Common Variants of the *EPDR1* Gene and the Risk of Dupuytren's Disease

Häufige Varianten des *EPDR1*-Gens und das Risiko für das Auftreten eines Morbus Dupuytren

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Key words
- M. Dupuytren
- genetic disposition
- ependymin-related gene 1

Schlüsselwörter
- M. Dupuytren
- genetische Disposition
- Ependymin-related-Gen 1

Abstract

The object of this study was the investigation of 3 common variants of single nucleotide polymorphisms of the ependymin-related gene 1 and its association with the occurrence of Dupuytren's disease. DNA samples were obtained from the peripheral blood of 508 consecutive patients. The control group comprised 515 healthy adults who were age-matched with the Dupuytren's patients. 3 common variants were analysed using TaqMan® genotyping assays and sequencing. The differences in the frequencies of variants of single nucleotide polymorphisms in patients and the control group were statistically tested. Additionally, haplotype frequency and linkage disequilibrium were analysed for these variants. A statistically significant association was noted between *rs16879765_CT*, *rs16879765_TT* and *rs13240429_AA* variants and Dupuytren's disease. 2 haplotypes: *rs2722280_C+rs13240429_A+rs16879765_C* and *rs2722280_C+rs13240429_G+rs16879765_T* were found to be statistically significantly associated with Dupuytren's disease. Moreover, we found that *rs13240429* and *rs16879765* variants were in strong linkage disequilibrium, while *rs2722280* was only in moderate linkage disequilibrium. No significant differences were found in the frequencies of the variants of the gene between the groups with a positive and negative familial history of Dupuytren's disease. In conclusion, results of this study suggest that *EPDR1* gene can be added to a growing list of genes associated with Dupuytren's disease development.

Zusammenfassung

Die vorliegende Studie untersuchte den Zusammenhang zwischen 3 häufigen Varianten des Ependymin-related-Gens 1 und dem Auftreten eines Morbus Dupuytren. DNA-Proben wurden aus dem peripheren Blut von 508 konsekutiven Patienten gewonnen. Die Kontrollgruppe umfasste 515 gesunde Erwachsene mit ähnlicher Altersverteilung wie die Patientengruppe. 3 häufige Varianten wurden mithilfe des TaqMan® Genotypisierungsassays und Sequenzierung analysiert. Patienten- und Kontrollgruppe wurden statistisch in Bezug auf die Häufigkeit des Auftretens der Varianten des Ependymin-related-Gen 1 miteinander verglichen. Außerdem wurden die Häufigkeiten der Haplotypen sowie die Kopplungsungleichgewichte für diese Varianten analysiert. Ein statistisch signifikanter Zusammenhang mit dem Auftreten eines Morbus Dupuytren wurde für die Varianten rs16879765_CT, rs16879765_TT und rs13240429_AA gefunden. 2 Haplotypen, nämlich rs2722280_C+rs13240429_A+rs16879765_C und s2722280_C+rs13240429_G+rs16879765_T, wiesen einen statistisch signifikanten Zusammenhang mit dem Auftreten eines Morbus Dupuytren auf. Des Weiteren wurde für die Varianten rs13240429 und rs16879765 ein starkes Kopplungsungleichgewicht gefunden, während für rs2722280 lediglich ein moderates Kopplungsungleichgewicht vorlag. Keine signifikanten Unterschiede im Auftreten der Genvarianten ergaben sich beim Vergleich von Patienten mit positiver und negativer Dupuytren-Familienanamnese. Zusammengefasst legen die Ergebnisse der Studie nahe, dass das *EPDR1*-Gen der wachsenden Liste von Genen hinzugefügt werden kann, welche mit dem Auftreten von Morbus Dupuytren assoziiert sind.

received 10.6.2013
accepted 6.9.2013

Bibliography
DOI http://dx.doi.org/10.1055/s-0033-1357119
Handchir Mikrochir Plast Chir 2013; 45: 253–257

ISSN 0722-1819

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Introduction

There is some evidence suggesting that ependymin-related gene 1 (*EPDR1*), also known as secreted frizzled-related protein gene 4 (*SFRP4*) may be considered a potential Dupuytren's disease (DD) susceptibility gene [1]. The gene is situated at 7p14.1 locus. Its protein product, an ependymin-related protein 1 (named also as secreted frizzled-related protein 4), appears to be a modulator of WNT-mediated signalling.

