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Chapter 6

Genetic contribution to development of radiographic knee osteoarthritis in a population presenting with non-acute knee symptoms a decade earlier

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Abstract

In this study the contribution of the OA susceptibility genes \textit{ASPN}, \textit{GDF5}, \textit{DIO2} and the 7q22 region to radiographic development of knee osteoarthritis (OA) in patients with a mean age of 40.6 years ± 7.9 (SD) who suffered from non-acute knee complaints a decade earlier was examined. Dose response associations of 4 SNP’s on the susceptibility genes were determined by comparing 36 patients who showed development of OA on radiographs (Kellgren&Lawrence score ≥1) with 88 patients who had no development of OA on radiographs and normal cartilage. Multivariate logistic regression analysis including the variables age, gender, body mass index (BMI) and reported knee trauma was performed. A dose response association of \textit{DIO2} SNP rs225014: odds ratio (OR) 2.3, 95% CI 1.1-4.5 (P=0.019) and \textit{GDF5} SNP rs143383: (OR) 2.0, 95% CI 1.1-3.8 (P=0.031) was observed with knee OA development. The \textit{ASPN} and 7q22 SNPs were not associated with OA development.
Introduction

Osteoarthritis (OA) of the knee is a common cause of musculoskeletal disability and is characterized by late-onset degeneration of articular cartilage, which is marked by the recapitulation of OA articular chondrocytes to a growth plate morphology and signalling and breakdown of matrix proteins. This leads to the development of fibrillations, fissures and ulcerations at the articular cartilage surface most sensitively detected by magnetic resonance (MR) imaging. To detect common underlying pathways that affect susceptibility, large scale genetic studies have been performed and have revealed a considerable number of robust OA susceptibility gene products which appear to be active in a shared pathway involving the developmental process of endochondral ossification. The susceptibility genes ASPN, GDF5, DIO2 and the 7q22 locus are considered consistent early OA susceptibility signals that have shown replication in several distinct studies for OA development and additionally showed functional follow up data that provided insight into underlying disease mechanisms. Progression of disease could result in reactivation of genes involved in endochondral ossification, leading to loss and mineralization of articular cartilage, a process known to contribute to OA.

The aim of this study is to examine the contribution of the risk alleles of ASPN, GDF5, DIO2 and the top SNP of the 7q22 locus to radiographic development of knee OA in a relatively young study population that presented with non-acute knee symptoms a decade earlier by comparing patients with radiographic OA development and patients without signs of OA in the knee on radiographs and MR images.

Methods

Study population

Study design: Case-control, Level of Evidence: 3. The current study was approved by the Medical Ethics Review Boards and written informed consent of each participant was obtained. The current study is a follow-up of a trial performed 10 years ago. The initial study consisted of 856 patients (mean age 31 years ± 8.0 standard deviation (SD)) with non-acute knee complaints, defined as persistent knee complaints i.e. pain, swelling and instability, lasting more than four weeks. After 10 years ± 0.90 (SD), all 856 patients of the initial study were contacted and invited for follow-up
To obtain DNA, all initial eligible 326 follow-up participants were contacted and sent a saliva collection container for DNA extraction. Eventually, 217 patients (67%) could be included. Of the 109 lost subjects, 21 patients refused participation and 88 did not respond to the contact letter sent by mail, a second letter sent after 1 month and three contact attempts by telephone. MR images and radiographs of the initial symptomatic knee were taken at inclusion and after 10 years. The presence of on radiographs detectable OA features was compared between the baseline and follow-up images. None of the patients showed radiographic knee OA at baseline. Due to different scanning techniques and other scoring methods used at baseline and after 10 year follow-up, the MR outcomes from baseline and follow-up outcomes could not be compared accurately to assess OA development. Therefore, a certain degree of OA development in those patients without radiographic OA development but with cartilage defects visible on MR images could not be ruled out. In order to compare patients with radiographic OA development to a control group without any signs of OA development, only patients without cartilage defects visible on MR images were used as controls. Ultimately, a total of 124 (15%) patients were included in the current study (Figure 1).
Figure 1 Flowchart response to follow-up

