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Research Article

Dynamics of Ontogenesis in The Phytosphere: Fluctuation of the Asymmetry of The Birch Leaf (Betula Pendula)

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Abstract

The method for measuring the morphological parameters of birch (Betula Pendula) leaves with-out cutting them in the dynamics of ontogenesis has been refined, the asymmetries and fluctua-tions of leaves have been compared, and patterns of oscillatory adaptation in the dynamics of ontogenesis have been identified. First, a sheet is selected, marked with a white thread with a tag (birch and leaf number). Before photographing, a transparent mesh (2×2 mm) is placed on the front side of the growing leaf so that the middle line along the mesh coincides with the main vein of the leaf. The palette sheet is then photographed with a digital camera with a photo stor-age function. On the computer, the photo is cropped and enlarged for measurements on the screen up to A4 format. The measured 25 accounting birch leaves were divided into groups ac-cording to the nature of their behavior in ontogenesis: 1) left asymmetry, when the left side of the leaf is predominant; 2) tendency of the accounting sheet by fluctuation from left to right asymmetry; 3) transition from left to right asymmetry; 4) transition from right to left asymmetry; 5) tendency of the growing leaf by fluctuation from right to left symmetry; 6) right asymmetry. New sheet parameters have been introduced: absolute asymmetry; relative asymmetry. The "+" sign will show left asymmetry, and the "–" sign will show right asymmetry. The wavelet signal identification method for each leaf revealed up to 6-7 quanta of behavior in ontogenesis in the form of wave equations. Birch is distributed throughout the Northern Hemisphere, so the phyto-sphere can be studied by fluctuating asymmetry of leaves in ont ogeny in different geograph-ical coordinates of the cities of the world.

Keywords: Birch; Leaves; Ontogeny; Morphoparameters; Dynamics; Patterns

Introduction

Osteoarthritis (OA) is a chronic, progressive and debilitating disease of the joint, characterized by degeneration of the different joint tissues, with loss of articular cartilage, remodelling of the subchondral bone and synovitis. Globally, OA is ranked as the 11th highest contributor to disability [1], leading to great costs for the individuals affected as well as for the society. Presently, there is no cure for the disease, and the only ways to help the patients are, firstly by lifestyle changes and/or pain relief, then, in more severe cases, with injections of corticosteroids and, ultimately, joint replacement surgery. Since these treatments are not able to modify the course of the disease, prevent the degenerative processes, or restore damaged cartilage, there is a large demand for disease modifying therapy of OA. Deeper knowledge about the factors of the pathophysiology of OA is essential for developing such treatments.

Osteoarthritis can be divided into primary (idiopathic) OA and secondary OA, which has a known trigger, such as traumatic injury or congenital diseases or developmental deformities. The risk for primary OA is multifactorial. Among the risk factors are age, gender, lifestyle and genetics. The hereditary component is of polygenetic nature.

Among the cytokines known to be involved in the pathophysiology of OA is interleukin 6 (IL-6). IL-6 is a pleiotropic cytokine that plays important roles in regulation of immunity, metabolism, bone homeostasis and neural functions. IL-6 has multiple important functions during innate and adaptive immune responses. In particular, it was shown to be involved in inflammatory and autoimmune processes, such as infectious diseases, diabetes, and rheumatoid arthritis (RA). In addition, it was suggested to be implicated in the development of cardiovascular disease, periodontitis, and some forms of cancer (for a review see refs [2-4]. Lastly, a recent meta-analysis of nine studies suggested a strong association between levels of circulating IL-6 and severity of Covid-19 [5].

In OA patients, IL-6 is found at elevated concentrations in both serum and synovial fluid [6,7]. This has been suggested as a potential biomarker for development of OA, and a predictor of the outcome of the disease [8]. IL-6 can promote synovitis and destruction of joint tissue in several ways. In the cartilage, IL-6 alters the extracellular matrix (ECM) by acting on catabolic and anabolic processes of the ECM turnover. IL-6 decreases the synthesis of proteoglycan [9] and type II collagen [10], and increases IL-1b induced degradation of proteoglycan [11]. IL-6 also promotes production of proteolytic enzymes like matrix metallopeptidases or proteinases? (MMPs) [9,12] and A disintegrin and metalloproteinase with thrombospondin type 1 motif (ADAMTS) [13]. The combined effect of these changes imposed on chondrocytes and ECM by IL-6, is a more brittle, stiff and erosive cartilage. IL-6 stimulates osteoblasts to express receptor activator of nuclear factor (NF)-kB ligand (RANKL). RANKL enhances the differentiation, activation and bone resorption by osteoclasts [14,15]. IL-6 is involved in the differentiation of macrophages from monocytes [16], which can mediate induction of inflammatory mediators, proteinases and growth factors within synovia [17,18]. Significantly higher in osteoarthritic than in healthy cartilage is the secretion of prostaglandin E2 (PGE2) and prostaglandin D2 (PGD2), the major PGs synthesized by chondrocytes [19]. In OA, PGE2 is shown to be involved in pain signalling [20,21] and oedema with erosion of cartilage and juxtaarticular bone [21,22]. In vitro, PGE2 stimulates IL-6 production in human chondrocytes [23].

