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Technical Notes

A multiplex single-base extension protocol for genotyping Cdx2, FokI, BsmI, ApaI, and TaqI polymorphisms of the vitamin D receptor gene

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ABSTRACT. The well-described role of the vitamin D endocrine system in bone metabolism makes its receptor a widely investigated candidate gene in association studies looking for the genetic basis of complex bone-related phenotypes. Most association studies genotype five polymorphic sites along the gene using PCR-RFLP and allele-specific amplification methods, which may not be the better choice in large case/control or cross-sectional studies. In this case, genotyping SNPs in parallel and using automated allele-calling methods are important to decrease genotyping errors due to manual data handling and save sample in cases where the amount of DNA is limited. The aim of this study was to present a straightforward method based on multiplex PCR amplification followed by multiplex single-base extension as a simple way to genotype five vitamin D receptor gene polymorphisms in parallel, which may be implemented in medium- to large-scale case/control or cross-sectional studies. The results regarding method feasibility and optimization are presented by genotyping eight paternity trios and seven samples of Bra-

zilian postmenopausal women who took part in an ongoing association study carried out by members of our group.

Key words: VDR, SNPs, Multiplex genotyping, Single-base extension

INTRODUCTION

The active metabolite of vitamin D, 1,25-dihydroxyvitamin D3, and the steroid/thyroid hormone nuclear receptor coded by the vitamin D receptor gene (VDR) are the two major players in the vitamin D endocrine system. Besides the essential role in calcium homeostasis and bone metabolism, research in the last two decades has shown that cell differentiation, inhibition of cell growth, immunomodulation, and control of other hormonal systems are also biological processes where the vitamin D endocrine system plays an important role (Dusso et al., 2005). The 1,25-dihydroxyvitamin D3 broad transcriptional action mediated through the vitamin D receptor makes it a strong candidate in association studies from bone phenotypes to cancer (Valdivielso and Fernandez, 2006).

The Cdx2, FokI, ApaI, TaqI, and BsmI polymorphisms are broadly genotyped in VDR/disease association studies (Uitterlinden et al., 2004). The Cdx2 polymorphism is an A to G transition named according to their location at the intestinal specific transcription factor Cdx2-binding site in the VDR 1e promoter region. The A allele is more active than the G allele regarding the Cdx2 transcription factor binding. Thus, the A allele is associated with more intestinal VDR gene transcription (Arai et al., 1997; Yamamoto et al., 1999). The FokI polymorphism is a T to C transition disrupting the start codon and resulting in a vitamin D receptor protein that is three amino acids shorter and more active as a transcription factor (Arai et al., 1997). The other three single nucleotide polymorphisms (SNPs) are in intron 8 and show strong linkage disequilibrium with each other and with other 3' end polymorphisms (Fang et al., 2005). There is no clear evidence of a functional role played by any of the three SNPs at the 3' end and the associations found in some studies probably are related to linkage disequilibrium among them and some functional polymorphic site (Ingles et al., 1997). Cross-sectional and case control studies regarding bone phenotypes where the five polymorphisms are investigated show discordant results. If the difficult to reproduce the associations is related to population genetic background or to poor study design will be cleared by well-designed large scale association study in different populations (Shen et al., 2005; Zmuda et al., 2005, 2006). The implementation of such studies will demand medium to highthrougput automated genotyping methods.

Besides the technical development experimented in the SNP genotyping field (Kwok and Chen, 2003; Syvanen, 2005), most of published association studies genotyping the FokI, BsmI, ApaI, and TaqI use polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). The Cdx2 investigation in association studies was first carried out by direct PCR sequencing (Arai et al., 2001), but since Fang et al. (2003) described an allele specific amplification protocol, it has been the method widely used. More recent association studies has genotyped the BsmI, ApaI, TaqI, and FokI using highthrougput TaqMan allelic discrimination assays (Fang et al., 2005). Both PCR-RFLP and allele-specific amplification methodologies are easily implemented when the number of samples to be genotyped is not too high. However, powerful association studies demand dozens of hundreds samples to be genotyped and non-automated meth-

ods turn to be difficult to use when planning such large scale association studies. As PCR-RFLP and allele-specific amplification methods genotype samples individually and normally there is no way to automate the allele-calling process the possibility to mess genotypes from different samples during the data handling must be considered. Another issue regarding methods that genotype one marker per time is DNA sample limitation, which sometimes may occur.