**INITIAL STUDY**
856 patients
mean age 31 years ± 8; 33% Women

5 died
21 excluded
87 refused participation

**10 year FOLLOW-UP STUDY**
326 patients eligible to participate
33% Women

21 refused DNA collection
88 non-responders

**PRESENT STUDY**
DNA collected in 217 patients
Mean age 42.1 years ± 8
33% Women

No radiographic OA development but cartilage defects present on MR images
N=84
Mean age 44.2 years ± 7.2
29% Women

No follow-up radiographs available N=9
Mean age 41.6 years ± 8.0; 22% Women

**OA DEVELOPMENT & CARTILAGE DEFECTS**
N=36
Mean age 44.5 years ± 6.7
31% Women

**NON-OA DEVELOPMENT & NO CARTILAGE DEFECTS**
N=88
Mean age 39.1 years ± 7.9
40% Women

DIO2 rs225014 C allele frequency
ASPN rs13301537 T allele frequency
GDF5 rs143383 T allele frequency
7q22 locus rs3815148 C allele frequency
Radiographic knee examination and assessment

Standardized weight bearing posterior-anterior knee radiographs next to supine lateral radiographs of the knee were made at baseline and at 10 year follow-up. At baseline one of six musculoskeletal radiologists with at least four years of experience scored the radiographs for overall severity of OA using the K&L system 15. The follow-up radiographs were scored by an experienced musculoskeletal radiologist and a research fellow using the same K&L scoring method 14. Individual development of OA was obtained by comparing the baseline K&L score to the follow-up K&L scores. Development of OA was considered to be present at a K&L score of 1 point or more.

Knee MRI

MRI examinations after 10 years of the initial affected knee were performed on a 3 T system (Achieva 3T, Philips Medical Systems, Best, the Netherlands). Due to different MRI scanning techniques used at baseline and at 10 year follow-up, only the follow-up scans were used to assess cartilage defects. Focal cartilage defects were defined as an abrupt transition between the defect and surrounding cartilage, a diffuse cartilage defect was defined as a gradual transition between normal and thinned cartilage 16. Cartilage defects outcome scores were used in a binary (absent vs. present) fashion.

DNA

DNA from patients was obtained using saliva collecting containers (Oragene OG-250, DNA Genotek, Ontario, Canada). Recent studies have shown that the use of saliva samples is a good alternative to blood samples to obtain genomic DNA of high quality 17. The containers were sent to all 326 eligible patients including instructions for use. All patients were asked to wash their mouth once with water and to wait at least 30 seconds. Then, the patients were asked to spit in the white container, to cap the container with the blue lid, and finally to gently shake the sample. The Oragene saliva samples were stored according to manufacturer at room temperature until DNA extraction. On average saliva samples were stored for 1.5 month.
DNA was extracted from saliva samples using the Oragene kit (DNA Genotek) as described by the manufacturer. The Oragene saliva samples were incubated at 50°C for 2 hours. The 0.5 ml samples were transferred to a 1.5 mL Eppendorf tubes; 20 μL of Oragene purifier was added, and the sample was mixed by inversion and incubated on ice for 10 minutes. The samples were then centrifuged in a small, bench top microfuge for 3 minutes at 18,000 g at room temperature and the supernatant was transferred to a new tube. 0.5 mL of 95% ethanol was added; the samples were mixed by inversion at least five times and incubated for 10 minutes at room temperature. The samples were then centrifuged in a small, bench top microfuge for 1 minute at 18,000g at room temperature, the supernatants were discarded, and the DNA was dissolved in 100 μL TE buffer (10 mmol/L Tris-HCl, 0.1 mmol/L EDTA (pH 8.0)) and quantified. The DNA samples were stored at 4°C until PCR analysis.

For the candidate gene association study we selected 4 SNPs on 4 candidate genes ASPN, GDF5, DIO2 and the 7q22 locus. These susceptibility genes have been shown to replicate in several distinct studies for primary OA. In the current study we hypothesize that these susceptibility genes already contribute to development of radiographic knee OA in relatively young patients suffering from knee complaints. The P-values of Hardy-Weinberg test were for DIO2: 0.385, for GDF5: 0.916, for ASPN: 0.033 and for the 7q22 locus: 0.242. All genotypes were included in the equation. Following is a description of the 4 candidate genes selected for the current study.