The genetic polymorphism rs1800795 (-174G>C) is a well-studied SNP located in the promoter region of the IL6 gene. This polymorphism is a marker of supposed DNA element, which modulates IL-6 production by affecting the level of the IL6-gene transcription. While the rs1800795 C allele marks lower production, the G allele identifies high-producers of the IL-6 [24,25]. In contrast to the Asian and African populations, which have predominantly the G allele, the European populations have almost balanced G:C ratios (i.e. 0.55 : 0.44) [26].

A study of the Han population in China showed associations between both the C allele and CC genotype of rs1800795 with the susceptibility to, and the severity of knee osteoarthritis (KOA). The study also showed an upregulation in the expression of the IL-6 that was significantly associated with the C allele [24]. A case-control study of the Thai population including 115 KOA cases and 100 healthy controls demonstrated that the rs1800795 GC genotype was significantly associated with the increased risk of developing KOA. The G allele was less frequent than the C allele in KOA patients compared to controls [27]. On the other hand, a study from Italy including 75 hip osteoarthritis (HOA) cases and 107 healthy controls, suggests a lower risk of developing HOA for individuals with the CC genotype of the rs1800795. In that study, the G allele was more frequent in patients compared to controls than the C allele [28].

MicroRNAs are short non-coding sequences of RNA (~22nt), highly conserved and involved in post-transcriptional regulation of many gene expressions. MiR-149 has been shown to be down-regulated in OA chondrocytes and plays a role on pathophysiology of OA [29]. The miR-149 has been suggested as a potential target for pharmacological treatment of OA [30-33].

In the present study we investigated the potential role of the rs1800795 polymorphism in susceptibility for KOA and HOA in the Croatian population. In addition, we studied the effect of miR-149 on the expression of IL-6 in fibroblasts, aiming to gain more insight into the aetiology of osteoarthritis.

Materials and Methods

Subjects

The patients were treated at the University Hospital for Orthopaedics and Traumatology Lovran, School of Medicine, University of Rijeka, Croatia. The criteria for participation in the study included clinically and radiologically confirmed diagnosis of primary HOA or KOA, with indication for partial knee arthroplasty (PKA) or total knee arthroplasty (TKA), or a total hip arthroplasty (THA). Included patients were informed that research data would be submitted for publication, and gave their consent. The patients were diagnosed using clinical guidelines from Western Ontario and McMaster Universities' Osteoarthritis (WOMAC) Index [34] and the American College of Rheumatology (ACR) for hip [35] and knee [36]. The Kellgren-Lawrence grading scale [37] was used for radiological assessment of the affected joint.

Excluded from the study were patients with primary OA without indication for PKA, TKA or THA, patients with secondary form of KOA or HOA, patients with rheumatoid arthritis of the hip or knee, and patients without a signed informed consent. The study was approved by the Institutional review boards (Medical ethics committees) at the University Hospital for Orthopaedics and Traumatology Lovran, Clinical Hospital Center Rijeka, and the School of Medicine, University of Rijeka, Croatia.

This case-control study included a total of 487 Croatian Caucasian patients diagnosed with primary OA, of which 255 HOA patients and 232 KOA patients. 255 HOA patients had a mean age of 67.80 years (range 31-90) with standard deviation [SD] - 9.64). Two thirds of HOA patients were female (171, 67.1%), and had the mean age of 68.85 years (range 31-87). One third, 83 (32.5%) were

male, with the mean age of 65.64 years (range 38-90). In the group consisting of KOA patients, the mean age was 69.65 years (range 47-86), SD - 7.25. There were 167 (72.0%) female patients, with a mean age of 69.54 years (range 47-86), and 65 (28.0%) male KOA patients, with a mean age of 69.92 years (range 52-82).

