

Research Article

Duplex Allele-Specific PCR Assay Simultaneously Detecting Genotype of Osteoarthritis Susceptibility Loci Within *GDF5* and *COL11A1* Genes

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Abstract

Introduction: Genome-wide association studies and meta-analyses have identified rs143383 *GDF5* (growth and differentiation factor 5) and rs2615977 *COL11A1* (collagen type XI alpha 1) as polymorphic loci among the most robust associations with osteoarthritis (OA). However, currently, there is a lack of available rapid, technically feasible, and cost-effective methods to detect OA susceptibility genotype, not linked with the specific descent of the patients.

Methods: Genomic DNA was extracted from the blood of OA patients and healthy individual using standard methods. Primers specific at the 3'-end for the polymorphic sites were designed. Direct sequencing was used to determine the genotype of the study subjects.

Results and conclusion: Here we describe one-step allele-specific PCR assay simultaneously detecting genotype of rs143383 *GDF5* and rs2615977 *COL11A1*. Duplex PCR enabled to obtain specific bands corresponding to the amplified product of interest. The genotypes called based on PCR results were consistent with the results of direct sequencing.

Keywords: Single nucleotide polymorphism; Osteoarthritis; Genetic susceptibility; Multiplex polymerase chain reaction; Growth and differentiation factor 5

Introduction

Osteoarthritis (OA) has a clear and detrimental impact on wellbeing, with up to one-fifth of affected individuals giving up work or retiring early [1]. Treatment options for severe steoarthritis (OA) beyond pain relief or total knee replacement are very limited. Because of this, attention has recently shifted to identifying which factors increase the risk of OA [2]. Among these factors, certain genetic susceptibility loci have been identified by association studies and meta-analyses [3]. More specifically, T-allele of polymorphic locus rs143383 *GDF5* (growth and differentiation factor 5) is one of the most robust OA predisposition loci across multiple populations [4]. Another strong association uncovered by arcOGEN genome-wide association study (GWAS) is rs2615977 (A-allele) within intron 31 of *COL11A1* gene ($p = 1.2 \times 10^{-5}$) [5]. The Arthrotest, a prognostic tool to predict progression towards severe knee osteoarthritis, has been developed based on genetic information from Spanish knee OA patients and is used at clinic [6]. However, currently, there is lack of available rapid, technically feasible, and cost-effective methods to detect OA susceptibility genotype independently upon genetic ancestry. We were keen therefore to develop duplex PCR assay simultaneously detecting genotype of rs143383 *GDF5* and rs2615977 *COL11A1*.

Materials and Methods

DNA samples

We collected samples of peripheral blood from patients diagnosed with severe knee OA (n=3) and healthy donor (n=1). The basic characteristics of the subjects are shown in Table 1. The study was approved by the local research ethics board (Rostov State Medical University Ethics Committee, Rostov-on-Don, Russia). Genomic DNA was isolated from peripheral blood leukocytes using a commercial kit (Litech, Moscow, Russia) according to manufacturer's instructions.

Sample ID	Group	Age	Gender	BMI (kg/m ²)	OA grade (Kellgren- Lawrence)	rs143383 <i>GDF5</i>	rs2615977 <i>COL11A1</i>
OA1	OA*	55	female	29.71	III	CT	CA
OA2	OA	56	male	29.3	III	TT	CA
OA3	OA	61	male	35.5	III	TT	AA
C1	control	36	male	25.6	-	CC	CC

*OA - patients diagnosed with osteoarthritis; control - healthy individual.

Table 1: Basic characteristics of the subjects

Allele-specific PCR

The primers, designed to be specific for either 3'-end rs143383 *GDF5* or rs2615977 *COL11A1*, are shown in Table 2 (synthesized by Eurogene, Moscow, Russia). In order to develop duplex PCR, the amplicon sizes were designed to be 307 bp for *GDF5* and 99 bp for *COL11A1*.

Primer	Sequence (5'-3')
rs143383 <i>GDF5</i> (C/T)	
Forward-C	5' – TGC AGG AGA AAG GGG GCG GTC – 3'
Forward-T	5' – TGC AGG AGA AAG GGG GCG GTT – 3'
Reverse	5' – CCA GCC AAG CCA GGT ACC AA – 3'
rs2615977 <i>COL11A1</i> (A/C)	
Forward-A	5' – CA CAT AAT GCC CCGTGT CTC TCA AA – 3'
Forward-C	5' – CA CAT AAT GCC CCG TGT CTC TCA AC – 3'
Reverse	5' – CT GGT ATT CAG GCC CAA CTT CAA GAG -3'