DIO2

Iodothyronine deiodinase enzyme type 2 (DIO2) is a regulator of thyroid hormone metabolism in the growth plate, where thyroid hormone triggers terminal maturation of growth plate chondrocytes. DIO2 has recently been shown to be a susceptibility gene for primary OA. In a genome-wide linkage scan and association analysis an association was found between OA and the minor C allele of SNP rs225014. We hypothesize that the DIO2 gene also contributes to development of radiographic knee OA in the current study population.

ASPN
Asporin (ASPN) inhibits transforming growth factor-β (TGF-β), which has a crucial role in the development and homeostasis of cartilage. ASPN is expressed at low levels in normal cartilage but is expressed abundantly in OA articular cartilage. Due to polymorphisms in the ASPN gene, variant ASPN proteins arise with a variable number of aspartic acid (D) repeats in the amino-terminal end of the protein. The T allele of SNP (rs13301537) is associated with variants of the ASPN gene encoding 13 (D13) and 14 (D14) aspartic acid repeats. In a Japanese and Han Chinese population, but not in a Caucasian population, D14 was associated with knee OA and in a Caucasian population D13 was associated with a decreased risk of knee OA.

**GDF5**

Growth differentiation factor 5 (GDF5) is a bone morphogenetic protein (BMP) involved in early development of joints in embryonic tissues and is expressed throughout the synovial joint tissues during life. The SNP (rs143383) is the most widely replicated genetic association with knee OA, with the risk-associated T allele showing reduced expression relative to the C allele in OA. The GDF5 rs143383 polymorphism is associated with knee OA in both Asians and Caucasians.

**7q22 locus**

Conserved oligomeric Golgi complex subunit 5 (COG5) was recently discovered in a genome-wide association study as a novel gene involved in OA. The C allele of SNP (rs3815148) was associated with an increased risk of knee OA.

**SNP genotyping**

Genotyping was carried out in 217 samples at OA susceptibility SNPs from 4 genes: ASPN, GDF5, DIO2 and the 7q22 locus. Selected SNPs were fit in a Sequenom multiplex assay designed by the Assay Designer software version 3.1 (Sequenom, San Diego, CA). SNPs were genotyped by mass spectrometry (the homogeneous MassARRAY system; Sequenom, San Diego, CA) using standard conditions. PCR reactions were carried out in a final volume of 5 µl and contained 2.5 ng of genomic DNA. Genotypes were assigned using Genotyper version 3.0 software (Sequenom, San Diego, CA). The genotyping success rate was 98.02% ± 0.75 (SD). Internal genotyping controls
were included, with a concordance rate of 100%. Genotype frequencies for the tested SNPs were all in Hardy-Weinberg equilibrium (Table 1).

Statistical methods
A case-control approach was also used to assess the association between the susceptible risk allele frequencies of the of the 7 selected SNP’s in patients with knee OA development and in patients without radiographic development of knee OA. Radiographic development of OA was defined a K&L score of ≥1. Patients who did not show radiographic OA development, but who did have cartilage defects detected on the follow-up MR images were excluded from further analysis (Figure 1). A logistic regression model was fitted to measure the strength of the association with the different genotypes (dose response), which is expressed as odds ratios (OR) with 95% confidence intervals (95% CI). In these analyses we adjusted for age, gender, body mass index (BMI) and reported knee trauma. P-values < 0.05 were considered to indicate statistical significance. In order to assess the discriminating power of the different genotypes studied, we generated receiving operator curves (ROC) using knee development as outcomes and age, sex, BMI and trauma as conventional risk factors.

Results
Population characteristics
Of the 124 patients included in the current study, 5 patients (4 %) were of non-Caucasian descent. At follow-up, the mean age of the 124 patients included for the association study was 40.6 years ± 7.9 (SD), 46 (37.1%) of the patients were female and the average body mass index (BMI) was 25.6 ± 3.5 (SD).

