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TNFA Genetic Polymorphism is Associated with Risk for Developing Hip but not Knee Osteoarthritis in Croatian Population

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Abstract

Aim: We studied genetic epidemiology of primary large-joint (hip and knee) osteoarthritis (OA), in order to find disease risk factors by a candidate-gene approach. We used *TNFA* gene SNP rs1800629 in a case-control study in the Croatian Caucasian population.

Method: We analyzed 225 hip and 205 knee OA patients (both with total joint replacements), and 554 healthy individuals, majority being blood donors. We genotyped for *TNFA* SNP rs1800629 (+308, C>T). Allelic and genotypic frequencies were compared between patients and controls.

Results: The minor allele (T) of rs1800629 significantly conferred susceptibility only to hip OA ($p < 5*10^{-5}$, OR = 1.81, CI 95% = 1.34 - 2.44). The major (C) allele was significantly associated with the protection to hip ($p < 5*10^{-5}$, OR = 0.63, CI 95% = 0.41 - 0.74), but not knee OA. Patients with the homozygous genotype C/C had significantly lower risk of developing hip OA ($< 3*10^{-4}$, OR = 0.53, CI 95% = 0.38 - 0.76), but had no association with knee OA. The homozygosity of the minor allele (T/T) has been significantly associated with susceptibility to only hip OA ($< 2*10^{-2}$, OR = 2.56, CI 95% = 1.09 - 5.57).

Conclusion: Our findings demonstrate that major and minor alleles of the rs1800629 are associated with lower or higher risk, respectively, to developing hip, but not knee OA in the Croatian population. The data suggest a difference in the etiology of hip OA from that of the knee, perhaps due to an unknown dissimilarity in vulnerability of these joints to the actions of *TNFA*. Alternatively, other genetic factors including long non-protein coding LOC100287329 and/or miR6832 in vicinity of rs1800629 might be involved in the observed risk.

Keywords: *Genetic Risk; TNFA; Osteoarthritis; Hip; SNP*

Introduction

Osteoarthritis (OA) is a chronic, progressive, and multifactorial disease of joints whose hallmarks are destruction of the articular cartilage, deformation of subchondral bone and inflammation of the surrounding synovia. Despite mostly affecting health problems in middle aged and older people, it has significant social and economic influence, which is an important argument and incentive for research into causes and exploration for novel therapies. OA, due to its wide clinical heterogeneity, might be a mix of different disorders similar in ensuing symptomatology. We distinguish primary OA, with the late beginning and unclear origin, from the secondary OA, having the early onset because of known causes such as developmental deformities, trauma, damage, or the like [1].

In the beginning of the osteoarthritic process, some important changes at the molecular level occur in the joint, and they were thought to happen long before clinical symptoms can be observed during diagnostic evaluations. First, changes in the organization of the extracellular matrix (ECM) results with weakening of the collagen network in the joint [2]. The chondrocytes become hypertrophic and initiate a reparative stage, but this eventually stops and catabolic processes supervene. It is marked by the decrease of the production of collagen type II. Also, expression of transcription factor Runt 2, collagen type X, and proteolytic enzymes - collagenases, like matrix metalloproteinases (MMPs, especially MMP-1 and MMP-13) are increasingly secreted by chondrocytes, synoviocytes and osteoblasts during the late stages of OA [2,3]. Similarly, proteoglycans are steadily being destroyed via extracellular proteases - agrecanases, also called "a disintegrin and metalloproteinase with thrombospondin motifs" (ADAMTS) enzymes, of which ADAMTS-4 and -5 are probably the most important in the OA pathogenesis [4]. The action of MMPs on degradation of the ECM is held in balance with "Tissue inhibitors of metalloproteinases" (TIMPs). Thus, the ratio of active MMPs versus TIMPs in joint cartilage is thought to be a critical factor for ECM degradation in the OA joint [5]. Ultimately, the described changes decrease water-binding capabilities of the ECM with a result that mechanical forces now have different (bent) axes of action on the joints' surfaces and subchondral bone. Such changes can break the cartilage disturbing the integrity of a joint. Subsequently, the chondrocytes undergo apoptosis leading to a definitive loss of joint cartilage. This eventually leads to incremental decrease of possible movements in affected joints, and possibly also pain. Similar process was observed in the joint cartilage during aging [2].