This study presents a straightforward protocol for genotyping the five most investigated VDR polymorphisms. The method is based on multiplex PCR, single-base extension methods and automated allele calling. The multiplex fashion and automated allele-calling process overcome issues with DNA sample limitation and possible genotyping errors due to mixing of different sample genotypes during manual data handling. The method was developed to work with minimum amounts of reagents, which also reduces the cost of genotyping.

MATERIAL AND METHODS

DNA samples

The DNA samples used in this study were extracted from peripheral blood using a modified salting out protocol (Miller et al., 1988). The DNA samples represent eight inclusion paternity trios and 7 samples of Brazilian postmenopausal women used in one ongoing project study of our group.

Vitamin D receptor single nucleotide polymorphisms

The five SNPs chosen to be part of the multiplex genotyping protocol described here are those broadly investigated in association studies between VDR gene and a diverse range of phenotypes. The SNPs appear in the literature as Cdx2, FokI, BsmI, ApaI, and TaqI. The sequence surrounding each of the five SNPs was easily retrieved from the dbSNP (http://www.ncbi.nlm.nih.gov/projects/SNP/) using respectively the following reference SNP identification numbers: rs11568820, rs10735810, rs1544410, rs7975232, and rs731236.

Polymerase chain reaction primer design

The sequences downloaded from dbSNP were used to design PCR primers in order to amplify fragments harboring each SNP. The primers were designed using Primer3 algorithm, which is freely available on the internet (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). As the ApaI and TaqI loci are only 80 bp apart from each other, PCR primers for amplification of only one fragment harboring the two SNPs were designed. The hairpin and primer dimer formation can potentially impair the PCR efficiency and multiplexing. In order to verify if the four primer pairs were not hairpin and primer dimer prone, they were tested using the Autodimer algorithm which was developed specifically to design primers maximizing the multiplexing success (Vallone and Butler, 2004). PCR primer sequences and melting temperatures are shown in Table 1.

Single-base extension primer design

The primers for single-base extension genotyping were designed manually in the sense

Table 1. Polymerase chain reaction (PCR) Primers designed to amplify fragments harboring the five vitamin D receptor single nucleotide polymorphosm.

Polymorphism	Forward PCR primer (5'-3')	Reverse PCR primer (5'-3')	Fragment size (bp)	Tm F/R (°C)
Cdx2	CATTGTAGAACATCTTTTGTATCAGGA	GACAAAAAGGATCAGGGATGA	224	59.9/59.0
FokI	GGCCTGCTTGCTGTTCTTAC	TCACCTGAAGAAGCCTTTGC	174	60.0/60.5
BsmI	CCTCACTGCCCTTAGCTCTG	CCATCTCTCAGGCTCCAAAG	209	60.1/59.9
ApaI TaqI	CTGCCGTTGAGTGTCTGTGT	TCGGCTAGCTTCTGGATCAT	242	59.9/59.9

Tm F/R = melting temperature for forward and reverse primers.

or antisense sequence, ending one base adjacent to the SNP to be genotyped. They were checked for primer hairpin and dimer formation with Autodimer. The SBE primer length was arranged in such a way as to distribute the fragments in the range of 20 to 60 bp adding a poly(T) tail on the 5' end of each primer (Table 2).

Table 2. Single-base extension primers for SNaPShot® multiplex reaction.

Polymorphism	Single-base extension primer	Primer size (bp)	Alleles genotyped	DNA strand direction	Reference alleles	Genotyped fragment size (bp)
Cdx2	CCTGAGTAAACTAGGTCACA	20	A/G	Forward	A/G	22/23
FokI	$(T)_{31}$GCTGGCCGCCATTGCCTCC	50	A/G	Reverse	C/T	51/52
BsmI	$(T)_{21}$CAGAGCCTGAGTATTGGGAATG	43	C/T	Reverse	A/G	45/46
ApaI	$(T)_{12}$GTGGTGGGATTGAGCAGTGAGG	34	G/T	Reverse	A/C	38/39
TaqI	$(T)_{9}$GCGGTCCTGGATGGCCTC	27	A/G	Reverse	C/T	29/31