OA development
Of the 36 patients who developed radiographic knee OA after 10 years follow-up 27 had a K&L score of 1, and 9 had a K&L score of 2. Of the 172 patients without radiographic development of OA, 84 (48.8%) showed cartilage defects detected on MR images, and 88 (51.2%) did not. The latter patients were used as controls. Patients with radiographic development of knee OA were
significant older and had significantly higher BMI’s than the controls without OA development and without cartilage lesions (Table 1).

As shown in Table 1, the minor C allele frequency of the rs225014 SNP in the control group without OA development and without cartilage lesions was 0.341 whereas, in the study group with OA development the minor C allele frequency was 0.471. There was a significant association for the presence of the minor C allele of SNP rs225014 of the DIO2 gene and radiographic knee OA development (OR 1.8, 95% CI 1.00-3.18; P value 0.049). This effect increased when corrected for Age, BMI and Trauma (OR 2.3, 95% CI 1.14-4.49; P value 0.019). The GDF5 T-allele frequency of the rs143383 SNP was 0.366 in the control group without OA development and cartilage lesions and 0.444 in the OA development group. Corrected for Age, BMI and Trauma, there was a significant association for the presence of the T allele of SNP rs143383 of the GDF5 gene and OA development (OR 2.0, 95% CI 1.07-3.78, P value 0.031). The OA susceptibility SNP in ASPN and at the 7q22 locus did not show association (Table 1).

Finally, we investigated whether the genes associated to increasing aspects of joint destruction as measured by radiographs and/or MRI characteristics after 10 years as defined by subjects without radiographic OA nor cartilage lesions visible on MR images (N=88), no radiographic signs of OA but with cartilage defects visible on MR images (N=84), and radiographic OA with cartilage defects visible on MR images (N=36). DIO2 showed significant association (P Value 0.020) with increasing signs of cartilage destruction visible on both radiographs and MR images (Table 2).
<table>
<thead>
<tr>
<th>Variables</th>
<th>No OA development &amp; No Cartilage defects N = 88</th>
<th>OA development N = 36</th>
<th>Uncorrected OR</th>
<th>95% CI</th>
<th>Uncorrected P-Value</th>
<th>Corrected OR</th>
<th>95% CI</th>
<th>Corrected P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td>35 (39.8%)</td>
<td>11 (30.6%)</td>
<td>0.67</td>
<td>0.29-1.53</td>
<td>0.336</td>
<td>0.75</td>
<td>0.28-2.01</td>
<td>0.561</td>
</tr>
<tr>
<td>Age</td>
<td>39.1 ± 7.9 (SD)</td>
<td>44.5 ± 6.7 (SD)</td>
<td>1.10</td>
<td>1.04-1.17</td>
<td>0.001*</td>
<td>2.00</td>
<td>1.03-1.17</td>
<td>0.004*</td>
</tr>
<tr>
<td>BMI</td>
<td>25.2 ± 3.6 (SD)</td>
<td>26.7 ± 2.7 (SD)</td>
<td>1.14</td>
<td>1.02-1.28</td>
<td>0.019*</td>
<td>1.10</td>
<td>0.96-1.27</td>
<td>0.162</td>
</tr>
<tr>
<td>Trauma</td>
<td>54 (64.3%)</td>
<td>20 (60.6%)</td>
<td>0.86</td>
<td>0.37-1.96</td>
<td>0.710</td>
<td>1.18</td>
<td>0.46-3.05</td>
<td>0.733</td>
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<tr>
<td>DIO2 rs225014 C allele frequency</td>
<td>0.341</td>
<td>0.471</td>
<td>1.79</td>
<td>1.00-3.18</td>
<td>0.049*</td>
<td>2.27</td>
<td>1.15-4.49</td>
<td>0.019*</td>
</tr>
<tr>
<td>ASPN rs13301537 T allele frequency</td>
<td>0.244</td>
<td>0.300</td>
<td>1.19</td>
<td>0.65-2.18</td>
<td>0.575</td>
<td>1.20</td>
<td>0.53-2.7</td>
<td>0.659</td>
</tr>
<tr>
<td>GDF5 rs143383 T allele frequency</td>
<td>0.366</td>
<td>0.444</td>
<td>1.40</td>
<td>0.81-2.39</td>
<td>0.225</td>
<td>2.01</td>
<td>1.07-3.78</td>
<td>0.031*</td>
</tr>
<tr>
<td>7q22 locus rs3815148 C allele frequency</td>
<td>0.241</td>
<td>0.264</td>
<td>1.15</td>
<td>0.60-2.20</td>
<td>0.684</td>
<td>1.13</td>
<td>0.51-2.52</td>
<td>0.760</td>
</tr>
</tbody>
</table>