Risk factors play an important role in development of primary OA. They include age, ethnicity, gender, obesity, occupation, and mechanical stress. All seem to be additionally changed by inheritance [6,7]. Indeed, subsequent studies showed that OA has complex (i.e. polygenic) non-Mendelian hereditary components affecting its development. Research into genetic risk factors showed familial aggregation of hand, hip, and spine but not knee OA in siblings with multiple joint involvements [8]. It is thought that 30 - 65% of overall risk for primary OA is genetic.

Linkage analysis studies have shown that chromosomes 2, 4, 6, 7, 11, 16 and the X may modify the risk for developing hip OA [1,8-13]. These early approaches revealed that most frequent associations between various phenotypes of osteoarthritis and genetic components were on chromosome 2q13-32 [1]. This region encompasses the IL1 gene cluster, "Frizzled related protein gene" (*FRZB*) and structural protein of cartilage matrix Matrilin-3 (*MATN3*). On chromosomes 6 and 16, potential loci were identified that could be linked with development of the hand and knee OA including Collagen type IX (*COL9A1*), bone morphogenetic protein 5 (*BMP5*) and Interleukin (IL)-4 receptor (*IL4R*) genes [11,14]. In addition, regions on the chromosomes 4 and 7 were found to be associated with development of hip, knee and hand OA [15-17]. Similarly, linkage studies in the Netherlands discovered an association between the region on chromosome 12 harboring DIO2 gene and susceptibility to hip OA in women [18].

It is assumed that complex genetic diseases could be caused by various gene combinations in different populations [19].

There are basically two kinds of approaches to assessing genetic risk in complex diseases, which could be mutually inclusive. Genome wide study (GWAS) approach scans millions of genomic markers (usually single nucleotide polymorphisms, SNPs) and provides the basis for further correlation assessments. The other approach is an educated guess study (or candidate gene method) that tries to follow etiological signs of the disease and searches for the proof of association. In the case of the primary OA, both approaches have been done and have yielded numerous correlations between genetic areas and the disease. Such a result is possibly due to a combination of two factors, the first being more complex nature of the OA in itself, and the second, a higher than expected genetic variability of studied human subpopulations.

Regarding the former approach, several GWAS studies have shown linkage with the disease at multiple and different chromosomal loci [1]. The most recent ones identified a defined genetic areas harboring genes like COMP and CHADL, which are important for cartilage/bone metabolism in the joint spaces [20] and other regions like Transforming growth factor beta 1 (*TGFB1*), Fibroblast growth factor 18

(*FGF18*), Cathepsin K (*CTSK*) and IL11 [21] possibly influencing development of OA. The latter, and the most recent GWAS identified in total 64 loci significantly associated with the OA, all that need further research about their possible roles in pathogenesis.

The candidate gene approach also resulted with a number of genes over the past decades, all of which seem to be distributed differently in human subpopulations. These included growth differentiation factor 5 gene - *GDF5*), asporin (*ASPN*), *FRZB* and prostaglandin-endoperoxide synthase 2 gene *(PTGS2*) [22-28].

One kind of the candidate gene approach focused on pro-inflammatory cytokines IL-1β, TNF-α, IL-6, IL-8, IL-15, IL-17, IL-18, IL-21, CC motif chemokine ligand 5 (CCL5) and Leukemia inhibitory factor (LIF), because they are thought to be involved in the development of cartilage damage and progress of OA [29,30]. Chondrocytes can produce them, albeit transiently, in response to activating signal. Regarding large joints, such signal could either be an environmental one (i.e. mechanical force) or endogenous signal like, for example, an initiation of inflammation in the course of infection or cellular stress. Cytokines secreted in synovial fluid might exert their effects on neighboring cells as well as on the producers having a complex interactive network of mediators that can modulate their own production, enhance or downregulate other cytokines, and influence inflammation and immune responses.

