The VEGFA gene and anterior cruciate ligament rupture risk in the Caucasian population

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ABSTRACT: The aim of the present study was to analyse VEGFA rs699947, rs1570360, and rs2010963 polymorphisms with susceptibility to anterior cruciate ligament rupture (ACLR) in a Polish population. The study included 412 physically active Caucasian participants. The study group consisted of 222 individuals with surgically diagnosed primary ACLR qualified for ligament reconstruction (ACLR group). The control group consisted of 190 apparently healthy participants without any history of ACLR (CON group). Three polymorphisms within the VEGFA (rs699947, rs1570360, and rs2010963) gene were chosen for investigation due to their significance in the angiogenesis signalling pathway and previous associations with risk of ACLRs. Both single-locus and haplotype-based analyses were conducted. No significant differences in the allele and genotype frequency distributions were noted for the rs699947 and rs1570360 polymorphisms. In contrast, rs2010963 was associated with risk of ACLR in the codominant (p=0.047) and recessive model (p=0.017). In the latter, the CC genotype was overrepresented among individuals with ACL rupture (23.4% vs 14.2%, OR=1.85 [1.11-3.08]). Two VEGFA haplotypes were associated with ACLR under the additive (global score=11.39, p=0.022) and dominant model (global score=11.61, p=0.020). The [C;G;G] haplotype was underrepresented in the ACLR group (52.2% vs 70.4%) and the [C;C;C] haplotype was overrepresented (23.4% vs 14.2%, OR=1.85 [1.11-3.08]). The results obtained suggest a potential correlation between the VEGFA rs2010963 polymorphism and ACLR risk, suggesting that harbouring this specific C allele may be an unfavourable risk factor for a knee injury in Caucasian participants from Poland.

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INTRODUCTION

Angiogenesis is described as the formation of new capillary blood vessels from existing microvessels. It plays a key role in numerous biological processes such as wound healing, embryological development, inflammation, and the pathogenesis of various diseases, e.g. cancer, diabetic retinopathy, and rheumatoid arthritis [1]. Angiogenesis has also been implicated in matrix remodelling following mechanical loading. Its signalling pathway is regulated by numerous growth factors, such as vascular endothelial growth factors (VEGFs) [2]. Studies have shown increased levels of angiogenic cytokines and growth factors after mechanical loading in ruptured tendons and ligaments [3,4], and after cyclic stretching of tendon fibroblasts [5]. These results have suggested that the angiogenesis signalling cascade may be upregulated after loading, to promote matrix remodelling in an effort to maintain homeostasis [2]. However, dysregulation of this process may potentially have negative consequences for tissue capacity [6].

VEGFs, a family of important growth factors, are key signalling proteins involved in both physiological and pathological angiogenesis because they play a role in endothelial cell proliferation and migration. The family contains at least 7 members with a homodimeric structure. In particular, the A isoform, usually referred to as VEGF, shows the highest vasculogenic and angiogenic potency. VEGFA exerts its biologic effect through interaction with cell surface receptors, VEGFR1 and VEGFR2, selectively expressed on vascular
endothelial cells. The protein is encoded by the VEGFA gene, which is located on chromosome 6 (6p21.1) and consists of 8 exons [7,8]. At least 30 single nucleotide polymorphisms (SNPs) in this gene have been described [9]. The gene variants may lead to differences in VEGFA expression between individuals and may influence the etiology of a variety of pathological conditions with which VEGFA has been associated [10].

Among genetic variants within the VEGFA gene which have been described in the context of genetic conditioning for angiogenesis, rs699947 (C/A), rs1570360 (G/A), and rs2010963 (G/C) are the most frequently investigated functional polymorphisms associated with a potential predisposition to anterior cruciate ligament rupture (ACLR) and Achilles tendinopathy (TEN). These associations have been recently replicated in some populations. They were recently implicated with risk of ACLR in a South African Caucasian population [11], a South African Coloured population [2], and also risk of TEN in a study investigating two populations from South Africa and the United Kingdom [12]. Unfortunately, there are currently no data available for the incidence rate of ACLR in an East-European population. It needs to be highlighted that investigation of genetic associations in various populations and related phenotypes to ascertain the important biological pathways underpinning susceptibility to musculoskeletal soft tissue injuries is necessary [13].

Taken together, the aforementioned findings suggest that VEGFA polymorphisms are associated with risk of ACLR in various populations. The aim of the present study was to analyse VEGFA rs699947, rs1570360, and rs2010963 polymorphisms with susceptibility to ACLRs in a Polish population.

