Title of the Study: Investigation of novel genetic and epigenetic factors mediating osteoarthritis

Study objectives, background, and design

Osteoarthritis (OA) affects more than 27 million people in the United States alone (Lubar et al. 2010) and approximately 40% of OA patients are older than 70 years (Zeggini et al., 2012). OA causes pain and loss of joint function and unfortunately, there is no fundamental treatment for this disorder (Dai J. and Ikegawa S. 2010). Age and genetic factors strongly contribute to the development of primary osteoarthritis, for which the direct cause is still unknown. The rationale for this proposal is that once the novel genetic and epigenetic factors for OA are known, the effect of these factors in the affected population could be analyzed and new methods of treatment explored based on the function of the coded proteins.

Objective 1
Our first objective is to verify if mutations in the new gene for multiple epiphyseal dysplasia (MED) also contribute to osteoarthritis. MED is a common genetic disorder of the skeleton and significantly overlaps genetically and phenotypically with osteoarthritis. We have strong preliminary data from linkage analysis, indicating a new candidate gene for this disorder. Our working hypothesis is that MED and OA share the same genetic background and to test our hypothesis we will analyze this new gene in 50 patients with primary osteoarthritis from Louisiana. The rationale for this objective is that, once we have identified the new gene contributing to OA, we can study its function and work towards a new treatment for this disorder. When the proposed study will be completed, it is our expectation to detect changes at the sequence level of the new gene in OA patients from Louisiana.

Objective 2
Our second objective is to identify genes affected by telomere shortening in human chondrocytes through expression profiling. We will measure relative telomere length in affected and unaffected chondrocytes collected from the same joint of 40 patients with knee OA. Based on these measurements we will select 20 tissue samples from ten individuals for RNASEq. Our working hypothesis is that patients with OA have shorter telomeres in affected chondrocytes and this causes over-expression of telomere-proximal genes, which contributes to OA. During the total knee replacement arthroplasty, articular cartilage that is routinely discarded will be placed in sterile containers. Articular cartilage samples will be harvested from areas with the most advanced degenerative changes and the intact area, which will serve as the control. The rationale for this objective is that, once the over-expression of telomere proximal genes is proven in chondrocytes with accelerated telomere shortening, we will be able to analyze the relationship between over-expressed genes and osteoarthritis.
Subject population

Patients will be informed about the study during the pre-operative visit for their total knee replacement. If agreeable, patients will be identified as potential participants and asked to enroll using informed consent. Fifty patients from the Department of Orthopedics, Louisiana State University who have been diagnosed with idiopathic osteoarthritis (OA) will be selected for these studies over the next year. Testing will be performed at Ochsner Medical Center in Kenner, Louisiana at the time of surgery to minimize patient discomfort. On the day of surgery "Anonymous Patient Data Collection Sheet" will be completed and blood will be drawn once the patient is under general anesthesia.

Subject testing will include:
- Demographics
- Medical History
- Anthropometrics
- Peripheral blood draw (10 ml)
- During the total knee replacement arthroplasty, articular cartilage that is routinely discarded will be placed in sterile containers. Articular cartilage samples will be harvested from areas with the most advanced degenerative changes and the intact area, which will serve as the control.

A subject identifier will be assigned to each subject; this number will identify all relevant samples and data. The subject identifier key will be kept separate from the study data in a secure file in a secure room. The patient population at Louisiana State University includes a broad range of adults of various races, ethnicities, genders, and ages (50-75 years). Race, gender, and/or ethnicity will not serve as criteria for either inclusion or exclusion. Patients included in this study reside within the Greater New Orleans Metropolitan area and surrounding parishes.

The blood and cartilage samples will then be transported to the Tulane University Center for Aging Laboratory, where the genomic DNA from the blood leucocytes and cartilage as well as RNA from cartilage will be extracted, and the molecular testing will be performed.

Procedures

A. Recruitment of subjects: Subjects were recruited as described in the subject population section above. Race, gender, and/or ethnicity will not serve as criteria for either inclusion or exclusion. We used the following criteria for the original testing:

Inclusion Criteria
1. Louisiana residents.
2. Diagnosis of primary osteoarthritis

Exclusion Criteria

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1. Patients who have confounding pathologic states (i.e. infection, cancer and avascular necrosis),
2. Secondary osteoarthritis and inflammatory arthropathy. The diagnosis of idiopathic knee osteoarthritis will be based on: patient’s medical history evaluation, physical examination, X-rays of knees (subchondral bone sclerotisation, joint space narrowing, osteophytes, subchondral cysts) and laboratory tests (erythrocyte sedimentation rate and CRP - C reactive protein).

B. Collection of biological samples and molecular analysis:
1. Collection of blood: Peripheral blood draw: approximately (10 ml) of blood will be drawn from each subject via venipuncture.