We previously reported analyses of the IL-1 gene cluster on the OA of hip (HOA) and knee (KOA) in our case-control study [31,32] and found that women with 4-marker haplotype 1-2-1-1 [IL1A(rs1800587) - IL1B(rs1143634) - IL1B(rs16944) - IL1RN(VNTR)] had six-fold lower risk to develop KOA, and that 1-1-1-2 predisposes for HOA in both sexes [33]. Similarly, we found that IL17 gene cluster harbors protective and predisposing genetic elements for both, hip and knee OA [34,35].

TNFα was shown to induce the secretion of other cytokines, and, if produced locally in the joint, it can increase the synthesis of ADAMTS-4, -5 and prostaglandin E2 [36]. TNFα can also diminish the production of proteoglycans and type II collagen with which both, cartilage matrix and bone degradation can take place [37]. Because of these effects, TNFα is thought to have a crucial role in developing OA.

Genetic polymorphisms can alter the expression of *TNFA* gene, and the most commonly studied one in the *TNFA* gene was the SNP rs1800629 (-308, C>T), which was investigated in patients with OA. Associations of the high-TNF-producer allele (T) with KOA were found in Mexican, Egyptian and Chinese populations [38-40]. Meta-analysis (of seven association case-control studies using four Caucasian and three Asian populations, including 983 patients with KOA and 1355 controls) showed an increased risk for development of KOA in T/T and T/C genotypes [41]. Hip OA patients were also included in this meta-analysis, however, with only one Caucasian case-control study having 55 patients, which was insufficient to draw any conclusions.

In view of these facts, we wanted to establish whether *TNFA* gene is associated with hip OA in our hospital-based case-control study in the Croatian Caucasian population. We also wished to determine whether primary knee OA has a similar risk compared to the populations studied so far. We used more stringent criteria for OA diagnosis than previously reported by others. We namely, included only primary OA patients with severe hip or knee OA, as they have undergone replacement of their large joints (hip or knee). We also excluded those having affected both (knee and hip) joints. We used the well-known marker for *TNFA* gene - SNP rs1800629 (-308 C>T) and explored its association with primary hip and knee OA.

Patients and Methods

Subjects

In this case-control study we admitted patients that had clinically and radiologically confirmed diagnosis of solely the HOA (N = 225) or the KOA (N = 205), an indication for either total hip replacement (THR) or total knee replacement (TKR). All had signed informed consent to donate blood for research purposes. Only those with particularly severe single location (hip or knee) OA (without signs of OA from other location) were eligible in the study. Some patients had both joints replaced by arthroplasty.

We used clinical guidelines from American College of Rheumatology (ACR) and Western Ontario and McMaster Universities' Osteoarthritis (WOMAC) index to evaluate symptoms in patients with hip and knee OA [42,43]. Radiological criteria for assessment of the HOA were Kellgren-Lawrence grading scale [44]. Indications for primary THR used in our study were according to National Institutes of Health (NIH) Consensus Statement on THR, and include: joint pain, functional limitation, and radiographic evidence of joint damage.

The study included 225 patients with HOA (mean age = 67.82 years, standard deviation (SD) = 9.61, range: 31 - 90), 205 with KOA (mean age = 69.7 years, SD = 7.24, range: 47 - 86) and 554 healthy persons (majority were blood donors and less than 20% were healthy hospital personnel) as control individuals (mean age = 41.58 years, SD = 11.72, range: 19 - 91).

The average age of the control group represents a random sample from a total population. The consequences of comparing the random population controls (as we did) with OA patients are that the potential differences might have been blurred, or in other words, the outcome we present is more on a conservative end of the results. We excluded from the study patients with primary OA without indication for THR, TKR or assigned informed consent. Similarly, patients with any secondary form of OA, as well as rheumatoid arthritis, were excluded too.