WNT Genes Family

The WNT family genes encode extracellular signalling proteins that influence gene expression leading to changes in cell proliferation and survival [1,2]. WNT signalling is reported to regulate the proliferation and differentiation of fibroblasts in fibromatosis and neoplasms [3]. Abnormal WNT signalling is associated with development of some diseases, particularly neoplasms. The model WNT signalling pathway is the canonical pathway, which activates the nuclear function of β-catenin, leading to changes in gene expression, influencing cell proliferation and survival. Abnormal proliferation of fibroblats is a key feature in the early development of the DD. Increased levels of β-catenin have been observed in cell cultures in vitro, suggesting that the WNT signalling pathway may be up-regulated in Dupuytren's disease [4]. Altered levels of WNT-activated proteins can cause abnormal proliferation of fibroblasts in DD patients, which is one of the key features of early DD development [1,5].

Single Nucleotide Polymorphism (SNP)

Single nucleotide polymorphism is a DNA alteration (point mutation) in which a single nucleotide is deleted or replaced by another nucleotide within the coding sequences of genes, non-coding regions of genes or in the intergenic regions. Use of SNP arrays allows the interrogation of a large number of genes simultaneously and the preselection of candidate genes of interest. We used this method in our previous study [6]. A recent genome-wide association scan study of Dutch patients identified 8 SNPs at 3 loci that showed significant association with DD [1]. The strongest association was found for *rs16879765* within the *EPDR1* gene. To the best of our knowledge this study is the only one to investigate any association of a common variant of the *EPDR1* gene and DD.

Having collected genetic material from our previous study, we intended to investigate 3 single nucleotide polymorphisms of the *EPDR1* gene and their association with the occurrence of DD in a series of consecutive patients and controls from the authors' native population.

Haplotype Frequency and Linkage Disequilibrium

Recent studies suggest that the genome is organised into blocks of haplotypes. As opposed to haplotypes, the genotype gives the bases at each SNP for both copies of the chromosome, but loses the information as to the chromosome on which each base appears. Haplotypes are a combination of alleles at different markers along the same chromosome that are inherited as a unit. The fundamental difference between haplotypes and individual genotypes at SNPs is that the alleles are assigned to a chromosome. In essence, each individual has 2 haplotypes for a given stretch of the genome, representing the maternal and paternal chromosomes. Assignment of alleles to the chromosome (haplotypes) can be powerful because it yields information about recombination, which is important for locating disease-causing mutations. If a haplotype is associated with a certain disease, then examining stretches of DNA near the SNP cluster would lead to identifying the gene (or genes) responsible for this disease. Particular alleles at neighbouring loci tend to be co-inherited. For tightly linked loci, this might lead to associations between alleles in the population: a property known as linkage disequilibrium (LD). LD has recently become the focus of intense study in the hope that it might facilitate the mapping of complex disease loci through whole-genome association studies.

This was the motivation that prompted us to enrich our study by additional haplotype frequency and linkage disequilibrium analyses.

Patients and Methods

Patients and controls

Over a period of 5 years (2008–2012), 508 patients with Dupuytren's disease, 410 men (81%) and 98 women (19%) with a mean age of 57 years (range: 38–86) were recruited. 112 patients underwent surgery for DD and were invited to participate by mail, whereas 396 were recruited during their stay in the hospital for operation. The approval of the Bioethical Council of the local Medical University was obtained and informed consent was obtained from all subjects before enrolment. During an interview the goals of the study were explained, genetic counselling was given and a blood sample was taken for DNA analysis. The detailed family history and the duration of DD were recorded.

The control group comprised 515 healthy adults, 410 men and 105 women with a mean age of 55 years (range: 36–74) who were age matched (±2 years) with the DD patients. The healthy adults were assessed as having a negative family history for cancer after answering a questionnaire about their family medical history, which was part of a population-based study of the 1.5 million residents of West Pomerania province to identify familial aggregations of malignancies. A blood sample was taken for DNA analysis from all controls.

