Rapid Screening of the Vitamin D Receptor *Bsm* I Gene Polymorphism by Real-Time PCR Using the LightCycler® Instrument

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A real-time PCR method was developed to facilitate genotyping of the vitamin D receptor (VDR) *Bsm* I gene polymorphism. Up to now, elaborate and time-consuming restriction fragment length polymorphism (RFLP) analysis has been the method of choice for this purpose. The new assay described in this article offers a simple time- and cost-saving alternative to the RFLP approach, with comparable sensitivity for genotyping of VDR *Bsm* I polymorphisms in human blood research samples.

Introduction

The vitamin D receptor VDR, a member of the nuclear receptor superfamily of transcriptional regulators, is involved in the mediation of cell growth and differentiation by 1,25-dihydroxyvitamin D3 in different target tissues [1, 2]. Gene expression is influenced by direct interaction of the VDR-vitamin D3 complex with specific sequence elements in the promoter region of hormone-responsive target genes. Transactivation or repression by VDR involves multiple interactions with protein cofactors, heterodimerization partners, and the transcriptional machinery [3, 4].

Several single nucleotide polymorphisms (SNPs) of the VDR gene are known [5], contributing to altered stability of VDR mRNA and altered expression of the receptor [6]. VDR gene polymorphisms account for many of the heritable factors influencing bone density [7-11]. Using linkage analysis, a significant correlation between bone density and the different allele types in 70 monozygous and 55 dizygous unisexual, concordant, but not discordant twins, was described [7]. Additionally, there was a correlation between bone density and circulating osteocalcin, a parameter for bone metabolism [12]. In several studies, different measures of bone density and bone metabolism were correlated with the *Bsm* I polymorphism [13-18]. VDR gene polymorphisms *Bsm* I, *Taq* I, and *Apa* I, located at the VDR 3´ untranslated region, were associated with low bone mineral density and osteoporosis [6, 19]. The VDR 5´ polymorphism *Fok* I seemed to be associated with peak bone mass [20]. In conclusion, the *Bsm* I polymorphism is regarded as one among multiple genetic risk factors for the development of osteoporosis. Thus, the analysis of the VDR *Bsm* I polymorphism may have the potential to become an additional tool in risk assessment of osteoporosis. Absence of the *Bsm* I restriction site (GAATGCN/N) is denoted by the B allele, presence by the b allele, resulting in a genotype BB (homozygous absence of the restriction site), bb (homozygous presence of the restriction site), or bB (heterozygous). The BB genotype is associated with low bone mineral density at both the lumbar spine and the femoral neck in healthy women from different ethnic backgrounds [7-11].

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Despite its implication for bone stability, the ubiquitous activity of vitamin D as transcriptional regulator supports the assumption that VDR gene polymorphisms may also play a pathogenic role for other diseases. VDR *Bsm* I polymorphism is associated with an increased susceptibility to type-I diabetes mellitus [21]. As there is a high VDR expression in the biliary tract, first attempts were made to correlate VDR polymorphisms with hepatobiliary diseases [22]. Furthermore, an association of VDR *Bsm* I gene polymorphism with hypertension was postulated [23].

Materials and Methods

A rapid one-step real-time PCR assay for analyzing the VDR *Bsm* I gene polymorphism was developed using fluorescence-labeled hybridization probes covering the *Bsm* I restriction site (Figure 1), and the LightCycler® Instrument. Classification was performed by melting-point analysis.

			T_m
VDR_b F	TAggggggATTCTgAggAACTA	S	56,8°C
VDR bsm A	AgTTTTgTACCCTgCCCgC	A	59,9°C
Sensor b	AgTATTgggAATgCgCAggCC X	A	64,0°C
Anchor	TCTgTggCCCCAggAACCCTg p	A	67,5°C

Figure 1: hu VDR *Bsm* I polymorphism (45082 GAATGC→T). Primers and probes for the real-time PCR approach are presented (S, sense; A, antisense).