2. Collection of cartilage: during the total knee replacement arthroplasty, articular cartilage that is routinely discarded will be placed in sterile containers. Articular cartilage samples will be harvested from areas with the most advanced degenerative changes and the intact area, which will serve as the control.

3. Molecular analysis:

Objective 1
Analysis of the new candidate gene for osteoarthritis in 50 unrelated individuals with OA from Louisiana will be completed in the Center for Aging by direct sequencing of PCR products using a dGTP BigDye Terminator v3.0 Ready Reaction Cycle Sequencing Kit and an ABI Prism 3130 Sequencer (Applied Biosystems/HITACHI).

Although our preliminary data, the current literature, and the overlapping phenotype strongly indicate that a new gene for MED can be linked to OA, there is a possibility that we will not detect changes in patients with OA. In this case we will focus on the screening of the known candidate gene MATN3 in patients with OA from the Louisiana population. MATN3 Mutations identified in patients with MED (Jackson et al., 2004) and common forms of OA (Stefánsson et al., 2003), suggest that these two disorders are genetically related.

Objective 2
Our in vivo approach will be to use RNA sequencing to analyze expression of telomere-proximal genes in cartilage of patients with knee osteoarthritis. First, we will measure relative telomere length in affected and unaffected chondrocytes collected from the same joint of 40 patients with knee OA. Our approach will be to use a quantitative polymerase chain reaction (q-PCR) as described by Cawthon, 2002 and Terry et al., 2008 with modifications to measure relative telomere length. Isolation of genomic DNA from cartilage will be performed with Wizard SV Genomic DNA Purification System (Promega). To estimate telomere length, two qPCRs will be performed on each DNA sample by using ABI 7300 real-time PCR system: a single-copy-gene specific q-PCR to β-globin and a telomere-specific q-PCR. A telomere/single-copy gene (T/S) ratio will be calculated for each sample using the q-PCR results and normalized to the T/S ratio of a reference sample. This normalized T/S ratio will be used as an estimate of the relative
telomere length. From these experiments we will obtain measures of the relative telomere length in affected and unaffected cartilage, and also a ratio of relative telomere length between affected and unaffected cartilage. We will use the paired t-test to determine if relative telomere length differs between affected and unaffected cartilage.

Next, we will identify genes affected by telomere shortening in human chondrocytes through expression profiling. Our working hypothesis is that patients with OA have shorter telomeres in affected chondrocytes and this causes over-expression of telomere-proximal genes, which contributes to OA. Our approach will be to analyze via RNA-Seq affected and unaffected cartilage tissue from the same joint of ten patients with knee OA with varying degrees of telomere shortening. We will select five patients with very short telomeres in affected cartilage and five patients with relatively long telomeres in affected cartilage. In addition, we will have control (unaffected cartilage from the same joint) for each of the selected patients. We will compare expression of telomere proximal genes with the grade of osteoarthritis and telomere length. RNA-Seq will be performed with the Ion Proton System (Ion Torrent/Life Technologies) in the Center for Aging, where this project will be conducted. The RNA-Seq procedure starts with total RNA isolation from the cartilage tissue by using PureLink RNA Mini Kit (Ambion). We will add to total gRNA a set of external RNA controls (ERCC Ex-Fold RNA Spike-In Mix, Ion Torrent) that enable performance assessment of a variety of technology platforms used for gene expression experiments. The depletion of up to 99.9% of the 5S, 5.8S, 18S, and 28S ribosomal RNA from total RNA (10-500 ng) will be performed with the RiboMinus™ Eukaryote System v2 (Ambion). Next, the whole transcriptome is fragmented by using enzyme RNase III. We will quantify the yield of fragmented RNA using the Qubit Fluorometer and Qubit RNA Assay Kit (Invitrogen).

The next step is a library preparation with Ion Total RNA-Seq Kit v2 (Ion Torrent), which contains reagents for RNA-based library construction and generates a representative cDNA library for discovery of small RNAs and isoforms, coding RNA, non-coding RNA, and alternative splice variants, while preserving strand orientation. We will hybridize and ligate up to 100 ng of the rRNA depleted total RNA with the Ion Adaptor Mix (Ion Torrent), which is a set of oligonucleotides with a single-stranded degenerate sequence at one end and defined sequence required for the Ion Proton at the other end. Then we will perform reverse transcription, purification and amplification of the cDNA. Amplification of cDNA will be conducted with barcoded primers by using Ion Xpress RNA-Seq Barcode 01–16 Kit (Ion Torrent). Barcoded cDNA libraries will allow us to pool libraries from different individuals and analyze them on one chip. We will assess the yield of the amplified cDNA with Qubit Fluorometer (dsDNA HS Assay Kit, Invitrogen) and the size distribution on agarose gel.