The control group of 591 healthy individuals consisted of several groups. The majority were voluntary blood donors, and the rest were medical personnel at the clinical hospital centre. In the control group, we have information about gender for 444 individuals, of which 348 (78.4%) are male and 96 (21.6%) are female. Only 396 of the controls were listed with age, having a mean age of 41.54 years (range 19-91, SD 11.83).

Isolation of DNA and SNP analyses

Extraction of genomic DNA was done by using patients' peripheral venous blood collected during the joint replacement surgery as described in the previous work by Jotanovic et al [38,39]. For SNP analysis, allele discrimination assays were performed by the TaqMan real time PCR method as described in earlier studies [38-42], using commercially available kits (Applied Biosystems, San Jose, CA, USA) with primers and probes for rs1800795 (-174G>C). The C probe was labelled with FAM, and the G probe with VIC. Each sample was run at initial incubation for 10 minutes at 95°C, followed by 40 cycles of 10 seconds at 95°C and 60 seconds at 60°C.

Cell culture

The human fibroblast cell line SW-1353 was obtained from the CLS Cell Lines Service. The cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco, St. Louis, MO, USA), supplemented with 5 % foetal bovine serum (FBS). The human immortal skin keratinocyte cell line HaCaT (Institute of Oral Biology, University of Oslo, Norway) was grown in keratinocyte serum free medium (KSFM) supplemented with 1 ng/ml human recombinant epidermal growth factor (EGF) and 25 mg/ml bovine pituitary extract (Gibco, St. Louis, MO, USA). The medium for both cell lines was further supplemented with 1 % (v/v) penicillin, 1% (v/v) streptomycin, and 0.25 mg/ml amphotericin B (Lonza, Basel, Switzerland). The cultured cells were split twice weekly at 80 % confluence.

Transfection of SW-1353 fibroblast cell line with microRNA

SW-1353 cells (250.000) were incubated in DMEM medium with 5 % FBS, without supplements and transfected using INTERFERin (Polyplus-Transfection, Illkirch, France) reagent according to the manufacturer's protocol. Transfections were carried out using 25nM, 50nM concentrations of either hsamiR-149-5p mimic or Allstar scrambled control (Qiagen, Hilden, Germany) microRNAs, respectively. Additional, negative, controls were done using the transfection reagent only (MOCK). The miRNA mimic was purchased from Gene Pharma, Shanghai, China. Transfection efficiencies were evaluated by flowcytometry using the FACSCalibur flow-cytometer (Becton Dickinson, Oslo, Norway) with FITC-labelled scrambled RNA and found to be > 95 % effective. Cell cultures were treated with 7,5 ng/ml IL-1b, and incubated at 37°C for six hours to stimulate the production of IL-6 after transfection experiments. After transfection and stimulation, the cells were centrifuged at 1000 rpm for 5 minutes, and both, the supernatant and pelleted cells, were harvested for analyses.

qRT-PCR on transfected cells and controls

RNA was extracted from the cells using RNeasy Mini Kit (Qiagen, Germany) according to the manufacturer's protocol. NanoDrop™ ND 1000 spectrophotometer (Thermo Fisher, MA, USA) was used to determine the concentration of purified RNA in the different samples. The RNA was diluted to 200ng/µl. Synthesis of cDNAs from mRNA templates obtained from transfected SW-1353 cell cultures was performed using Reverse Transcription Core Kit (Eurogentec, Seraing, Belgium). Quantitative real-time PCR (qRT-PCR) was conducted on Aria Mx Real-Time PCR System (Agilent Technologies, Santa Clara, USA) with predesigned IL-6, TNF-α and TATA binding protein (TBP) TaqMan primer probes (Applied Boisystems, Forster City, CA, USA). All samples were run in triplicates. TBP was used as a reference gene for normalization of mRNA expression. The amplification was initiated with denaturation at 95°C for 5 minutes, followed by 40 cycles of 95°C for 15s and 60°C for 60s.

Enzyme-linked immunosorbent assay (ELISA)

To measure the level of IL-6 in the supernatants, the human IL-6 Pre-Coated ELISA kit (BGK05231) from BioGems (BioGems, California, USA) was used according to the protocol from the manufacturer. The different samples were run in duplicates. The ELISA plates were run on an Epoch Microplate Spectrophotometer (Epoch BioTek, Winooski, VT, USA), and the absorbance was read by the program Gen5 Microplate Reader and Imager Software version 3.05 (BioTek Instruments, Winooski, VT, USA).