Multiplex polymerase chain reaction amplification

The PCR was optimized to co-amplify the four fragments in one single reaction. The protocol was carried out in 12.5 µL as follows: 1X Taq polymerase buffer, 2.5 mM $MgCl_2$, 250 mM dNTPs, 1.6 mg/mL BSA, 0.50 µM of each primer, 10-40 ng DNA, 1 U of Taq polymerase. PCR amplification was performed in an ABI9700 thermocycler using the following cycles: denaturation at 95°C for 5 min, followed by 15 cycles of 40 s at 95°C, 40 s at 62°C decreasing 0.5°C per cycle, 40 s at 72°C, and 15 cycles of 40 s at 95°C, 40 s at 54°C, 40 s at 72°C, and final extension step at 72°C for 5 min.

Multiplex genotyping by minisequencing

The PCR-amplified products were purified with exonuclease I (ExoI) and shrimp alkaline phosphatase (SAP) enzymes in order to eliminate non-incorporated dNTPs and primers. The enzymatic purification was carried out in 3 µL PCR mix by adding 1 U ExoI, 0.95 U SAP and 0.5X SAP reaction buffer, which were incubated for 90 min at 37°C following 20 min at

80°C for enzyme denaturation. The minisequencing was performed using 1.25 µL SNaPshot® Multiplex minisequencing kit reaction mix (Applied Biosystems), 1.25X Big Dye Sequencing Buffer, 1 µL purified PCR product, 1 µL Multiplex primer mix containing 0.2 µM of each single-base extension primer, and sterile autoclaved Milli-Q water up to 5 µL. Single-base extension was performed as follow: 25 cycles of 96°C for 10 s, 50°C for 5 s, and 60°C for 30 s. Single-base extended products were enzymatically purified in order to degrade fluorescent ddNTP not incorporated in the reaction by adding 0.5 U SAP diluted 1:1 with 10X SAP reaction buffer for each reaction, followed by incubation at 37°C for 60 min and a step at 75°C for 20 min. The sample to be electrophoresed on ABI3100 was prepared adding 1 µL purified SBE products in 8.85 µL Hi-Di formamide and 0.15 µL GS120 Liz internal size standard. Samples were electrophoresed on an ABI prism 3100 Genetic Analyzer (Applied Biosystems), setting the equipment to use the SNP36_POP4 default module. Our group uses the ABI3700 POP-6 polymer instead of ABI3100 POP-4 polymer. The electropherograms were analyzed with GeneMapper 3.5 or Genescan Analysis 3.7/Genotyper 3.7 software (Applied Biosystems).

Polymerase chain reaction product sequencing reaction

Direct PCR product sequencing reactions were carried out for samples representing both homozygous and heterozygous genotypes for each VDR SNP. This procedure was performed in singleplex PCR using the same protocol described above. Each singleplex reaction was enzymatically purified using 0.5 U SAP and 0.5 U ExoI over 5 µL PCR product. The sequencing reactions were carried out using 1 µL ExoI/SAP purified PCR amplicons, 0.5 µL Big Dye® Terminator Kit, 3.2 pmol PCR primer, 1.75 µL Big Dye Sequencing Buffer (Applied Biosystems) in a total volume of 10 µL. The sequencing reaction was performed as follows: 96°C for 1 min and 25 cycles of 96°C for 10 s, 50°C for 5 s, and 60°C for 4 min. The sequencing reaction fragments were purified using EDTA/ethanol protocol, where 2.5 µL 125 mM EDTA and 30 µL 100% ethanol were added to each sample. The samples were mixed by inversion and incubated at room temperature for 15 min. After incubation, samples were centrifuged at 4°C at 1650 *g* for 45 min. The supernatant was discarded by inversion and 30 µL 70% ethanol was added to each sample. The samples were centrifuged at 4°C at 1650 *g* for 15 min. The supernatant was discarded by inversion and each sample was allowed to dry at room temperature for 30 min. Afterward, 10 µL Hi-Di formamide was added and samples denatured at 96°C for 3 min and immediately chilled at 4°C for 2 min. Samples were electrophoresed on an ABI prism 3100 Genetic Analyzer (Applied Biosystems), setting the equipment to use the RapidSeq36_POP6 default module. The electropherograms were analyzed using the Seqscape® 2.1 software.