*Statistically significant difference. OA = osteoarthritis, OR = Odds ratio, CI = Confidence Interval, SD = Standard Deviation
**Table 2**

**Multivariate regression analysis outcomes of OA development and cartilage lesions visible on MR imaging**

<table>
<thead>
<tr>
<th>Variables</th>
<th>No OA development &amp; No Cartilage defects on MRI (N = 88)</th>
<th>No OA development but Cartilage defects on MRI (N = 88)</th>
<th>Radiographic OA development &amp; Cartilage defects (N = 36)</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>DIO2 rs225014 C allele frequency</strong></td>
<td>0.341</td>
<td>0.409</td>
<td>0.471</td>
<td>0.020*</td>
</tr>
<tr>
<td><strong>ASPN rs13301537 T allele frequency</strong></td>
<td>0.244</td>
<td>0.244</td>
<td>0.300</td>
<td>0.411</td>
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<tr>
<td><strong>GDF5 rs143383 T allele frequency</strong></td>
<td>0.366</td>
<td>0.378</td>
<td>0.444</td>
<td>0.206</td>
</tr>
<tr>
<td><strong>7q22 locus rs3815148 C allele frequency</strong></td>
<td>0.241</td>
<td>0.244</td>
<td>0.264</td>
<td>0.586</td>
</tr>
</tbody>
</table>

OA = osteoarthritis, OR = Odds ratio, CI = Confidence Interval. Outcomes were corrected for Age, Sex, BMI and Trauma.

*Statistically significant difference.
**Increased risk prediction of DIO2 and GDF5 genotypes**

To investigate the possibility to use the DIO2 and GDF5 genotypes as a predictive tool, we next performed a receiver operating characteristics (ROC) analysis. Two models were fitted with knee OA development after a non-acute knee symptoms as the outcome. Model one consisted only of age, gender, BMI and trauma, model two included age, gender, BMI, trauma and DIO2 and GDF5 genotypes. The predictive value of the anthropometric traits alone as reflected by the area under the curve (AUC) was 0.647 (95%CI 0.530-0.764), however, when genotypes of the DIO2 and GFD5 gene were added the AUC improved to 0.697 (95%CI 0.582-0.812) (Figure 2).

**Figure 2**

Receiver operating characteristics (ROC) analysis diagrams. The outcome is osteoarthritis (OA) development a decade after knee complaints. In Figure 2A the model included age, gender, trauma and BMI. In Figure 2B the model included age, gender, BMI, trauma, DIO2 and GDF5 genotypes.

*In Figure 2A the area under the curve (AUC) is 0.647 (95%CI 0.530-0.764). In Figure 2B the AUC is 0.697 (95%CI 0.582-0.812).*
Discussion

In the current study we showed that the DIO2 OA susceptibility SNP rs225014 and the GDF5 susceptibility rs143383 SNP are significantly associated with development of knee OA (OR 2.3; 95% CI 1.14-4.49 and OR 2.0; 95% CI 1.07-3.78, respectively) in a relatively young patient group with a mean age of 44 years with a history of knee complaints a decade ago. This effect appeared independent of other factors related to knee OA development such as knee trauma, age and BMI. Prediction of knee OA development improved from an average AUC of 0.647 for age, sex, BMI and trauma alone to an AUC of 0.697 when including DIO2 and GDF5 genotypes in the risk prediction model, almost reaching clinical relevant AUC value (≥0.7). Furthermore, an increased minor allele frequency of DIO2 is related to signs of OA development visible on both radiographs and MR images (P Value 0.020) and a similar trend is visible for GDF5, however, not significant (P Value 0.206).