Statistics

Allelic and genotypic differences between cases and controls at the rs1800795 (-174G>C) loci were done by chi-square method using an online software [26]. A statistically significant difference was defined when p was <0.05. The statistical analyses of the data outcome from the qRT-PCR were performed using Agilent Aria Software v1.5.

Results and Discussion

Allelic and genotypic frequencies of the IL-6 rs1800795 SNP

(Table 1) shows allelic frequency analysis for the rs1800795 SNP in HOA. The G allele is 5,5% more frequent in HOA patients than in healthy controls with significance of p = 0.034, odds ratio (OR) 1,27, and 95% confidence interval (CI) between 1.02-1.57. The minor, C allele, is also significantly (5,5%) less frequent in HOA patients than in controls (p=0.34; OR=0.79, 95% CI: 0.64-0.98).

(Table 2) shows genotypic frequency analysis for the rs1800795 SNP in HOA. The GG genotype is 10% more frequent in HOA patients than controls, being significant at the level of p=0.006 (OR=1.53, 95%CI 1.13-2.06). The GC genotype is significantly less frequent in HOA patients than controls, with a p value of 0.02 (OR=0.70, 95% CI 0.52-0.94).

(Tables 3,4) show the frequencies and statistical calculations in comparing KOA patients with healthy controls in regard to allelic and genotypic frequencies, respectively. None of the allelic frequency differences were significant (Table 3). Similarly, in genotypic analyses, no significant changes were observed (Table 4).

Table 1:

rs1800795 -174G>C	HOA Patients (n=255)	Controls (n=591)	p	OR (95%CI)	Chi-square	Possible association
G	0.651 (332)	0.596 (704)	0.034	1.27 (1.02-1.57)	4.603	Predisposition
C	0.349 (178)	0.404 (478)	0.034	0.79 (0.64-0.98)	4.603	Protection

Table 2:

rs1800795 -174G>C	HOA Patients (n=255)	Controls (n=591)	p	OR (95%CI)	Chi-square	Possible Association
GG	0.431 (110)	0.332 (196)	0.006	1.53 (1.13-2.06)	7.674	Predisposition
CC	0.129 (33)	0.140 (83)	0.744	0.91 (0.59-1.40)	0.183	-
GC	0.439 (112)	0.528 (312)	0.02	0.70 (0.52-0.94)	5.607	Protection

Tables 3:

rs1800795 -174G>C	KOA Patients (n=232)	Controls (n=591)	p	OR (95%CI)	Chi-square	Possible Association
G	0.603 (280)	0.596 (704)	0.78	1.03 (0.83-1.29)	0.085	-
C	0.397 (184)	0.404 (478)	0.78	0.97 (0.78-1.21)	0.085	-

Table 4:

rs1800795 -174G>C	KOA Patients (n=232)	Controls (n=591)	p	OR (95%CI)	Chi-square	Possible Association
GG	0.358 (83)	0.332 (196)	0.513	1.12 (0.82-1.54)	0.507	-
CC	0.151 (35)	0.140 (83)	0.74	1.09 (0.71-1.67)	0.147	-
GC	0.491 (114)	0.528 (312)	0.353	0.86 (0.64-1.17)	0.891	-