Methods

All 3 common variants of single nucleotide polymorphisms were analysed by real-time polymerase chain reaction (PCR), using the LightCycler480 (Roche, Rotkreutz, Switzerland). The analyses were carried out using TaqMan® genotyping assay (Life Technologies Corp, Foster City, CA, USA), consisting of sequence specific primers and oligonucleotide fluorescent labelled probes, which enabled amplification of the examined fragments and further allele discrimination. We investigated 3 common changes in *EPDR1* gene: intronic *rs16879765, rs13240429* (present at the exon1/intron1 boundary) and *rs2722280* (located in 3'UTR).

Statistical methods

The basic statistical analysis included comparison of the allele frequencies in DD patients and in controls. The disease risk associated with each factor was calculated using a logistic regression

model adjusted by sex and year of birth on an R software environment (version 2.15.0).

Over and above this we analysed the frequency of the variants in selected subgroups of DD patients comparing them to the similarly matched controls: (i) Age subgroups: ≤60 years (n=168) and >60 years (n=340). The age determinant of 60 years has been chosen since in our set of cases there were few patients with DD diagnosed before 50 years of age. (ii) Positive (n=128) and negative (n=255) DD familial history subgroups (familial history for the DD was recorded in 383 of 508 patients).

Estimation of haplotype frequencies and their potential association with the disease risk was performed using the haplo.stats CRAN package, version 1.6.3 by Sinnwell and Schaid for R [7]. Linkage disequilibrium (LD) between SNPs for a given haplotype was calculated using the software JLIN by Carter et al. [8]. All statistical analyses were performed using the R software environment, version 2.15.2.

Results

Logistic regression model

The assessed allele distributions for all analysed SNPs were in Hardy-Weinberg equilibrium for both the DD and control groups. The Hardy-Weinberg equilibrium model assumes that both allele/genotype frequencies in a population remain constant from generation to generation, unless specific disturbing factors are introduced. A study population is in Hardy-Weinberg equilibrium, if the occurrence of some combinations of alleles or genetic markers is of the same frequency as would be expected from a random formation of haplotypes from alleles based on their frequencies.

As shown in ○ **Table 1**, the frequencies of the *EPDR1* variants in the unselected DD group when compared to the respective control population revealed an association between *rs16879765_CT, rs16879765_TT* and *rs13240429_AA* and Dupuytren's disease. We found also statistically non-significant over-representation of minor allele of *rs 2722280_TT* among Dupuytren's patients (the term "minor allele" means an allele occurring less commonly in a given population).

The patient cohorts were further analysed in age subgroups (≤ 60 and >60 years) by comparing the frequency of the variants observed in this subgroups to matched controls. We found moderately increased prevalence of the risk alleles of *rs 13240429 and rs 16879765* among both early onset (≤ 60 years) patients and late-onset patients; the effect seems to be stronger among younger individuals (○ **Table 2**).

We found a non-significant tendency of *rs16879765_CT* overrepresentation among young heterozygous carriers. This may be explained by smaller numbers of young patients (n=168) when compared to late-onset cases (n=341). Both type 1 and 2 statistical errors cannot be excluded, the results need thus to be verified by examination of additional larger series of patients.

We compared also frequency of SNPs in DD patients having positive family history (n=128) with those with negative familial history (n=255). We failed to find any statistically significant differences in the allele distribution.

Table 1 Frequency of the examined SNPs in *EPDR1* gene among DD patients and controls. Statistically significant differences are in bold.

DD patients n=508	Controls n=515	Odds ratios and p-coefficients	
rs 2722280 n=502	**rs 2722280 n=515**	**p**	**OR**
CC 417 (83%)	CC 418 (81%)	baseline	baseline
CT 79 (16%)	CT 95 (18%)	0.73	0.35
TT 6 (1%)	TT 2 (0.4%)	0.15	1.44
rs 13240429 n=496	**rs 13240429 n=511**	**p**	**OR**
GG 272 (55%)	GG 289 56%)	baseline	baseline
AG 164 (33%)	AG 196 (38%)	0.52	0.65
AA 60 (12%)	**AA 26 (5%)**	**<0.0001**	**4.08**
rs 16879765 n=504	**rs 16879765 n=512**	**p**	**OR**
CC 310 (62%)	CC 378 (74%)	baseline	baseline
CT 163 (32%)	**CT 122 (24%)**	**0.0001**	**3.81**
TT 31 (6%)	**TT 12 (2%)**	**0.0006**	**3.41**

