genes of interest (TGFβ receptor genes) a search was performed of the publicly available databases. Polymorphisms in TGFβRI and TGFβRII genes were identified from the National Centre for Bioinformatics (NCBI) SNP consortium database (http://www.ncbi.nlm.nih.gov/SNP/index.html). A novel polymorphism in TGFβRIII was also identified by comparing the available TGFβRIII gene sequence alignments found in the databases located in the 3′ untranslated region (3′-UTR) of the TGFβRIII gene at position 1068 from the transcription initiation site (Genbank accession no. L07594). The presence of the TGFβRI and RIII SNPs were confirmed by DNA sequencing the polymerase chain reaction (PCR) products. SNPs chosen for these studies were named according to their relative position on the TGFβ receptor genes; TGFβRI (Genbank accession ID 7046); TGFβRII (Genbank accession D50683). TGFβ receptors polymorphisms were typed using the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method.[17] The sequences of the primers used and the product fragment size are given in Table 1.

PCRs were carried out in 96-well plates. Each PCR consisted of 1 μl of DNA (100 ng/μl), 2.5 μl of ×1 NH_4 buffer (Bioline), 2.5 μl of each 200 mM d'NTP (Behring), 0.1 μl of 0.5 unit Taq polymerase (Bioline), 1 μl $MgCl_2$ at either 1 mM, 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 1.

PCR was carried out under the following conditions:

Two minutes of denaturation at 95 °C; followed by 39 cycles of further denaturation of 45 s at 95 °C; 1 min at either annealing temperature (TGFβRI-3′ at 72 °C; TGFβRII-3′ UTR at 56 °C; TGFβRII-e5 at 59 °C and TGFβRIII-3′ at 59 °C) followed by 45 s of extension at 72 °C; a final elongation step of 5 min at 72 °C was also included. Amplified DNA (5 μl) was digested with the appropriate enzyme including buffer and made up to a 10 μl reaction mixture using distilled water. Digestions were carried out overnight in a Hybaid Omnigene thermal cycler. The enzymes were as follows, BsrI for TGFβRI, BsmI and BfaI for TGFβRII and BfaI for TGFβRIII. Enzymes were all purchased from New England Biolabs. The digested products were fractionated in 4% polyacrylamide gels and visualised by ethidium bromide and ultraviolet light.

Statistical analysis

For each SNP, association with DD was investigated by comparing the distribution of genotype frequencies of DD cases with controls using a chi-square test. STATA 7 statistical analysis programme was used to calculate *p* values and odds ratios for risk associated with individual genotypes under a risk model where the risk allele is considered as either co-dominant, dominant or recessive and the *p* values and odds ratios were calculated using a chi-square test.

Table 1 SNP position, primer sequences and product fragment sizes used for the detection of the four TGFβRI,-RIIR gene polymorphisms

Gene	SNP position	Primers	Product fragment sizes
TGFβRI-3′UTR	9q22 3′-UTR A/C	PF-: 5′-CTTGTAAGCCAAGTTTTCACCC-3′ PR: 5′-CTCCACATGCTTAGGGGTGT-3′	219 bp (96 + 123) T-219 G-96, 123
TGFβRII-3′UTR	3p22 3′-UTR C/G	PF-: 5′-TCTCACTTCTGGGTTATCAGCA-3′ PR: 5′-CATGAGTACAGCTGAAGTGTTCC-3′	201 bp (57 + 144) C-201 G-57, 144
TGFβRII-e5	3p22 Coding-e5 C/T	PF-: 5′-TGATGGCCTCACTGTCTGTT-3′ PR: 5′-ACCTCCCACTGCATTACAGC-3′	174 bp (117 + 57) C-117,57 T-174
TGFβRIII-3′UTR	1p32 3′-UTR G/C-1068	PF-: 5′-TGCATACACCACTGCAAAATG-3′ PR: 5′-TCTTGGTGGAATTGGTGACA-3′	456 bp (76 + 380) C-380,76 G-456