A template preparation will be performed with Ion PI Template OT2 200 Kit (Ion Torrent). In this step, each library template is clonally amplified on Ion Sphere Particles (ISPs). The Ion PI Template OT2 200 Kit is the integral component of the Ion OneTouch(TM) 2 System, designed to make high quality templated beads for sequencing on the Ion Proton. After amplification we will recover and wash the template-positive Ion Sphere Particles. Next we will enrich them with the Ion OneTouch ES system and wash the enriched ISPs. We will determine the appropriate library dilution and/or the enrichment
efficiency by using the Qubit Fluorometer (Invitrogen). Ion PI Sequencing 200 Kit (Ion Torrent) is an integral component of the Ion Proton(TM) sequencing workflow and is designed to sequence Ion Sphere Particles deposited on Ion Proton I Chip in 2 hours. Ion Proton I Chip is a high-density array of wells (165M) used to perform massively parallel sequencing. We will be able to obtain 60-80 million aligned reads per run on Ion Proton I Chip (Ion Torrent).

C. Collection of basic demographic and anthropometric data

To gather information on patients, the following data will be collected:

1. Questionnaire:

   Anonymous Patient Data Collection Sheet (Surgeon: Dr. Dasa)

   Patient’s ID: ________________

   Date of Surgery/Collection: ________________

   Articular cartilage from the knee, ID: ________________

   Blood, ID: ________________

   Patient Age (Years): ________________

   Patient Gender (Circle): Female Male

   Place of birth (country, state) ________________

   How long has patient had symptoms of osteoarthritis? ________________

   Indicate age of patient when diagnosis of osteoarthritis was confirmed____

   Kellergen-Lawrence Score (Circle): 1 2 3 4

   Outerbridge Score (Circle): 1 2 3 4

   Patient Ethnicity (Circle):
   - African American
   - Asian
   - Caucasian
   - Hispanic
   - Other: ________________

   Patient Height (Feet/Inches): ________________

   Patient Weight (Pounds): ________________

2. Banked specimens

   a. DNA
   b. Cartilage

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D. Analysis of data: The genetic data will be analyzed using appropriate genetic methods and statistical tools.

Risks
The risks associated with blood drawing by venipuncture include a short moment of discomfort, infection, and ecchymosis at the needle injection site. There is a risk that the confidentiality of the subjects' personal, medical, and genetic information collected could be compromised. The survey involves minimal risk to participants.

Protections Against Risk
Phlebotomy:
Anesthesiologists will use an aseptic technique and universal precautions to minimize pain and the risk of bruise formation and infection. Subjects will be under general anesthesia during the blood draw to avoid dizziness, syncope, or loss of consciousness. We will draw 10 ml of blood for this study. Phlebotomy will be performed at Ochsner Medical Center in Kenner, Louisiana. This is a location where a rapid, appropriate response to an emergency is possible.

Psychological/Social Risks:
All patients will be assured of the confidentiality of their specimens and data both verbally and in the informed consent. The archiving of DNA and RNA and the potential availability of this material, along with limited information, to other investigators will be explicitly described in the informed consent process. HIPAA authorization will be separately obtained from each patient. To ensure confidentiality and anonymity during the study, each subject will be assigned a confidential study number. Access to the subject study identification codes or other information is restricted to the clinician. Access to data or materials generated from this study for other researchers will be permitted only after the data and materials have been adequately de-identified so as to not compromise subject confidentiality. Patients will be informed that the results may be published in a manuscript, but their identities will remain anonymous.

Benefits
The knowledge gained through this project may benefit society by identification of genetic factors associated with osteoarthritis. The discovery of a gene that has influence on osteoarthritis development may improve our understanding of the pathogenesis of this disease, may lead to earlier diagnosis and treatment, and may lead to the development of new treatments. Finally, testing for osteoarthritis susceptibility alleles may have prognostic value and allow physicians to more effectively guide medical therapy.

Remuneration
There will be no compensation for this study.

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Costs
There will be no costs to the subject for participating in this research study.

Alternatives
The subjects do not have to participate in the research.

Consent process and documentation
Recruitment and Informed Consent:
All protocols incorporated in this study will be approved by the Tulane University, Louisiana State University, and Ochsner Institutional Review Boards. A group of patients with idiopathic osteoarthritis without evidence of secondary osteoarthritis, infection or cancer will be recruited from the patients of the Louisiana State University orthopedic practice in Kenner, Louisiana. Dr. Vinod Dasa, an orthopedic surgeon, will speak with patients preoperatively providing a description of the study in layman’s terms (research purpose, personnel, procedures, risks and benefits to each participant) and will obtain consent following that conversation. The consent process will be an ongoing process, and patients have the opportunity to withdraw their participation in the study at any time. Afterwards, "Anonymous Patient Data Collection Sheet" will be completed (attached above).

Qualifications of the investigators
Dr. Dasa, who will be responsible for clinical part of this project, principal investigator and other study personnel are professionals with credentials and many years of interest and experience.

Bibliography

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