**MATERIALS AND METHODS**

**Ethics committee**

The procedures followed in the study were conducted ethically according to the principles of the World Medical Association Declaration of Helsinki and were approved by the Ethics Committee of the Pomeranian Medical University in Szczecin (approval number 09/KB/IV/2011).

**Participants**

A total of 412 physically active, unrelated, self-reported Caucasian participants were recruited for the study between 2009 and 2016. The study group consisted of 222 (66 females and 156 males) individuals with surgically diagnosed primary ACLR who qualified for ligament reconstruction (ACLR group). All 222 participants from the ACLR group sustained their injury through non-contact mechanisms [14]. The control group consisted of 190 (83 females and 107 males) apparently healthy participants without any history of ACLR (CON group).

The ACLR participants were soccer players from the Polish 1st, 2nd, and 3rd division soccer league (training 11-14 hours per week). The control group comprised healthy, physically active individuals with the majority playing soccer as their main sport, who self-reported no history of ligament or tendon injury. All the male participants (ACLR and CON groups) were from the same soccer teams, of the same ethnicity (all self-reported Polish, East-Europeans for ≥3 generations), of similar age (ACLR group = 26 ± 4 years, CON group = 25 ± 3 years), and had a comparable level of exposure to risk of ACLR (same volume and intensity of training and match play). The ACLR female participants (mean age: 25 ± 4 years) consisted of 41 soccer players from the Polish 1st division soccer league (training 10-12 hours per week) and also included amateur skiers (n = 25). The female control participants from the CON group (age 29 ± 2 years) were recruited from sports clubs and wellness centres and self-reported as being physically active for a minimum of 7 hours per week.

**Genetic analyses**

DNA was extracted from the buccal cells using a GenElute Mammalian Genomic DNA Miniprep Kit (Sigma, Germany) according to the manufacturer’s protocol. All samples were genotyped in duplicate using an allelic discrimination assay on a StepOne Real-Time Polymerase Chain Reaction instrument (Applied Biosystems, USA) with TaqMan probes. To discriminate VEGFA rs699947, rs1570360, and rs2010963 alleles, TaqMan Pre- Designed SNP Genotyping Assays were used (Applied Biosystems, USA) (assay ID: C___8311602_10, C___1647379_10, and C___8311614_10 respectively), including primers and fluorescently labelled (FAM and VIC) MGB probes to detect alleles.

**Statistical methods**

Statistical analysis was conducted with the SNPassoc package for R (version 3.4.0, The R Foundation for Statistical Computing, https://cran.r-project.org). Genotype models were constructed with respect to the minor allele. Haplotype analysis was conducted with

**TABLE 1.** Minor allele frequencies (MAF) and the probabilities that the genotype frequencies do not differ from Hardy-Weinberg expectations for the rs699947, rs1570360 and rs2010963 VEGFA polymorphisms

| SNP       | MAF (%) | ACLR+CON (n=412) | CON (n=190) | ACLR (n=222) |
|-----------|---------|------------------|-------------|--------------|
| rs699947  | A (42.2)| 0.069            | 0.226       | 0.174        |
| rs1570360 | A (27.2)| 1.0              | 1.0         | 0.873        |
| rs2010963 | C (43.9)| 1.0              | 0.449       | 0.686        |

Note: ACLR – anterior cruciate ligament rupture, CON – control group.
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R (https://cran-r.project.org, version 3.1.0) using the haplo.stats package and haplo.glm regression function. Percentage change over training was used as the dependent variable, while the PPARD haplotypes were used as the independent variables. P values <0.05 were considered statistically significant.

**RESULTS**

The genotype frequencies did not differ significantly from the expectations of Hardy-Weinberg equilibrium in the control group, in the ACL group or in the pooled case-control sample (Table 1).

The results of single-locus association analysis are summarized in Tables 2-4. No associations were found between the rs699947 and rs1570360 SNPs and ACL status in all models (Table 2 and 3). However, there was a significant association of the rs2010963 with ACL rupture in the codominant (p=0.047) and recessive model (p=0.017). In the latter, the CC genotype was overrepresented among individuals with ACL rupture (23.4% vs 14.2%, OR=1.85[1.11-3.08]). In addition, a haplotypic association between the VEGFA gene and ACL rupture was detected (Table 5). Four VEGFA haplotypes ([rs699947; rs1570360; rs2010963]) were inferred with frequencies >1% ranging from 1.8% ([C;G;C]) to 55.9% ([C;G;G]). The global score test indicated the association between VEGFA haplotypes and ACL rupture under the additive (p=0.022) and dominant (p=0.020) model. In each model, the [C;G;G] haplotype was as-

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**TABLE 2.** Association analysis of **VEGFA** gene rs699947 polymorphism with anterior cruciate ligament rupture.