Table 2: Primers used in the study

First, we identified the most robust conditions to detect genotype of either rs143383 *GDF5* or rs2615977 *COL11A1*. PCR was carried out using GeneAmp® PCR System 9700 thermal cycler (ThermoFisher, Waltham, MA, USA). The reaction mixture (total of 25 µl) contained 1x PCR buffer (2.5 µl), 1.5 mM Mg²⁺ (*GDF5*) / 2 mM Mg²⁺ (*COL11A1*), 200 µM (*COL11A1*) / 300 µM (*GDF5*) each of dNTPs, 1.5 U thermostable *Taq* polymerase (all reagents by Syntol, Moscow, Russia), 1 µM each forward and reverse primers, and 2 µl of DNA solution. PCR conditions were as follows: initial denaturation for 3 min at 95°C, then 30 cycles of 95°C for 10 s, 64 °C (*GDF5*) / 66 °C (*COL11A1*) for 10 s, and 72 °C for 30 s. Amplicons were analyzed in 2% agarose gels using GelDoc System (Bio-Rad, Hercules, CA, USA).

Modified PCR conditions (duplex allele-specific reaction)

Next, we defined the most robust conditions for simultaneous genotyping of *GDF5* and *COL11A1*. The reaction mixture (total of 25 µl) contained 1x PCR buffer (2.5 µl), 2 mM Mg²⁺, 200 µM each of dNTPs, 2 U thermostable *Taq* polymerase (Syntol), 1 µM each forward and reverse primers, and 2 µl DNA solution. PCR conditions were as follows: after the initial denaturation step for 3 min at 95 °C, we consecutively performed 6 cycles (10 s at 96 °C, 10 s at 70 °C, 30 s at 72 °C), 6 cycles (10 s at 95 °C, 10 s at 69 °C, 30 s at 72 °C), 6 cycles (10 s at 94 °C, 10 s at 68 °C, 30 s at 72 °C), followed by 17 cycles of 10 s at 94 °C, 10 s at 67 °C, and 30 s at 72 °C (35 cycles in total).

Direct sequencing

GDF5 and *COL11A1* amplicons were purified using GeneJet PCR Purification Kit (ThermoFisher) and sequenced with the BigDye™ Terminator V 1.1 Cycle Sequencing Kit (ThermoFisher) on an automatic ABI Prism 3130xl Genetic Analyzer (ThermoFisher) using Sequencing Analysis software version 5.4.

Results and Discussion

DNA sequences of target regions of interest for the samples OA1, OA2, OA3 (patients with OA), and C1 (healthy individual) are presented in Figure 1a-d (rs143383 *GDF5*) and Figure 1f-i (rs2615977 *COL11A1*), respectively. First, genotypes called based on uniplex PCR results for these two genes (Figure 1e, j) were compared with DNA sequencing

results, which confirmed the specificity of PCR (Table 1). After that, we proceeded with duplex PCR (Figure 1k), which could enable to obtain specific bands corresponding to both *GDF5* (307 bp) and *COL11A1* genes (99 bp). In case of *GDF5*, the weak band in the second lane can be referred to as non-specific amplification when compared to other much thicker bands corresponding to *GDF5* alleles. In overall, this duplex PCR performed well to call the genotypes for analyzed samples.