The study was approved by the Medical ethics committees of the University Hospital for Orthopaedics and Traumatology Lovran and School of Medicine, University of Rijeka, Croatia.

DNA isolation

Genomic DNA was isolated as described previously [31,32]. Briefly, 200µl of whole blood was mixed with 400 µl of sucrose buffer (0.32M sucrose, 10 mM Tris-HCl pH 7.5, 5 mM $MgCl_2$, 1% v/v Triton X-100) prompting cells to be lysed, and then cell nuclei were precipitated (and subsequently washed twice) by centrifugation for 2 minutes at 5000g. After washing, the nuclei were taken in 400 µl of DNAzol (Invitrogen Corporation, Carlsbad, California, USA) and incubated at room temperature for 5 minutes. Genomic DNA was precipitated with 200 µl of 100% ethanol and collected by centrifugation (2 minutes at 5000g). The precipitate was washed twice with 1 ml of 75% ethanol and centrifuged (1 minute at 5000g). Genomic DNA was then resuspended in 100 µl of 8 mM NaOH and allowed to solubilize for 15 minutes at room temperature. HEPES buffer (16 mM HEPES in 8 mM NaOH, pH 7.5) was used to adjust the pH 7-8.4. The average concentration of genomic DNA was around 30 µg/ml with 260/280 OD ratio higher than 1.7.

SNP analysis

Allele discrimination assays were performed by the PCR method as in our previous studies [31-35]. In short, the alleles of the *TNFA* gene at -308(C>T) (rs1800629) were detected by the Taqman method (Applied Biosystems, San Jose, CA, USA) using the primers and a probe sold as a commercially available custom-made kit by Applied Biosystems, in the real-time-qPCR machine MX3005P with its software (Agilent, Stratagene, CA, USA).

Statistical analysis

Statistical analyses were done by Chi-square method (Statcalc program, Acastat software and software found on the Internet as contingency tables http://statpages.info/ctab2x2.html) comparing genetic typing of the SNP rs1800629 near the TNFA gene between cases and controls. Power analyses were done using G*Power calculator 3.1 [45]. For post hoc analyses, statistical power (1-β) was calculated as a function of significance level α, sample size, and population effect size. The rs1800629 heterozygotes were in the Hardy-Weinberg equilibrium according to Arlequin 3.5 software [46]. A statistically significant difference was defined when p was less than 0.05. The post hoc power (1-β) of the study for HOA or KOA was 0,85 or 0,73, respectively ($\alpha < 0,05$). In other words, we had 85% (or 73%) chance to find a statistically significant difference of 8% comparing cases (n = 225 HOA, or n = 205 KOA) and controls (n = 554). For KOA, a priori power calculations have shown that in order to find a statistically significant difference of 10% between cases and controls with 88,5% chance, we needed 206 patients and 557 controls, which was almost as many individuals as we had in our study.

Results

Table 1 and 2 summarize frequencies of allelic/genotypic variants of the *TNFA* gene rs1800629 polymorphism for HOA, and table 3 and 4, for KOA in our case-control study. Statistical analyses were done by comparison of the frequencies in the healthy blood donor group with those of patients that have surgically replaced their hip or knee joints.

In the table 1, allelic frequencies for *TNFA* SNP rs1800629 differ significantly between patients and controls. Minor allele (T) was associated with the susceptibility to hip OA ($p < 5*10^{-5}$) having an odds ratio (OR) of 1.81, and 95% confidence index (CI) limits ranging from 1.34 to 2.44. In contrast, the major allele (C) was associated with protection against the disease ($p < 5*10^{-5}$, OR = 0.63, CI 95% = 0.41 - 0.74).