Effect of microRNA-149 on IL-6 expression by connective tissue cell lines

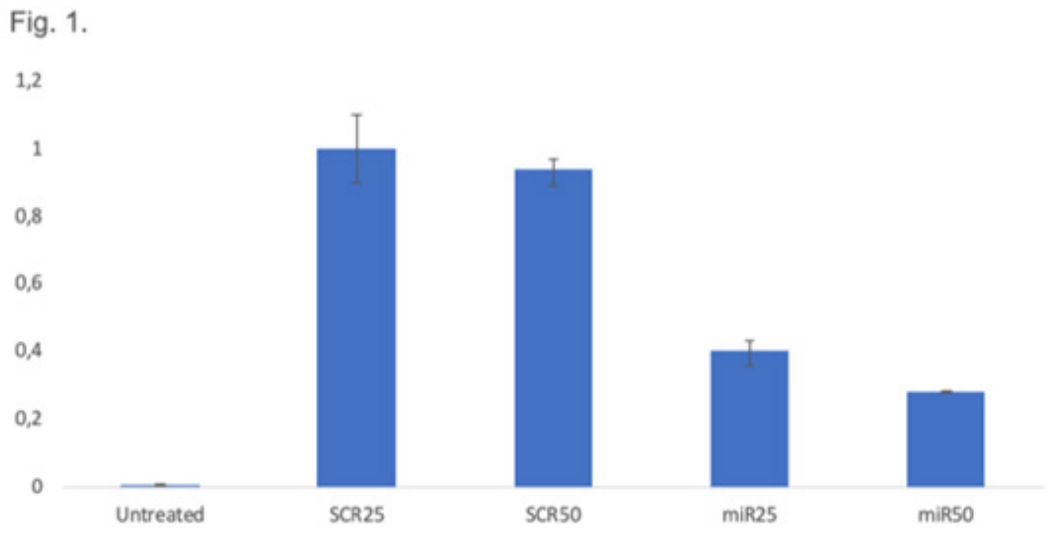

Figure 1: Quantitative real-time (qRT) polymerase chain reaction (PCR) measurement of Interleukin-6 (IL-6) gene transcripts in SW-1353 fibroblasts transfected with microRNA. SCR, indicates scrambled control; miR, denotes microRNA-149 at indicated nM concentrations. The y-axis shows relative quantity of the mRNA content.

To investigate the effect of miR-149 on the level of IL-6 produced by cells of the connective tissue, we transfected HaCaT (keratinocyte) and SW-1353 (fibroblast) cell lines with hsa-miR-149-5p mimic. We then measured the quantity of IL6 mRNA by qRT-PCR, and the amount of IL-6 protein by ELISA. The SW-1353 cells showed a reduction in the transcription (Figure 1) compared to a scrambled control (25nM), being 57% for the 25nM, and 69% for the 50nM miR-149 (Figure 1).

Figure 2 shows detection of IL-6 gene expression by ELISA in SW-1353 cells. We found a notable decrease in IL-6 protein level with a mean of 60.2% for the cells transfected with 25nM miR-149, and 72.4% for the cells transfected with 50nM miR-149. These findings indicate that miR-149 has reducing effects on IL-6 expression in SW-1353 fibroblasts at both, transcription and translation levels. For transfected HaCaT cells, only a trend towards downregulation of the IL6 transcripts caused by miR-149 (data not shown) was observed. Likewise, using ELISA for detection of IL-6 protein, we find no significant differences (data not shown) (Figure 2).

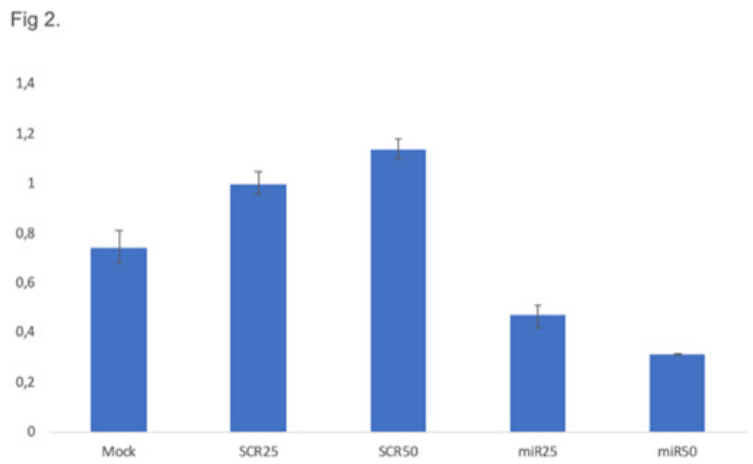

Figure 2: Enzyme-linked immunosorbent assay (ELISA) determination of Interleukin-6 (IL-6) protein in SW-1353 fibroblasts transfected with microRNA. SCR, indicates scrambled control; miR, denotes microRNA-149 at indicated nM concentrations. The y-axis shows relative quantity of the protein content.

Correlation with other studies

Our results indicate that the major allele (G) of the IL6 gene rs1800795 SNP might be a marker for predisposition to development of HOA in the Croatian Caucasian population. The rs1800795 minor allele (C) is possibly a marker for protection in developing HOA (Table 1). Furthermore, homozygous individuals carrying rs1800795 GG genotype have approximately 53% higher risk in developing HOA. Individuals with heterozygous GC genotype of rs1800795 have about 30% less chance to develop OA (Table 2). In contrast, the results from KOA case-control group indicate that IL6 rs1800795 SNP has no association with the risk in developing knee osteoarthritis in Croatians (Tables 3,4). Differences in the genetic risk between two large joint OAs were observed previously in the studies with IL17 and IL1 gene polymorphisms [39,41].