Haplotype frequency

Haplotype frequency analysis (○ **Table 3**) revealed 2 haplotypes significantly associated with DD: *rs2722280_C+ rs13240429_A+ rs16879765_C* (OR=1.6, 95% CI 1.25–2.06, $p=0.38$) and rs2722280_C+ *rs13240429_G+ rs16879765_T* (OR=2.2, 95% CI 1.67–2.95, $p<0.0001$).

Linkage disequilibrium

Linkage disequilibrium (LD) analysis revealed that *rs13240429* and *rs16879765* are in strong LD (D'=0.996), while *rs2722280*, (which is further apart) is only in moderate LD with the rest: D'=0.25–0.51. This means that the former 2 SNPs are strongly

Table 2 Frequency of the examined SNPs among DD patients and controls in age groups. Statistically significant differences are in bold.

DD patients <60 years n=168	Controls <60 years n=168	Odds ratio and p coefficient	DD patients >60 years n=340	Controls >60 years n=340	Odds ratio and p coefficient
rs 2722280	**rs 2722280**		**rs 2722280**	**rs 2722280**	
CC 128 (81%)	CC 136 (81%)	OR=1.0 p=0.99	CC 287 (84%)	CC 282 (81%)	OR=1.2 p=0.36
CT 28 (18%)	CT 30 (18%)	OR=1.0 p=0.97	CT 51 (15%)	CT 65 (19%)	OR=0.8 p=0.18
TT 2 (1%)	TT 2 (1%)	OR=1.1 p=0.95	TT 4 (1%)	TT 0	-
rs 13240429	**rs 3240429**		**rs 13240429**	**rs 13240429**	
GG 82 (53%)	GG 90 (56%)	OR=0.9 p=0.59	GG 190 (56%)	GG 199 (57%)	OR=1.0 p=0.83
AG 53 (34%)	AG 67 (41%)	OR=0.7 p=0.17	AG 109 (32%)	AG 129 (37%)	OR=0.8 p=0.19
AA 21 (13%)	**AA 5 (3%)**	**OR=4.9 p=0.0007**	**AA 39 (12%)**	**AA 21 (6%)**	**OR=2.0 p=0.01**
rs 16879765	**rs 6879765**		**rs 16879765**	**rs 16879765**	
CC 102 (63%)	CC 122 (74%)	OR=0.6 p=0.04	CC 206 (60%)	CC 256 (77%)	OR=0.5 p=0.0002
CT 47 (29%)	CT 40 (24%)	OR=1.3 p=0.31	CT 116 (34%)	CT 82 (24%)	OR=1.7 p=0.003
TT 12 (7%)	**TT 3 (2%)**	**OR=4.4 p=0.02**	**TT 19 (6%)**	**TT 9 (3%)**	**OR=2.2 p=0.048**

EPDR1 gene variants					
rs 2722280	rs 13240429	rs16879765	p coefficient	OR	95% CI
C	G	C	5.03×10^{-7}	reference	
T	G	C	0.39	1.10	0.69 - 1.75
T	A	C	0.79	1.33	0.79 - 2.21
C	A	T	0.38	0.90	0.44 - 1.83
C	A	C	**0.04**	**1.60**	**1.25 - 2.06***
C	G	T	**2.46×10^{-6}**	**2.22**	**1.67 - 1.95***

*Note that the list of haplotypes is not equivalent to the list of all theoretically possible haplotypes. Most of the latter were estimated by the software as too improbable (as there were too few cases with these haplotypes) to be considered in the analysis

Table 3 Haplotype frequency of examined *EPDR1* variants between DD patients and controls. Statistically significant differences are in bold.

associated to the rest of risk alleles for DD and tend to be co-inherited.