	Allelic frequencies (number)					
TNFA[2]	**rs1800629**	**Patients OA hip[1]**	**Controls[1]**	**p**	**OR**	**Association**
	SNP (C>T)	n = 450	n = 1108			
1	C	0.791 (356)	0.873 (967)	$<5*10^{-5}$	0.63 (0.41 - 0.74)	Protection
2	T	0.209 (94)	0.127 (141)	$<5*10^{-5}$	1.81 (1.34 - 2.44)	Predisposition
[1] Frequency (number)						
[2] Allele designations (1 - major, 2 - minor allele)						

Table 1: *Allelic association analysis of TNFA SNP (rs1800629) in hip OA patients in the Croatian population.*

Similarly, the genotype frequency (Table 2) for homozygotes C/C (1/1) at rs1800629 was significantly associated with the lower risk for development of hip OA in the Croatian population ($< 3*10^{-4}$, OR = 0.53, CI 95% = 0.38 - 0.76). Furthermore, the homozygous genotype T/T (2/2) also showed a significant difference OA ($< 2*10^{-2}$) between cases and controls, and its OR = 2.56 (CI 95% = 1.09 - 5.57) suggested association with higher risk for developing hip OA (Table 2).

	Genotypic frequencies (number)					
TNFA[2,3]	**rs1800629**	**Patients OA hip[1]**	**Controls[1]**	**p**	**OR**	**Association**
	SNP	n = 225	n = 554			
1/1	C/C	0.640 (144)	0.769 (426)	$<3*10^{-4}$	0.53 (0.38 - 0.76)	Protection
1/2	C/T	0.302 (68)	0.208 (115)	$5*10^{-3}$	1.65 (1.15 - 2.38)	
2/2	T/T	0.058 (13)	0.023 (13)	$<2*10^{-2}$	2.56 (1.09 - 5.57)	Predisposition
[1] Frequency (number)						
[2] Allele designations (1 - major, 2 - minor allele)						
[3] Genotype designations						

Table 2: *Genotype analysis of TNFA SNP (rs1800629) in hip OA patients in the Croatian population.*

Interestingly and at variance from hip OA, allelic frequencies of rs1800629 were not significantly different between knee OA patients and controls (Table 3).

Lastly, in table 4, comparison of genotypic frequencies of the SNP rs1800629 also showed no significant differences between knee OA patients and healthy controls.

	Allelic frequencies (number)					
TNFA[2]	**rs1800629**	**Patients (Pt) OA knee[1]**	**Controls [1]**	**p**	**OR**	**Association**
	SNP (C>T)	n = 410	n = 1108			
1	C	0.846 (347)	0.873 (967)	0.18		None
2	T	0.154 (63)	0.127 (141)	0.18		None
[1] Frequency (number)						
[2] Allele designations (1 - major, 2 - minor allele)						

Table 3: *Allelic association analysis of TNFA SNP (rs1800629) in knee OA patients in the Croatian population.*

	Genotypic frequencies (number)					
TNFA[2,3]	**rs1800629**	**Patients (Pt) OA knee[1]**	**Controls [1]**	**p**	**OR**	**Association**
	SNP	n = 205	n = 554			
1/1	C/C	0.741 (152)	0.769 (426)	0.43		None
1/2	C/T	0.210 (43)	0.208 (115)	0.95		
2/2	T/T	0.049 (10)	0.023 (13)	0.07		
[1] Frequency (number)						
[2] Allele designations (1 - major, 2 - minor allele)						
[3] Genotype designations						

Table 4: *Genotype association of TNFA SNP (rs1800629) in knee OA patients in the Croatian population.*

Discussion

In comparison between hip OA patients and controls, we have identified a statistically significant difference ($p < 5*10^{-5}$) in frequency of the SNP near the *TNFA* gene (rs1800629, -308; C>T) at the position 31575254 on chromosome 6 (Figure 1). The difference is associated with higher risk for HOA in persons carrying the minor allele (T), and conversely, with lower susceptibility for HOA in those having the major allele (C). We found that homozygotes for T (T/T) carry a predisposition for hip, but interestingly, not for KOA. The latter result is in contrast to meta-analysis reported previously that found the minor allele homozygotes and heterozygotes associated with higher risk for developing KOA [41].