| Model       | CON (n=190) % | ACLR (n=222) % | OR  | 95% CI   | p*    |
|-------------|---------------|----------------|-----|----------|-------|
| Codominant  |               |                |     |          |       |
| C/C         | 66 34.7       | 62 27.9        | 1.00|          |       |
| A/C         | 99 52.1       | 121 54.5       | 1.30| 0.84     | 2.01  | 0.231|
| A/A         | 25 13.2       | 39 17.6        | 1.66| 0.90     | 3.06  |       |
| Dominant    |               |                |     |          |       |
| C/C         | 66 34.7       | 62 27.9        | 1.00|          |       |
| A/C-A/A     | 124 65.3      | 160 72.1       | 1.37| 0.90     | 2.09  | 0.137|
| Recessive   |               |                |     |          |       |
| C/C-A/C     | 165 86.8      | 183 82.4       | 1.00|          |       |
| A/A         | 25 13.2       | 39 17.6        | 1.41| 0.82     | 2.42  | 0.216|
| Overdominant|               |                |     |          |       |
| C/C-A/A     | 91 47.9       | 101 45.5       | 1.10| 0.75     | 1.62  | 0.627|
| A/C         | 99 52.1       | 121 54.5       | 1.10| 0.75     | 1.62  |       |

**TABLE 3.** Association analysis of **VEGFA** gene rs1570360 polymorphism with anterior cruciate ligament rupture.

| Model       | CON (n=190) % | ACLR (n=222) % | OR  | 95% CI   | p*    |
|-------------|---------------|----------------|-----|----------|-------|
| Codominant  |               |                |     |          |       |
| G/G         | 110 57.9      | 108 48.6       | 1.00|          |       |
| A/G         | 69 36.3       | 95 42.8        | 1.40| 0.93     | 2.11  | 0.147|
| A/A         | 11 5.8        | 19 8.6         | 1.76| 0.80     | 3.87  |       |
| Dominant    |               |                |     |          |       |
| G/G         | 110 57.9      | 108 48.6       | 1.00|          |       |
| A/G-A/A     | 80 42.1       | 114 51.4       | 1.45| 0.98     | 2.14  | 0.061|
| Recessive   |               |                |     |          |       |
| G/G-A/G     | 179 94.2      | 203 91.4       | 1.00|          |       |
| G/G         | 11 5.8        | 19 8.6         | 1.52| 0.71     | 3.29  | 0.277|
| Overdominant|               |                |     |          |       |
| G/G-A/A     | 121 63.7      | 127 57.2       | 1.00|          |       |
| A/G         | 69 36.3       | 95 42.8        | 1.31| 0.88     | 1.95  | 0.180|
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would modulate the risk of ACLRs in Caucasian participants from Poland. The main finding of this study was the observation that harbouring a specific VEGFA genotype may be associated with different risk of non-contact ACLRs in the recruited Polish population. When tested individually, no significant differences in allele and genotype frequency distributions were noted for two of three investigated VEGFA SNPs, rs699947 and rs1570360. However, our statistical analyses revealed that the third one, rs2010963, was linked with risk of ACLRs in the studied population. We described the overrepresentation of the rs2010963 CC genotype in the ACLR group compared to the CON group, suggesting that harbouring this specific C allele may be an unfavourable risk factor for a knee injury. To the best of our knowledge, our research group is the first team to explore the association of VEGFA rs2010963 polymorphism with ACLR risk. Moreover, when the results obtained in the present study were incorporated in the complex haplotype analysis, the novel important

**DISCUSSION**

Recently, numerous studies have investigated the associations between ACLRs and various genetic markers, highlighting the importance of genetic predisposition as a risk factor in ACLRs [11,15-18]. Due to their regulatory role in the angiogenesis signalling pathway, the genes encoding VEGFA growth factors are promising candidates for the regulation of risk of musculoskeletal soft tissue injuries. Considering previous studies, VEGFA polymorphisms, tested individually or in haplotype combination, have been associated with increased or reduced risk of non-contact ACLRs [12,18]. We hypothesized that the three functional SNPs within the VEGFA gene would modulate the risk of ACLRs in Caucasian participants from Poland.