RESULTS

The primers designed to co-amplify four PCR fragments harboring the five SNPs allowed for easy amplification optimization using standard PCR protocol conditions. Enzymatic clean up of PCR products was optimized to work with 3 µL, reduced from the 10 µL recommended by the SNaPshot® Multiplex minisequencing kit. Genotyping the five SNPs using the SNaPshot® Multiplex minisequencing kit was also optimized to minimize the amount of kit spent in each reaction. The protocol described here works with one-fourth of the kit reaction mix volume in one-half of the final reaction volume recommended by the manufacturer. The single-

base extension reaction buffering unbalancing due to the kit reaction mix cut down was fixed using the Big Dye Sequencing Buffer (Applied Biosystems). The amount of SAP used to remove the fluorescent ddNTP excess after the single-base extension reaction was also reduced to one-half as a consequence of the reduction in the final reaction volume. Electrophoresis in the ABI3100 was optimized to run with ABI3700 POP™-6 polymer, which costs roughly 50 times less than the ABI3100 POP®-4 polymer.

None of the eight paternity trios showed genotypes that were not in agreement with Mendelian transmission (Table 3). The peak height and fragment size for each allele of the five SNPs genotyped are shown in Figure 1. Direct comparisons of SBE products and sequencing reactions show no difference in homozygous and heterozygous samples for all five loci (Figure 2).

Table 3. Genotype from eight inclusion trios and seven samples of Brazilian postmenopausal women.

Sample	VDR polymorphism genotypes				
	Cdx2	FokI	BsmI	ApaI	TaqI
332M	A/A	C/T	G/A	A/A	C/T
332C	G/A	C/T	G/A	A/A	C/T
332P	G/A	C/C	G/A	C/A	C/T
336M	G/A	C/C	G/A	C/A	C/T
336C	G/A	C/T	G/A	C/A	C/T
336P	G/A	C/T	G/A	C/A	C/T
371M	G/A	C/T	A/A	A/A	C/C
371C	G/G	C/T	A/A	A/A	C/C
371P	G/A	C/T	G/A	C/A	C/T
383M	G/A	C/T	G/A	A/A	C/T
383C	G/A	C/T	G/A	C/A	C/T
383P	G/A	C/C	G/G	C/C	T/T
414M	G/A	C/C	G/G	A/A	C/T
414C	G/G	C/C	G/G	A/A	T/T
414P	G/A	C/C	G/A	A/A	T/T
431M	G/A	C/C	G/A	C/A	C/T
431C	G/A	C/C	G/A	C/A	C/T
431P	G/A	C/T	G/A	C/C	T/T
447M	G/A	C/T	G/A	C/A	T/T
447C	G/A	C/C	G/G	C/A	T/T
447P	G/A	C/C	G/A	C/A	C/T
451M	G/G	C/C	A/A	A/A	C/C
451C	G/G	C/T	G/A	C/A	C/T
451P	G/G	C/T	G/G	C/C	T/T
009	G/A	C/C	G/A	C/A	C/T
013	G/G	C/T	G/G	C/A	T/T
014	G/A	T/T	G/G	A/A	C/T
029	G/A	C/T	A/A	A/A	C/C
041	G/A	C/C	G/A	A/A	C/C
042	G/A	T/T	G/G	C/C	T/T
051	A/A	C/C	G/A	C/A	T/T

VDR = vitamin D receptor. Sample number indicates the case, and the letter "M" indicates mother, "C" child, and "P" father. Alleles refer to the dbSNP reference allele.