The reasons for disagreement could be numerous. First of all, in complex hereditary diseases, it was a commonplace to find diverse alleles associated with predispositions for the disease in various populations. Meta analyses tend to mix different populations that normally have completely different population frequencies for many SNPs. Perhaps the one that reported *TNFA* SNP rs1800629 associated with KOA [41] might be explained with this argument as it was a mix of 3 Asian and 4 Caucasian population studies. Since no studies reported the connection of this SNP with hip OA, this is the first report linking it.

However, apart from the influence of the *TNFA* gene on the HOA, the influence of other nearby DNA elements should not be ignored. Namely, as shown in the figure 1, there are at least three elements of interest that might contribute or somehow count as risk factors for development of OA: 1) The microRNA (miR6832), 2) the long non-protein coding RNA (lnc at LOC100287329) and 3) the 2-Amino-purine regulation element (2-APRE) in the 3'UTR of the *TNFA* gene [47]. In addition, there are in vicinity of *TNFA* three small nucleolar RNAs (SNOR) like SNORA38, SNORD117 and SNORD84, and numerous other genes that could, although less likely than the previously mentioned ones, be involved in affecting the risk to OA.

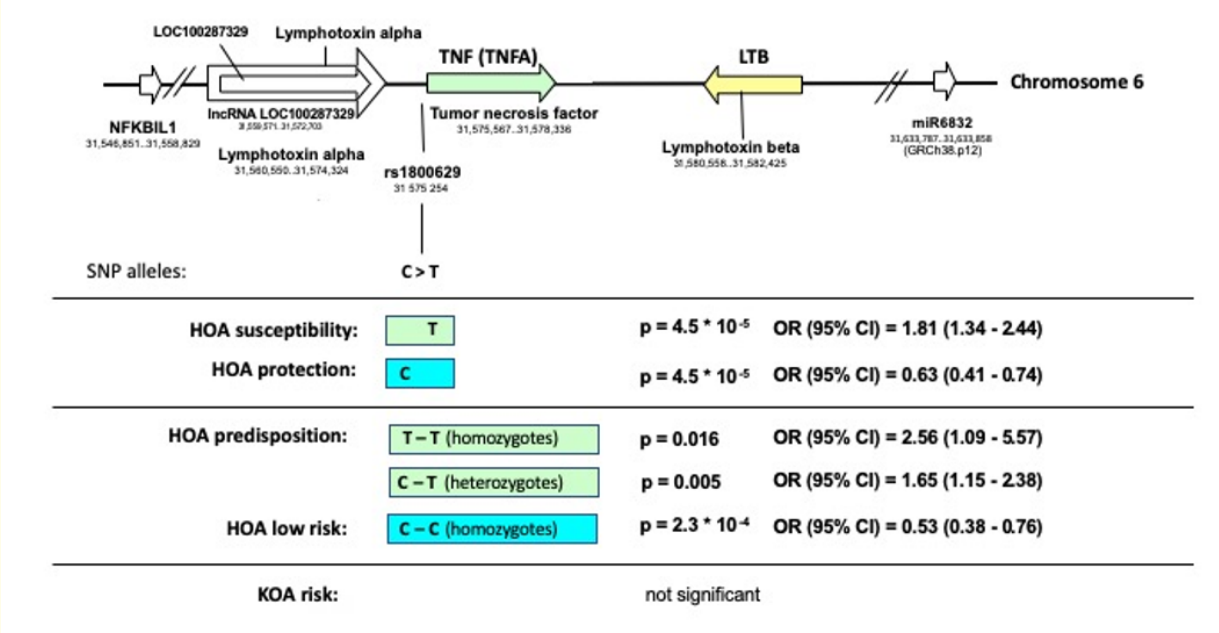

Figure 1: *Summary of TNFA locus analysis - hip and knee OA susceptibility.*

The 2-APRE can act as a positive feedback loop element that can increase the splicing of the *TNFA* pre-mRNA, thus potentiating pro-inflammatory aspect of TNFα action during cellular stress in peripheral blood mononuclear cells [48]. Enhanced splicing via such ancient pseudoknot RNA-activator mechanism links cellular stress response with protective immunity [47,48]. Perhaps some of the genotyped patients carry a different sequence in their 2-APRE DNA element that might always yield higher levels of this cytokine, and thus give rise to larger damage every time the gene become activated via, for example, an innate immunity type of provocation, or mechanical stress-related induction of *TNFA* gene.