The main finding of this study was the observation that harbouring a specific VEGFA genotype may be associated with different risk of non-contact ACLRs in the recruited Polish population. When tested individually, no significant differences in allele and genotype frequency distributions were noted for two of three investigated VEGFA SNPs, rs699947 and rs1570360. However, our statistical analyses revealed that the third one, rs2010963, was linked with risk of ACLRs in the studied population. We described the overrepresentation of the rs2010963 CC genotype in the ACLR group compared to the CON group, suggesting that harbouring this specific C allele may be an unfavourable risk factor for a knee injury. To the best of our knowledge, our research group is the first team to explore the association of VEGFA rs2010963 polymorphism with ACLR risk. Moreover, when the results obtained in the present study were incorporated in the complex haplotype analysis, the novel important

**TABLE 4.** Association analysis of VEGFA gene rs2010963 polymorphism with anterior cruciate ligament rupture.

| Model          | CON (n=190) | ACLR (n=222) | %   | %   | OR  | 95% CI  | p*   |
|----------------|-------------|--------------|-----|-----|-----|---------|------|
| Codominant     |             |              |     |     |     |         |      |
| G/G            | 66          | 63           | 34.7| 28.4| 1.00|         |      |
| C/G            | 97          | 107          | 51.1| 48.2| 1.16| 0.74    | 1.80 | 0.047|
| C/C            | 27          | 52           | 14.2| 23.4| 2.02| 1.13    | 3.60 |
| Dominant       |             |              |     |     |     |         |      |
| G/G            | 66          | 63           | 34.7| 28.4| 1.00|         |      |
| C/G-C/C        | 124         | 159          | 65.3| 71.6| 1.34| 0.88    | 2.04 |
| Recessive      |             |              |     |     |     |         |      |
| G/G-C/G        | 163         | 170          | 85.8| 76.6| 1.00|         |      |
| C/C            | 27          | 52           | 14.2| 23.4| 1.85| 1.11    | 3.08 |
| Overdominant   |             |              |     |     |     |         |      |
| G/G-C/C        | 93          | 115          | 48.9| 51.8| 1.00|         |      |
| C/G            | 97          | 107          | 51.1| 48.2| 0.89| 0.61    | 1.31 | 0.563|

**TABLE 5.** Haplotype-based association of VEGFA gene polymorphisms (rs699947, rs1570360, rs2010963) with anterior cruciate ligament rupture

| Haplotype (rs699947; rs1570360; rs2010963) | Additive (Score=11.39, p=0.022) | Dominant (Score=11.61, p=0.020) | Recessive (Score=3.05, p=0.384) |
|-------------------------------------------|----------------------------------|----------------------------------|----------------------------------|
| [C;G;G]                                   | 55.9                             | -2.32                            | -2.37                            | -2.37                            | 1.00 |
| [A;G;C]                                   | 15.0                             | -0.13                            | 0.895                            | -0.60                            | 0.551 |
| [A;A;C]                                   | 27.1                             | 1.86                             | 0.062                            | 1.78                            | 0.074 |
| [C;G;C]                                   | 1.8                              | 2.59                             | 0.009                            | 2.60                            | 0.009 |

Note: NA – Not applicable.
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Previously, some studies have revealed that an increase in the association between the rs2010963 C allele and risk of ACL haplotype. It needs to be highlighted that haplotype analysis confirmed that some individuals may benefit from carrying the [C;G;G] type has a protective effect. On the other hand, the [C;G;C] haplotype finding was that carriers of the rs2010963 C allele displayed higher risk of ACL ruptures in the Polish population. However, more experimental studies are needed to confirm the association described in this study. The differences and similarities observed between the above-mentioned results highlight the role of hypothesis testing in various groups to reduce confounding due to population stratification. Thus the association of a genetic marker in numerous studies supports the evidence for a potential biological role rather than this being a statistical association due to heterogeneity in allele frequencies [18].

The main limitation of the genetic association study is the relatively small sample size; therefore the results should be repeated in bigger, independent groups. The insufficient number of female cases included in the study is in part due to limited numbers of women who reported to the hospital for ACL reconstructive surgery. Soccer is also more popular amongst males [23]. Moreover, sports participation data were self-reported and the mechanism of ACL rupture was categorised based on injury details reported by the participants to the investigator. As there is no clinical tool to confirm the mechanism by which ACL rupture occurred, this constitutes the next limitation of our study. On the other hand, a strength of the present study was the inclusion of surgically diagnosed primary ACLR patients who sustained their injury through non-contact mechanisms, as these injuries are considered to have a larger genetic component.

CONCLUSIONS

In conclusion, this is the first study to investigate a potential correlation between the VEGFA rs2010963 polymorphism and ACLR risk, suggesting that harbouring this specific C allele may be an unfavourable risk factor for a knee injury in Caucasian participants from Poland. Although other associations between rs699947 as well as rs1570360 and ACLR risk were not confirmed in this study, VEGFA remains a significant candidate gene requiring further exploration due to its key role in angiogenesis.

Conflict of interest

Authors reported no conflict of interest.

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