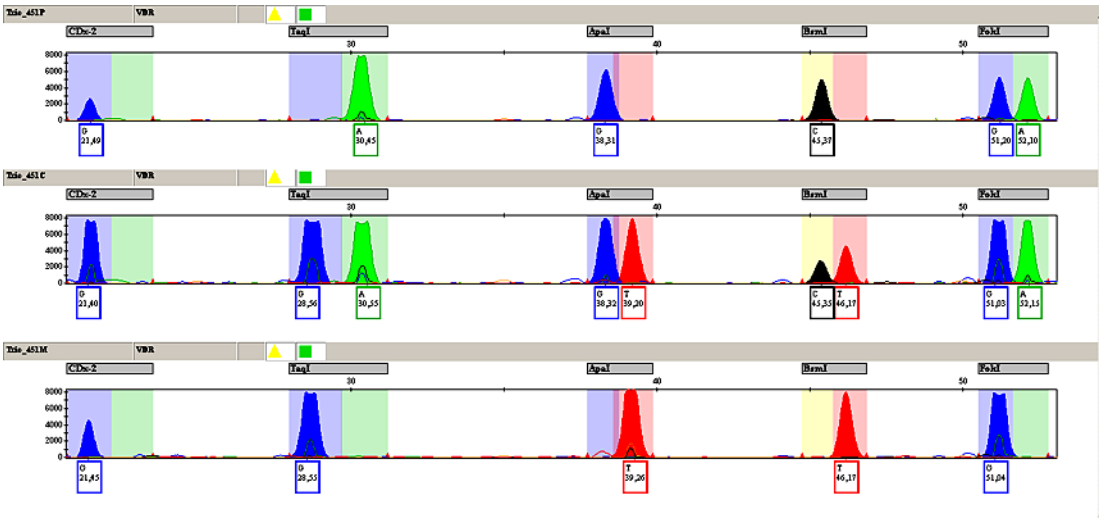

Figure 1. Electropherogram demonstrating allele calling in one genotyped trio (451). Labels below peaks indicate allele calling with respective size in base pairs. The letter "P" indicates father, "C" indicates child, and "M" indicates mother.

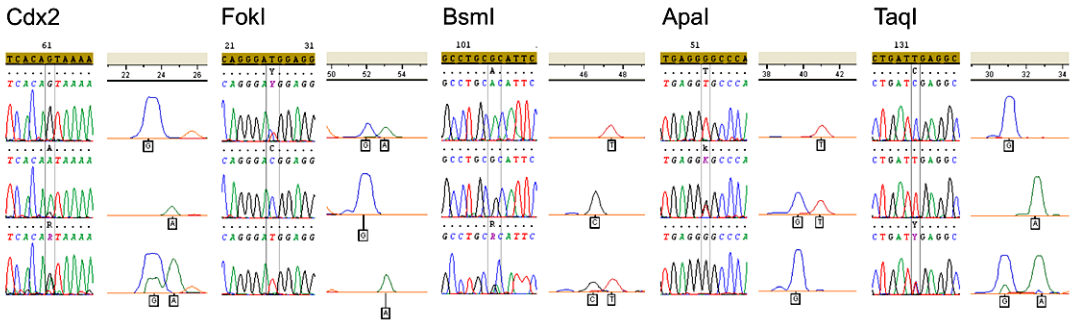

Figure 2. Direct comparison of PCR amplicon sequencing and SNaPshot reaction fragments. Genotyped samples are displayed as follows: Cdx2 (013 G, 051 A, 009 G/A), FokI (013 C/T, 009 C, 042 T), BsmI (029 A, 042 G, 009 G/A), ApaI (014 T, 009 G/T, 042 G), TaqI (041 C, 042 T, 009 C/T).

DISCUSSION

Single-base extension has been shown to be a very straightforward method for SNP genotyping, allowing medium and high throughput method development (Syvanen, 1999, 2005). Here, we present an optimized protocol based on fluorescent single-base extension combined with capillary electrophoresis that allowed for the multiplex genotyping of the five most often used VDR gene polymorphisms in association studies, from bone phenotypes to different kinds of cancer. Besides optimizing a set of PCR and single-base extension primers that worked in a multiplex fashion the method presented here introduces protocol changes in the commercial single-base extension kit in such a way as to reduce genotyping costs. Amounts of SAP and ExoI were reduced as the volume of the PCR product enzymatically cleaned up was reduced to one-third. Genotyping of the five VDR SNPs worked well even reducing the amount of kit

reaction mix to one-fourth and final reaction volume to one-half. The use of ABI3700 POP™-6 polymer instead of the recommended ABI3100 POP™-4 also helped to decrease the genotyping costs, which is essential when planning medium- to large-scale association studies.

The genotypes obtained by single-base extension were in agreement with those obtained by direct PCR amplicon sequencing, showing that the new primers and the protocols optimized during the development of this study are a good alternative for those who plan to use the VDR polymorphisms in large genetic association studies. Besides the advantage of saving time, genotyping the five SNPs in parallel and in an automated way may also be important in decreasing the rate of genotyping errors due to allele calling and data handling.

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