At the allele level (Table 1), our findings are in agreement with the study by Pola et al. [28] in the Italian population. At the genotype level, we could not detect a statistically significant association between the CC genotype and a decreased risk of developing KOA in our samples (Table 4), as was found in the study in the Italian population [28]. We did, on the other hand, find a possible protective role of the genotype GC, and a possible predisposing role of the genotype GG for HOA (Table 2), whereas the Italian study did not show statistical significance for these genotypes [28].

Regarding KOA cases, studies by Honsawek et al. [27] and Sun et al. [24] of the Thai and Han populations, respectively, found the CC genotype and the C allele to be potentially predisposing factors for developing KOA in contrast to our results (Tables 3,4). An explanation might be the difference in allelic frequencies between studied populations. While the European Caucasian population is quite polymorphic for the rs1800794, with a G/C ratio of 1.25:1 [26], the Asian population is almost monomorphic to the G allele. Another reason could be potential differences in inclusion criteria for the cases and controls. Our study, as well as the study by Sun et al., recruited patients undergoing artificial joint replacement, whereas in the study by Honsawek et al., the patients were recruited based on radiographs evaluated according to the Kellgren-Lawrence classification. Regarding controls, in the studies by both Honsawek et al. and Sun et al. the control group and the case group were similar in both number, gender and age. In addition, the control group from the study by Sun et al. underwent x-ray analysis to rule out any subjects with radiological sign of OA. In our control group, we chose to include even subjects without information on gender and/or age. This may have skewed our results towards a conservative conclusion. A genome-wide association study using data from the UK Biobank did not identify rs1800795 as a locus significant for susceptibility to HOA and/or KOA. A reason for this

may be the much higher significance threshold used (i.e. p<5´10-8) [26].

In the present study, we found significant association between the rs1800795 polymorphism located within the IL-6 gene and susceptibility to HOA. However, although less likely, this association may also point to genes neighbouring the IL6 gene, or perhaps some unknown cis-regulatory element in its vicinity, because OA is a multifactorial disease. As we cannot explain why there are large-joint (knee-hip) OA differences at all, we imagined that they might be due to putative local factors affecting the expression of particular inflammatory genes (which in turn would contribute to OA pathology). Hypothetically, the local change in a regulating factor, like for example a specific microRNA, could make a difference in aetiology between the hip and knee OA. Thus, we tested the influence of one microRNA known to influence the expression of the IL6 gene – miR-149 [43]. The miR-149 was analysed for its influence on the IL-6 mRNA production in the fibroblast (SW-1353) cell line, hopefully drawing a parallel to the local environment in a large-joint capsule tissue. In the present report, the IL6-gene expression can be regulated at the transcriptional and translational levels

(Figures 1 and 2). We propose that the variations in concentrations of microRNAs that regulate the local IL-6 expression are causing anatomical differences in primary OA. Support for our conjecture could be found in the work of Frank-Bertoncelj et al, demonstrating that epigenetic factors can guide joint-specific fibroblast functions and mark anatomical diversity of synovial fibroblasts [44]. Further, Latourte et al. described an inhibition of the IL-6 signaling as a protection against experimental OA [45], suggesting a pathophysiological role of IL-6 in its development.

Elevated levels of IL-6 have been found in both serum and synovial fluid in patients suffering from OA [6, 7], and IL-6 has been suggested as a potential biomarker of knee radiographic OA (together with TNF-alpha), and also as a predictor of the progression and outcome of the disease [8, 46]. IL-6 trans-signaling was found to directly and indirectly (with the help by TNF-alpha and IL-17) induce factors like RANKL on fibroblast-like synovial cells [15] supporting hypothesized pathological role of the IL-6 cytokine. However, there is a controversy, because male IL6 gene knockout mice get more advanced OA upon aging [47], suggesting the opposite (protection). Unless these transgenic knockout mice have more defects in genome due to genetic manipulation, in addition to the targeted IL6 gene, we have no rational explanation for this disagreement.

Conclusion

The frequencies of the IL-6 rs1800795 SNP alleles and genotypes were significantly different in HOA, compared to controls, in the Croatian population. However, these comparisons were not significant at all, when extended to KOA patients. We hypothesize that intra-synovial differences (i.e., hip versus knee) in concentrations of microRNA regulating IL-6 production is additional, discriminating risk factor for the localization of the primary OA. The molecular events involved might resemble our in vitro results (Figures 1 and 2), which show miR-149 -mediated downregulation of the IL-6 expression in fibroblasts.

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Conflict of Interest

The authors declare that there are no professional or financial conflict of interest associated with the manuscript.

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