On the other hand, the influence of the miR6832 and lncRNA could be unrelated to the function of the *TNFA*, yet they might be factors that regulate the balance between pro-inflammatory versus anti-inflammatory factors within the synovial tissues, which could potentiate local (joint-related) damage to cartilage and bone. Thus, the lncRNA segment close to *TNFA* could also potentially have an effect on hip OA. If so, then some of these factors might be eventual targets for pharmaceutical handling of primary OA.

Therapeutic targets might also include the anti-inflammatory cytokines like IL-4, IL-10, IL-13 and TGFβ. Their action is thought to be in antagonizing synthesis and production of IL-1β, TNFα and IL-6. The action of IL-13 includes decrease of synthesis of IL-1β, TNFα and MMP3, as well as increase of IL-1Ra [29]. Several other pro-inflammatory cytokines have been also implicated in pathogenesis of OA like IL-15, IL-17, IL-18, IL-21, Leukemia inhibiting factor (LIF) and IL-8 [30]. Besides targeting IL-1 and TNFα, the treatment of OA by antagonizing the action of the above-mentioned additional pro-inflammatory cytokines might be beneficial.

Genetic association studies by candidate gene approach that have focused on hip, knee and hand osteoarthritis have identified 21 independent susceptibility loci for osteoarthritis [49]. However, with the help of Human Genome Epidemiology (HuGE) Navigator almost 200 candidate genes could be identified to be associated with development of OA. Interestingly, meta-analyses of the nine GWAS (all utilizing

persons of European descent) remarkably pointed only to two of them. Namely, the association was found in genetic areas that contained Collagen 11A1 (*COL11A1)* and Vascular endothelial growth factor *(VEGF) genes. In detail, two SNPs (*rs4907986 and rs1241164) near *the COL11A1 gene were associated significantly with hip OA, and a single SNP near the VEGF gene* (rs833058) showed association with the knee OA [50].

Case-control studies that investigated genetic susceptibility to OA in separate joints (knee, hand or hip) showed numerous loci being associated with different locations of the disease, but all depended on the population that was studied [26].

Interestingly, the two most recent GWAS that were done by total genome DNA sequencing, have identified association with OA of genetic areas harboring *COMP* and *CHADL* genes, important for healthy cartilage and bone metabolism in the joints [20]. The other recent GWAS identified in total 64 genetic loci significantly associated with the OA, near genes like *TGFB1, FGF18*, *CTSK* and *IL11* [21].

Despite all these associations, the roles of the majority of mentioned genes (or perhaps factors nearby like miRs and lncRNAs) in the etiology of primary osteoarthritis remain still elusive. The solution might lie in the way these factors interact. Perhaps investigating intracellular pathways of identified factors might show the link between cellular stress and joint damage. Alternatively, we must await functionally relevant studies in animal models with gene-targeting techniques in order to decipher a complex genetic disease such as primary osteoarthritis.

Conclusion

Osteoarthritis (OA) is the most frequent large joint pathology associated with health problems in elderly people.

Etiology of primary OA has not been completely understood and many factors were implied, including genetics.

Genetic risk to primary OA varies among disease locations (i.e. hip versus knee) and between different human subpopulations.

This is the first report that the risk for hip OA is significantly associated ($p < 10^{-5}$) with the SNP rs1800629 located in the *TNFA* gene on chromosome 6, in the Croatian Caucasian population. For knee OA, we found no association with the risk, in contrast to previous positive findings in other subpopulations.

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