These data are in line with recently published data showing that articular cartilage expression of DIO2 is epigenetically regulated and that particularly DIO2 rs225014 risk allele carriers are less able to maintain cartilage homeostasis due to the fact that subtle changes in methylation, generally occurring upon environmental changes such as micro traumas, resulted in detrimental upregulation of DIO2. Type II deiodinase (D2), expressed by the DIO2 gene regulates the bioavailability of intracellular T3 in specific tissues such as the growth plate and facilitates terminal maturation of hypertrophic chondrocytes. Functional genomic studies showed high expression of DIO2 mRNA and D2 protein levels in osteoarthritic as compared to healthy cartilage. Furthermore, DIO2 allelic imbalance was assessed and showed that the OA risk allele ‘C’ was more abundantly present in articular joint tissues than the wild-type allele ‘T’. Up regulation of DIO2 expression in a human in vitro model resulted in a marked reduction of the capacity of chondrocytes to deposit ECM components, including type II and type X collagen, while inducing OA-specific markers of cartilage matrix degeneration and mineralization. In mice undergoing a forced running regime it has been shown that DIO2 deficiency has a protective effect on the homeostasis of articular cartilage in the knee joints.
It may be that trauma at relatively early age affects the propensity of the highly specialized, maturational arrested articular chondrocytes to lose their maturational arrested state and loss of epigenic control, among others, of the DIO2 gene.

Our results show that the T allele of the GDF5 SNP rs143383 is significantly associated with development of knee OA with an OR of 2.0 (95% CI 1.07-3.78). This is in line with earlier findings that GDF5 rs143383 polymorphism is associated with knee OA in both Asians and Caucasians. Recent research showed that GDF5 stimulation of human chondrocytes inhibits expression of cartilage ECM degrading enzymes MMP13 and ADAMTS4 and stimulates the expression of cartilage anabolic genes ACAN and SOX9. GDF5 stimulation also inhibits the canonical Wnt signaling pathway through expression of the DKK1 and FRZB inhibitors. The Wnt signaling pathway plays an important role during cartilage development, and activation of the pathway in the adult cartilage tissue leads to hypertrophy, initiation of calcification and tissue degradation via increased expression of ECM degrading components. Therefore altered expression of the GDF5 gene may result in ECM degradation and decrease of cartilage quality which may induce OA development.

It is commonly accepted that a clinically useful diagnostic markers should have an AUC of 0.7 or higher. Adding DIO2 and GDF5 genotypes in the model resulted in a significant increase of the AUC from 0.647 to 0.697. The latter underscores, that although patient characteristics and environmental factors are important, genetic factors play a substantial role in knee OA development. Non of the investigated SNP’s of the ASPN, and the 7q22 locus genes were significantly associated with OA development in the current study population. These findings are in line with outcomes of recent studies which report conflicting evidence about these genes in OA development. In total five patients of non-Caucasian descent were included in this study, however, upon discarding these patients, DIO2 and GDF5 outcomes remained the same, indicating that these patients did not drive our associations and possibly that these genes confer risk to OA development in both Caucasian and non-Caucasian populations as previously shown.

Several limitations of the study should be mentioned. Due to different scanning techniques and other scoring methods used at baseline and after 10 year follow-up, the MR outcomes from baseline and follow-up outcomes could not be compared accurately to assess OA development.
Therefore, a certain degree of development in those patients without radiographic OA but with cartilage defects visible on MR images could not be ruled out. In order to compare patients with radiographic OA development to a control group without any signs of OA development, only patients without cartilage defects visible on MR images were used as controls. This restriction resulted in exclusion of 43% of the initial study group, leading to a significant smaller study population. Being aware of the tendency of association studies to produce false-positive results, additional replication is necessary. In the current study gender differences were not related to OA development. This may be explained by the fact that the female participants in the relatively young study population were mostly pre-menopausal.

In conclusion, the presence of the minor C-allele of the DIO2 SNP rs225014 and the T allele of the GDF5 SNP rs143383 are associated with OA development in a relatively young study population 10 years after knee complaints. Subsequent ROC analyses showed that determining DIO2 and GDF5 genotypes significantly improves risk prediction towards clinical relevant values.

Reference List


