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Linkage Mapping and Whole Exome Sequencing Identify a Shared Variant in *CX3CR1* in a Large Multi-Generation Family

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ABSTRACT

Developmental dysplasia of the hip (DDH) is a crippling condition that affects children and adults, with an average incidence of 1–1.5 cases per 1000 live births. It results in disabling arthritis of the hip in up to 60% patients in the 20–40 year age group. There is no accurate diagnostic test available for newborns. The purpose of our study is to develop a sensitive and specific genetic test for DDH by identifying causative mutations. Linkage analysis and whole exome sequencing of 4 severely affected individuals of a 4 generation 71 member family was performed. The damaging rs3732378 variant in the CX3CR1 chemokine receptor was shared by all affected family members and by 15% of 28 sporadic dysplastics.

Developmental dysplasia of the hip (DDH) is a debilitating condition characterized by incomplete formation of the acetabulum leading to dislocation of the femur, suboptimal joint function, and accelerated wear of the articular cartilage resulting in arthritis [1]. DDH affects 1–1.5 in 1000 newborns in the United States with well defined "pockets" of high prevalence in Japan, Italy and other Mediterranean countries [2]. Because of its high prevalence and undesirable consequences, screening programs involving manipulation of the femur or ultrasound imaging of the hip in infants are in place in most countries [3]. Although reasonably accurate for detecting gross forms of hip dysplasia, existing screening techniques fail to find milder forms of dysplasia [4]. Undetected hip dysplasia is the leading cause of osteoarthritis of the hip in young individuals causing over 40% of cases in this 20–40 year old age group [4]. A sensitive and specific test for DDH has remained a desirable yet elusive goal in orthopaedic medicine for a long time.

DDH is a complex disorder having an etiology that is both environmental and genetic [5–7]. Environmental risk factors include breech presentation (with feet toward cervix), oligohydramnios (deficiency of amniotic fluid), and primiparity (first born) [3,8,9]. Evidence for a genetic cause is well established and includes a higher concordance between monozygotic (41%) than dizygotic (2.8%) twins, and a 12-fold increase of DDH among first degree relatives of those affected by the disorder [10,11]. DDH appears to be transmitted in an autosomal dominant fashion in several families and, perhaps because of its complex etiology, exhibits incomplete penetrance [12]. Our hypothesis is that DDH affected individuals have mutation(s) or genetic variants that make them susceptible to the disorder. The goal of this study is to identify genetic susceptibility factors for DDH, and in so doing, lay the foundation for a genetic test to accurately identify susceptible newborns so that intervention with a device such as a Pavlik harness can be used to allow complete development of the acetabular labrum. Should this goal be attained our understanding of molecular pathways responsible for acetabular development will be enhanced.

Methods

Clinical Diagnostic Criteria

Before the initiation of this study, approval was obtained from the Institutional Review Board of Thomas Jefferson University and informed consent was obtained from each participant. We have recruited a large family from Utah that shows transmission of DDH through four generations and isolated DNA from 72 family members (Fig. 1). Individual family members were diagnosed using clinical examinations and supine anterior posterior radiographs of the pelvis. Imaging of the hips was evaluated by three orthopaedic surgeons, with clinical opinions of two additional surgeons elicited in any cases of disagreement. Shenton's line (disrupted = affected), center edge angle (<20° = affected), Tonnis angle (>10° = affected), extrusion distance (>10 mm = affected) and femoral neck angle were

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measured in each radiograph and compared to control values derived from 11 independent studies. Detailed clinical evaluation and criteria for diagnosis of this family have been described [13]. In general those individual family members with one or two signs of DDH were deemed questionable, those with three or more signs affected.

Linkage Analysis and Whole Exome Sequencing

Four severely affected family members with three or more signs of the disorder were selected for whole exome sequencing and for analysis of shared exonic variants. Control reference sequence was derived from the 1000 Genome project (http://www.1000genomes.org) and from the GRCh37 assembly of NCBI. The protocols used for linkage analysis, high throughput sequencing and filtering criteria for candidate single nucleotide polymorphisms for biological relevance to chondrogenesis have been previously described in detail [14].

Validation of the sequencing results in the four severely affected members and in other family members, in-laws (individuals married-into the family) and in sporadic dysplastic individuals was performed using Sanger DNA sequencing. Sanger sequencing is a method of DNA sequencing based on the selective incorporation of chain-terminating dideoxynucleotides by DNA polymerase during in vitro DNA replication. It produces sequencing results by a method completely unrelated to high-throughput sequencing and can thus be used for verification of the results.