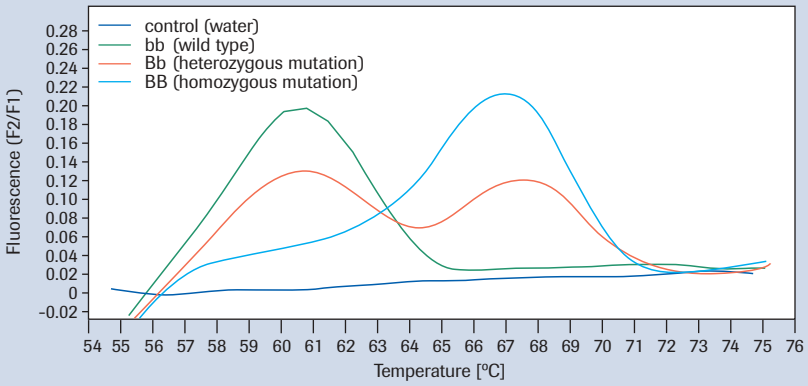

Figure 2: Real-time PCR. LightCycler® melting-curve analysis was performed to analyze the VDR *Bsm* I polymorphism.

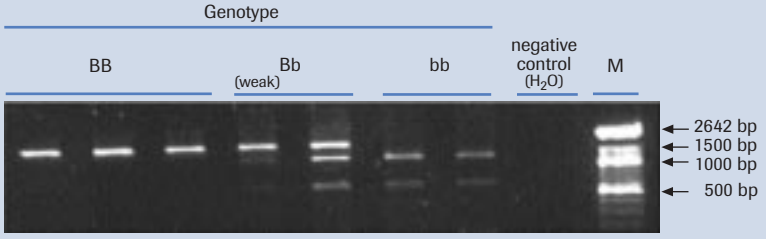

Figure 3: RFLP analysis. The classical RFLP approach was used to genotype the VDR *Bsm* I gene polymorphism. Samples were analyzed on a 1% agarose gel (M, DNA Molecular Weight Marker XIV).

Blood samples were drawn by atraumatic venipuncture into trisodium citrate tubes. All centrifuged blood samples were kept at -80°C until genomic DNA was isolated from leukocyte nuclei using a commercially available kit. DNA isolates were stored at 4°C. Real-time PCR was performed in capillaries with a reaction volume of 10 µl, containing 1 µl of DNA (40-80 ng), 0.3 µM sense primer VDR_b F (TAG GGG GGA TTC TGA GGA ACT A) and antisense primer VDR bsm A (AGT TTT GTA CCC TGC CCG C), 1 µl reaction buffer (LightCycler® DNA Master HybProbe 10 x buffer), 1.25 µl 25 mM $MgCl_2$, 10 mM of each dNTP, 1.9 µl H_2O, 0.2 µl bovine serum albumin (BSA, 240 ng/l stock) and 1.25 U Taq polymerase. Sensor b (0.6 µM) was 3´ modified with fluorescein (AGT ATT GGG AAT GCG CAG GCC-F). Anchor (0.6 µM) was 5´ labeled with LightCycler® Red 640 and 3´ terminally blocked by phosphorylation (Red640-TCT GTG GCCCC AGG AAC CCT G-P). Sensor b covers the *Bsm* I restriction site (Figure 1).

PCR conditions were as follows: initial denaturation at 97°C, 2 minutes; 40 cycles denaturation (97°C, 5 seconds, 20°C/seconds), annealing (62°C, 5 seconds, 20°C/seconds), extension (72°C, 10 seconds, 20°C/seconds); melting curve analysis: 95°C, 2 seconds, 20°C/seconds; 52°C, 1 second, 20°C/seconds, ramping to 80°C at 0.2°C/seconds, continuous monitoring of fluorescence signals. Derivative melting curves [(-dF/dT) vs T] were plotted for melting-point analysis.

Results and Discussion

Real-time PCR

The derivative melting curves shown in Figure 2 allow rapid and highly reproducible discrimination of the vitamin D receptor *Bsm* I polymorphism.

Methodological comparison

A methodological comparison between melting-curve analysis (Figure 2) and RFLP analysis (Figure 3) was performed with 28 randomized samples. Block cycler PCR using *Bsm* I restriction with subsequent electrophoretical analysis [24] and real-time PCR followed by melting-curve analysis gave identical results for all tested samples.

To show a causal correlation between genetic polymorphisms and the development of a pathological condition, genotyping of large numbers of affected individuals is necessary. So far, merely tedious classical approaches for VDR *Bsm* I classification such as RFLP analysis have been on hand. Therefore, the availability of a rapid time- and cost-saving analysis method is advantageous. The newly developed real-time PCR method for the classification of VDR *Bsm* I genotypes presented in this article offers an interesting alternative to the classical RFLP approach, and may be used as a valuable technological basis for investigations on pathogenetic mechanisms of different diseases. ■

Acknowledgements

We thank Helga Schlawatzki for her skillful technical assistance.

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