Discussion

Results of our logistic regression and haplotype analyses point at an association of *EPDR1* gene with Dupuytren's disease. Minor alleles of all 3 variants were more frequent among DD patients. The strongest, significant linkage was observed for *rs16879765* (OR = 3.4, $p = 0.0006$) and *rs13240429* (OR = 4.1, $p < 0.0001$). The *rs16879765* is an intronic variant located in the middle of intron 2. So far it is the only SNP of the *EPDR1* gene implicated in DD development, herein we replicate this finding of Dolmans et al. [1]. Our findings suggest that above-mentioned common variants result in down-regulation of the *EPDR1* gene in DD patients.

The 2 remaining variants, *rs13240429* (present at the exon1/intron 1 boundary) and *rs2722280* (located in 3'UTR of the gene) have not been studied up to now. For the third variant, due to very low frequency of the minor allele of *rs2722280* and limited numbers of cases and controls, we were unable to reach statistical power. The *rs13240429* and *rs16879765* are in a strong linkage disequilibrium. This means that they are co-inherited as one block associated with the disease risk.

Results of our study point at a stronger association of *EPDR1* germline variants with early onset (< 60 years) of disease. Such a finding is consistent with the 2-hit hypothesis by Knudson (1971) to explain the early onset of hereditary disease [9]. It is suggested that inheriting one germline copy of a damaged gene is the first hit. A second hit to the good copy in the gene occurs somatically earlier than 2 somatic changes.

Dolmans et al. (2011) investigated 35 SNPs most strongly associated with Dupuytren's disease. They found 9 different loci involved in susceptibility to the disease, of which 6 carried genes encoding proteins in the WNT signalling pathway [1]. These proteins such as WNT 2, WNT 4 and WNT 7b bind the frizzled receptors, leading to a cascade of events that eventually result in a decrease in the rate of β-catenin degradation. Secreted frizzled-related proteins such as SFRP4 (EPDR1), antagonise the WNT signalling pathway by binding to either WNTs or frizzled receptors, thereby affecting receptor occupancy. In the absence of active WNTs, β-catenin is degraded and potential target genes will not be activated. Another DD risk locus R-spondin family contains the *RSPO2* gene, encoding an R-spondin protein. The authors suggest that R-spondins interact with frizzled receptors and LRP5/6 to induce β-catenin signalling. Furthermore, R-spondins induce canonical WNT signalling by competing with the dickkopf (DKK) protein for binding to LPR5/6. The dickkopf protein is an inhibitor of WNT signalling. It hinders the formation of a complex among WNT, frizzled receptor and LRP5/6. The authors suggest that aberrations in this pathway (i.e., down-regulation of the *EPDR1* or *RSPO2* genes) may be responsible for the induction of fibromatosis and the further development of DD [1].

Ojwang et al. attempted to identify regions that may harbour Dupuytren's disease susceptibility genes by scanning the entire genome in a group of 40 DD patients and 40 controls. They used both log regression and mapping for analysis of single nucleotide polymorphism genotyping data. SNP analysis revealed a significant association with disease in regions for chromosomes 1, 3 through 6, 11, 16, 17, and 23 [10]. There are numerous candidate DD susceptibility genes present in these regions, which should be evaluated by next association studies [11]. Using our institutional register of DD patients we will continue to investigate this issue and, furthermore, the possible association of DD with common malignancies, such as digestive tract cancer, breast cancer and malignant melanoma.

In conclusion, the results of this study suggest that the *EPDR1* gene can be added to a growing list of genes associated with Dupuytren's disease development. There is a need to perform large multi-centre studies focused on identification of next candidate DD susceptibility genes.

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Age 42 years. Born in 1971 in Zielona Góra. Graduated in 1996 in Pomeranian Medical University in Szczecin, Poland. MD thesis 2001, habilitation 2008 in Pomeranian Medical University, Szczecin, Poland. Assistant Professor in Hereditary Cancer Division, Pomeranian Medical University, Szczecin. Since 2012 Professor in Department of Genetics and Patomorphology, Pomeranian Medical University, Szczecin. Author of 110 published papers in international journals, including prestige Nature Genetics, Cancer Research, Journal of National Cancer Institute, American Journal of Human Genetics, Journal of Medical Genetics, International Journal of Cancer. Main topic of scientific interest: clinical genetics, molecular backgrounds of cancers.

Acknowledgements

This research received grant from National Science Center, Republic of Poland [N N403 172839].

Conflict of interest: None

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