Table 2 TGFβRI genotypes and allele frequencies

	DD cases ($n = 183$)	Control ($n = 181$)
Allele frequency		
1 (A)	259 (71%)	271 (75%)
2 (C)	107 (29%)	91 (25%)
Genotype frequency		
1 (A/A)	98 (54%)	101 (53%)
2 (A/C)	63 (34%)	69 (38%)
3 (C/C)	22 (12%)	11 (6%)

Results

Three TGFβR SNPs in the 3′ UTR and one in exon 5 of TGFβRII were genotyped. TGFβRII SNPs were found to be extremely rare and we were unable to detect either the 3′-UTR or the exon 5 SNPs in any of our cases and controls. Therefore only data for SNP genotyping in TGFβRI and TGFβRIII are presented (Tables 2 and 3). The genotype distributions in both cases and controls for both TGFβRI and TGFβRIII SNPs were examined and were found to be in Hardy–Weinberg equilibrium (HWE).

Genotype frequencies of both SNPs were compared using chi-square analysis (Tables 4 and 5). Using a risk model in the statistical analysis of the data, the frequency of the genotypes for TGFβRI polymorphism between DD cases and controls was statistically significant ($p = 0.048$) in the recessive model. The co-dominant ($p = 0.064$ and 0.787) and dominant ($p = 0.66$) genotypes were not statistically significant. Presence of the C allele vs. the A allele is associated with an increased risk of developing DD (odds ratio = 2.11 and 95% CI = 0.94–4.97).

The frequency of the genotypes for TGFβRIII polymorphism was similar between DD cases and controls in all risk models. The co-dominant ($p = 0.346$ and 0.291), dominant ($p = 0.577$) and the recessive ($p = 0.21$) genotypes, did not demonstrate a statistically significant difference.

Table 3 TGFβRIII genotypes and allele frequencies

	DD cases ($n = 167$)	Control ($n = 177$)
Allele frequency		
1 (C)	236 (71%)	252 (71%)
2 (G)	98 (29%)	102 (29%)
Genotype frequency		
1 (C/C)	89 (53%)	89 (50%)
2 (G/C)	58 (35%)	74 (42%)
3 (G/G)	20 (12%)	14 (8%)

Table 4 TGFβRI genotype table

TGFβRI		OR	95% CI	p
Co-dominant	C/C vs. A/A	2.06	0.89–4.96	0.064
	A/C vs. A/A	0.94	0.59–1.49	0.787
Dominant	C/C & A/C vs. A/A	1.09	0.71–1.69	0.66
Recessive	C/C vs. A/C & A/A	2.11	0.94–4.97	0.048

Discussion

The aetiopathogenesis of DD remains an enigma. The relevance of some of the various implicated aetiologic factors such as age, sex, alcohol abuse, cirrhosis of the liver, smoking, diabetes, anticonvulsant medication in DD formation has been questioned.[18] However, two elements in the aetiology of DD stand out clearly. One is the familial nature of the disease and the other is that DD is an extremely common disorder that mainly affects Caucasians of northern European ancestry.[1,19] However, genetic studies have yet to detect a gene or genes involved in DD. Using a polymorphism case control association study approach, it is now possible to attempt to test candidate loci that may be involved in DD pathogenesis.

In view of the pathogenic role attributed to TGFβ cytokines and receptors in DD tissue demonstrated previously,[20] the TGFβR genes were selected as ideal candidate genes for the purpose of identifying the genetic regulation of this condition. Previously we had investigated the association between common $TGF\beta_1$ and $TGF\beta_2$ polymorphisms and DD. Our studies demonstrated that there was no statistically significant association between DD and either of these common polymorphic genes.[21,22] The next logical step following investigating $TGF\beta_{1-2}$ isoforms was to consider the receptors involved in the TGFβ pathway.