Results

Description of the Affected Family

Diagnostic criteria for the family recruited for this study have been previously described in detail [13]. Briefly, this four generation, 72 member family from Utah shows transmission of DDH in a manner consistent with an autosomal dominant mode of inheritance with incomplete penetrance (Fig. 1). Eleven patients had 3 or more signs of DDH and were considered to be unequivocably affected (see Methods). Thirteen individuals had one or two signs of DDH and had questionable diagnoses. Originally individual 27, an adolescent, was classified as affected. Subsequent review by a panel of orthopaedic surgeons changed this diagnosis to questionable. She was therefore classified as unknown for the purpose of linkage analysis in the current study. Poor quality of DNA in affected individuals 2 and 6 resulted in missing genotype rate >0.1 and caused them to be excluded from the analysis.

Linkage Analysis and Whole Exome Sequencing

Linkage analysis determines the likelihood that a given DNA variant will be co-inherited by affected individuals within a pedigree. As has been reported previously genome-wide analyses revealed a candidate region on chromosome 3 with analyses producing a maximum LOD score of 3.31 in an interval delimited by DNA single nucleotide polymorphisms (SNPs) or variants rs4481097 and rs4626072 at 38.91 to 40.66 Mb from the p terminal end of chromosome 3 [14]. LOD stands for "logarithm of the odds." In genetics, the LOD score is a statistical estimate of whether two genes, or a DNA variant and a disease gene, are likely to be located near each other on a chromosome and are therefore likely to be inherited together. A LOD score of 3 or higher is generally understood to mean that in this case a DNA single base variant and the disease causing gene are located close to each other on human chromosome 3. In terms of significance, a LOD score of 3 means the odds are a thousand to one that the two genes are linked, and therefore inherited together. High throughput sequencing revealed that a potentially harmful DNA variant (rs3732378) caused amino acid coding changes in the chemokine receptor Cx3CR1 that made it a likely DDH susceptibility inducing candidate [14].

Fig. 2 shows individuals in this family who have the rs3732378 variant in their DNA.

Testing of Unrelated DDH Sporadics for the Presence of the rs3732378 Variant

The DNAs of unrelated individuals with 3 or more signs of DDH were tested for the presence of this variant. This analysis revealed an adenine (A) base in the affected individual substituted for a guanine (G) found in the unaffected individual. It was determined that the A/G or A/A variant genotype occurred in 6 of 23 affected individuals tested. The allele frequency was found to be 15.2% (7 "A" alleles/46 total alleles from all sporadics). This compares to an allele frequency in the general population of 7.085% (194 "A" alleles/2738 total alleles tested) [15]. While these preliminary results suggest overrepresentation of this allele in the dysplastic population we are pursuing further DNA testing in other DDH sporadics to attain statistical significance for this result (Table 1).

Discussion

We have identified and retrieved DNA from one of the largest documented families showing inter-generational transmission of DDH. The goal of this study is to identify the molecular basis of the disease in this family using the approach of genome-wide linkage analysis together with whole exome sequencing. By performing genome-wide linkage analysis, which makes no assumptions about where a mutation might reside, a 2.61 Mb candidate region on chromosome 3p22.2 has been identified with a high degree of certainty. However, classical linkage analysis requires unambiguous knowledge of who is affected in a given pedigree. Because this disorder is complex, with both environmental and genetic causes, and

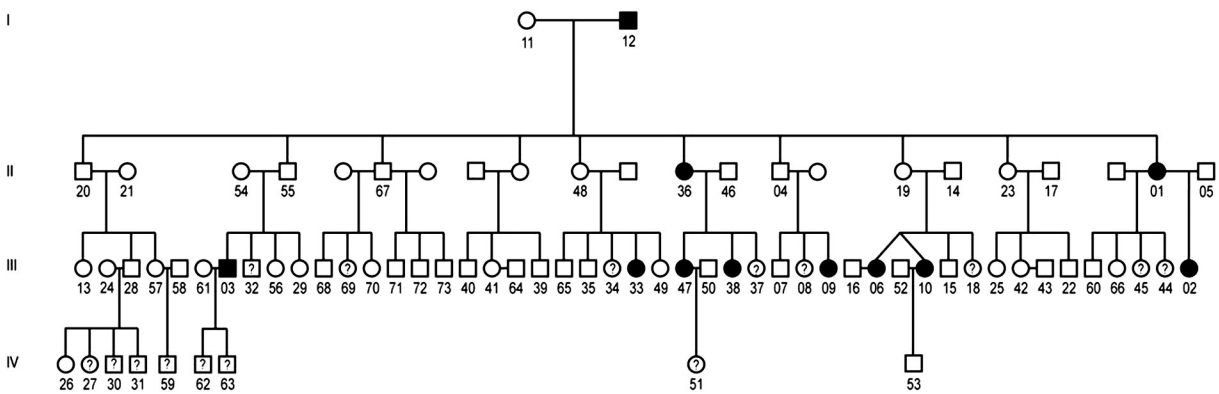

Fig. 1. Pedigree of a 71 member affected family. Filled in symbols denote individuals with 3 or more signs of DDH. Symbols containing question marks denote individuals with 1 or 2 signs of DDH.