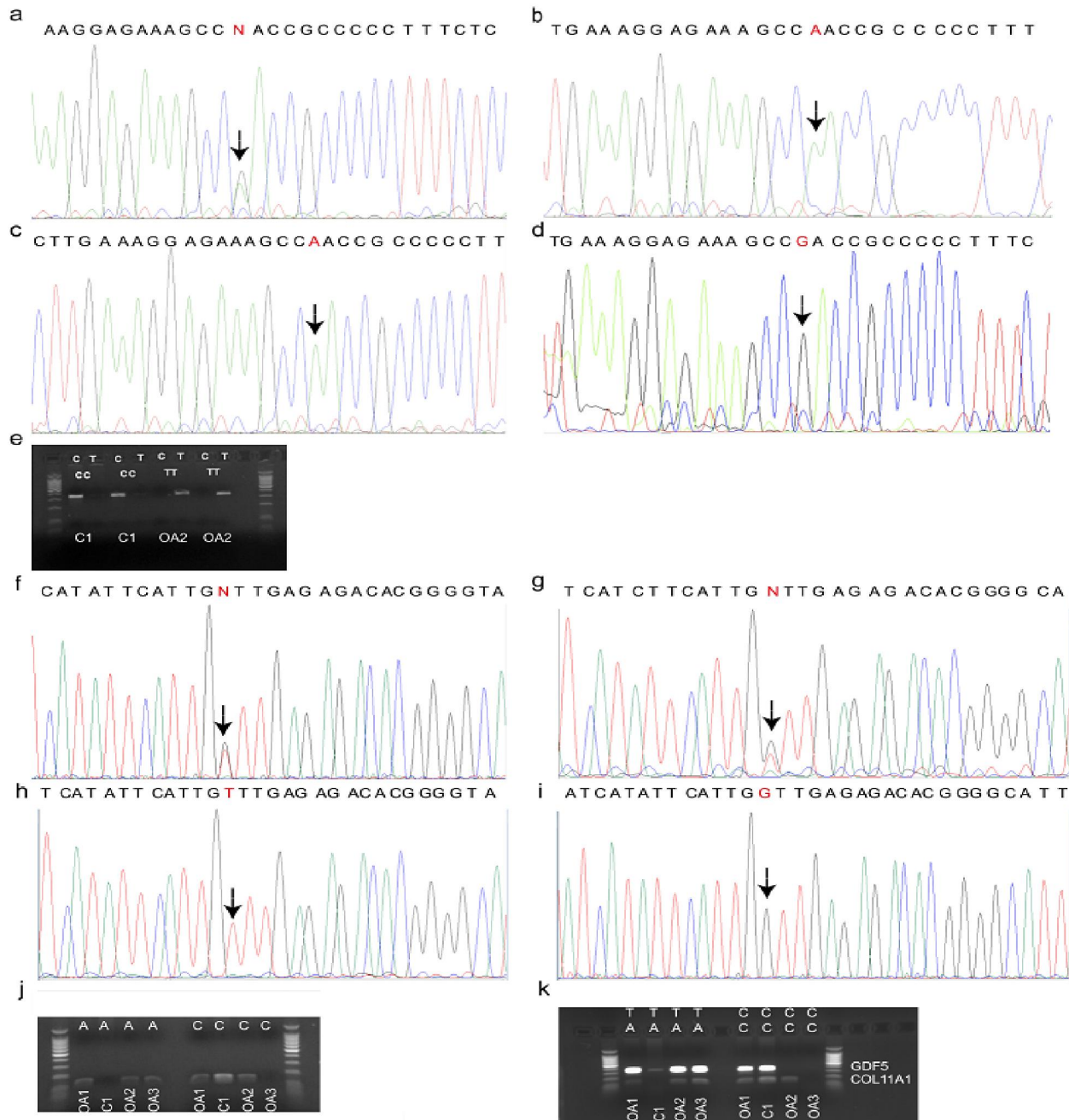


Figure 1: Detection of OA susceptibility genotype. (a-e) *C>T GDF5* (rs143383) genotypes: results of direct sequencing for OA patients (a) OA1, (b) OA2, (c) OA3 and healthy individual (d) C1. The positions of SNP are highlighted with

arrows. **(e)** Uniplex PCR amplicons (rs143383) for the samples OA2 and C1 (in duplicates). **(f-j)** *C>A COL11A1* (rs2615977) genotypes: results of direct sequencing for OA patients **(f)** OA1, **(g)** OA2, **(h)** OA3 and healthy individual **(i)** C1. **(j)** Uniplex PCR amplicons (rs2615977) for the samples OA1-3 and C1. **(k)** Duplex allele-specific PCR for all four samples OA1-3, C1. *C* and *T* – alternate alleles for rs143383 *GDF5*; *C* and *A* – alternate alleles for rs2615977 *COL11A1*.

Currently, multiple methods can be applied for genotyping known single nucleotide polymorphisms (SNPs): hybridization of allele-specific probes, primer extension, multiplex allele-specific and real-time PCR etc, each of them having its pros and cons [7]. Allele-specific PCR is a relatively simple technique where DNA polymerase amplifies target DNA only if allele-specific primers are perfectly complementary to it [7]. Despite allele-specific PCR being laborious and low throughput, it is a rapid, widely-used, and technically feasible genotyping method that is suitable for moderate-throughput laboratories [8]. Previously, multiplex allele-specific PCR assays have been used for detection of polymorphic loci associated with response to long-term anti-inflammatory treatment in patients with rheumatoid arthritis [9], with schizophrenia and Parkinson's disease [10], Alzheimer's disease [11]. Here, we developed and described duplex PCR assay detecting risk alleles of rs143383 *GDF5* and rs2615977 *COL11A1* genes, associated with OA. According to the recent reviews and meta-analyses, rs143383 *GDF5* is significantly associated with radiographic defined knee OA [12], and probably, hip and hand OA [13], whereas rs2615977 *COL11A1* has been mostly discussed as association with hip OA [5]. This duplex assay might be useful for developing more complex allele-specific genotyping methods.

Conflict of interest

The authors have no conflicts of interest to declare.

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