In our search for candidate SNPs in the TGFβ receptors, we were unable to detect any useful common polymorphisms in the TGFβRII gene. However, we identified two informative SNP markers in the 3′-UTR (untranslated region) of TGFβRI and RIII. The significance of the identification of a positive association with a SNP in the 3′-UTR of a gene can be interpreted by the fact that an

Table 5 TGFβRIII genotype table

TGFβRIII		OR	95% CI	p
Co-dominant	G/G vs. C/C	1.43	0.64–3.26	0.346
	C/G vs. C/C	0.78	0.49–1.26	0.291
Dominant	G/G & C/G vs. C/C	0.89	0.57–1.38	0.577
Recessive	G/G vs. C/G & C/C	1.58	0.73–3.52	0.21

alteration in this region of a gene can have an affect on mRNA stability and processing.[23]

The PCR-RFLP genotypic analysis in this study was performed on clinically confirmed advanced DD cases only. Early DD patients with nodules only and atypical DD cases were excluded from our patient group. The sample size calculations in this study were based on frequency of the allele of interest in the control population, to achieve a strength of 80% and a 0.05 significance level to achieve an odds ratio of 2. This is a commonly chosen value for odds ratios in case control-association studies for the purpose of complex genetic disease analysis.

Our results demonstrated that there is a significant result for the recessive model ($p = 0.048$) in the TGFβRI SNP. A recessive influence is observed in this cohort of DD cases, as is evidenced by the significant finding in C/C vs. A/C and A/A alleles. Presence of the C allele versus the A allele is associated with an increased risk of developing DD (odds ratio = 2.11 and 95% CI = 0.94–4.97). There was, however, no difference in disease severity with the different genotypes as all our cases were advanced stages of DD with the presence of both Dupuytren's nodules and contracture of the digits.

This result is interesting as there have been no previous positive case–control association studies performed in DD. Nevertheless, in view of its marginally significant value, there would be a need for another separate cohort of DD cases to be tested for this particular polymorphism to further validate this positive data. Following a statistical confirmation of this finding using another set of DD cases and controls, it would be important to further test the functional significance of this finding. It may be that the TGFβRI SNP is not itself the disease causing gene but in linkage disequilibrium with the actual disease causing gene.

In case–control association studies, SNPs are generally used to map potentially functional domains of candidate genes. It is assumed in these studies that a disease-causing variant is one of the SNPs tested, thus an association will be detected. However, if a candidate disease causing gene polymorphism has not yet been identified, SNPs can be used to investigate association indirectly. The strategy here is that the chosen SNP is a potential marker of the disease-causing gene of interest and lies very close to this gene, and as a result they will be inherited together. Therefore if one allele of a marker is always associated with the true disease allele then it will also show association to the disease and the marker is said to be in linkage disequilibrium with the disease gene.

DD appears to be a complex oligogenic rather than a monogenic condition that segregates into multiple modes of inheritance.[1] These findings do not necessarily exclude direct or indirect involvement of other relevant gene polymorphisms in the formation and progression of DD. It is therefore important to continue to look for other novel polymorphisms in the transforming growth factor beta signalling system as well as other candidate gene families for significant association with DD. Understanding the genetic basis of DD is important for developing novel diagnostic and therapeutic regimes for a more efficacious treatment in the future.

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Glossary of Genetic Terms

Allele One of several alternative forms of a DNA sequence at a specific chromosomal location.

Association The simultaneous occurrence of two characteristics (e.g. disease and a particular allele of a gene at a frequency greater than would be predicted by chance.

Case-control studies Allele frequencies compared between cases and unrelated controls.

Hardy-Weinburg equilibrium Populations that conform to Hardy–Weinburg law are in a nonevolving state called the Hardy–Weinburg equilibrium.

Hardy-Weinburg law Under certain conditions, the frequencies of genotypes and alleles in a population of sexually reproducing organisms will remain constant over time.

Linkage disequilibrium Alleles at two or more loci occurring together more frequently than would be expected by chance alone.

Oligogenic Determined by a small number of genes acting together.

Polymerase chain reaction (PCR) Allows a fast and selective amplification of specific regions of DNA. The reaction involves repeated cycles of three stages of denaturation, annealing and extension.

Polygenic disease Diseases which aggregate in families but do not segregate in a Mendelian fashion.

Polymorphism Variation in the DNA sequence at a particular chromosomal location.

Restriction fragment polymorphism (RFLP) Digestion of DNA containing a chosen site with a specific restriction enzyme could distinguish alleles based on fragment sizes by electrophoresis.

SNPs Single nucleotide polymorphisms, single base changes in a DNA sequence.