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Vitamin D receptor gene polymorphisms in relation to Vitamin D related disease states[☆]

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Abstract

The role in skeletal metabolism of the steroid hormone Vitamin D and its nuclear receptor (VDR) is well known. In addition, however, Vitamin D is also involved in a wide variety of other biological processes including modulation of the immune response and regulation of cell proliferation and differentiation. Variations in the Vitamin D endocrine system have thus been linked to several diseases, including osteoarthritis, diabetes, cancer, cardiovascular disease and tuberculosis. Evidence to support this pleiotropic character of Vitamin D has included epidemiological studies on circulating Vitamin D hormone levels, but also genetic epidemiological studies. Genetic studies provide excellent opportunities to link molecular insights with epidemiological data and have therefore gained much interest. DNA sequence variations which occur frequently in the population are referred to as "polymorphisms" and are usually suspected of having only modest and subtle effects. Recent studies have indicated many polymorphisms to exist in the VDR gene, but the influence of VDR gene polymorphisms on VDR protein function are largely unknown. Sofar, three adjacent restriction fragment length polymorphisms (RFLP) for *Bsm*I, *Apa*I and *Taq*I, respectively, at the 3′ end of the VDR gene have been the most frequently studied sofar. But because these polymorphisms are probably non-functional, linkage disequilibrium (LD) with one or more truly functional polymorphisms elsewhere in the VDR gene is assumed to explain the associations observed. Research is therefore focussed on documenting additional polymorphisms across the VDR gene to verify this hypothesis, and on trying to understand the functional consequences of the variations. Substantial progress has been made including the discovery of novel polymorphisms in the large promoter region of the VDR gene. Eventually, results of this research will deepen our understanding of variability in the Vitamin D endocrine system and might find applications in risk-assessment of disease and in predicting response-to-treatment.
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1. Introduction

The secosteroid hormone Vitamin D, its receptor (VDR) and the metabolizing enzymes involved in the formation of the biologically active form of the hormone, together are major players in the Vitamin D endocrine system. This system plays an important role in skeletal metabolism, including intestinal calcium absorption, but has also been shown to play an important role in other metabolic pathways, such as those involved in the immune response and cancer [1]. In the immune system, for example, Vitamin D promotes monocyte differentiation and inhibits lymphocyte proliferation and secretion of cytokines, such as IL-2, interferon-γ and IL-12. In several different types of cancer cells Vitamin D has been shown to have anti-proliferative effects.

One approach to understand inter individual differences in the Vitamin D endocrine system is to study the influence of variations in the DNA sequence of important proteins of this system. For example, deleterious mutations in the VDR gene cause 1,25-diydroxyvitamin D resistant rickets, a rare monogenetic disease. More subtle sequence variations (polymorphisms) in the VDR gene occur much more frequently in the population but they have not been systematically analysed and their effects on VDR function are poorly understood. Their influence on the Vitamin D endocrine system is currently under scrutiny in relation to a number of so-called complex diseases and traits, such as osteoporosis. This so-called candidate gene approach in the genetic dissection of complex traits is currently gaining increased importance over genome search approaches using linkage analysis [2,3].
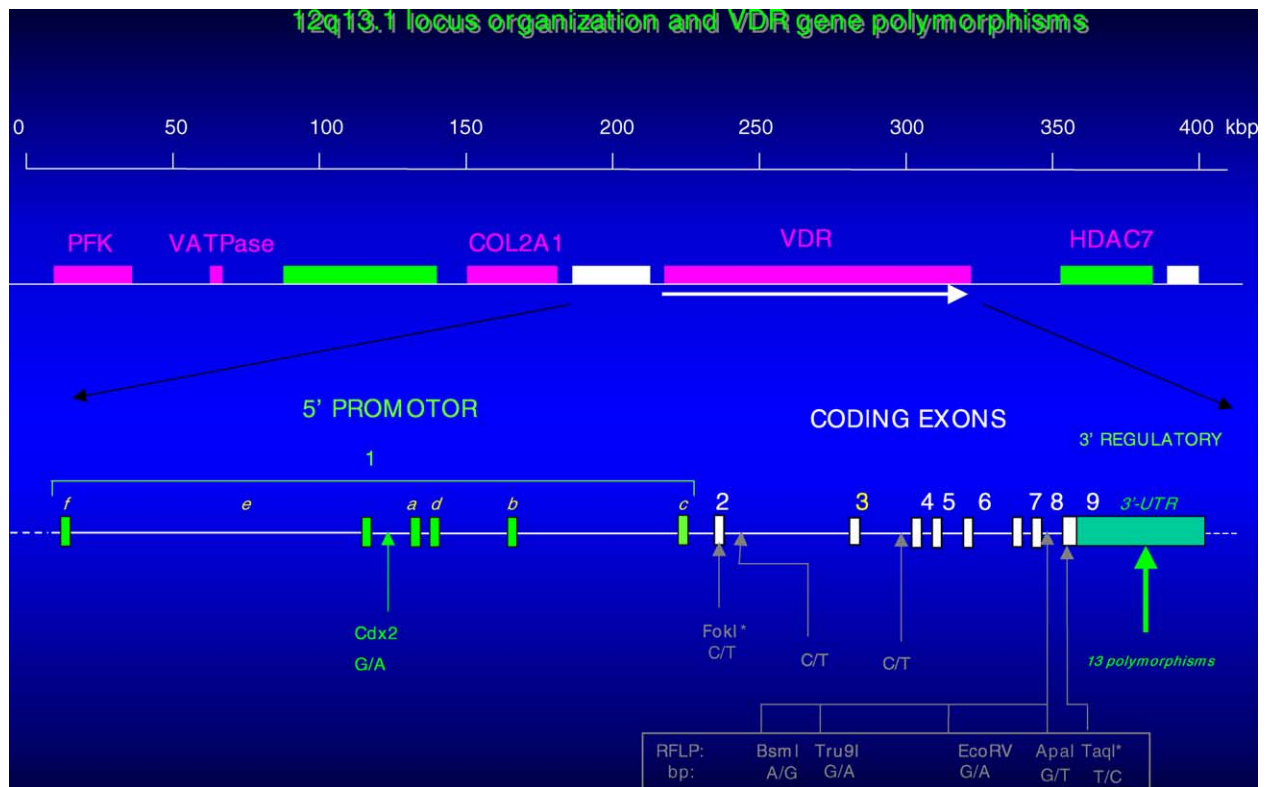
Fig. 1. Genomic structure of the VDR–COL2A1 locus on chromosome 12q13 and position of some polymorphisms in the Vitamin D receptor gene. * Indicates that these polymorhisms are in the coding sequence. For the *Fok*I RFLP see text. The *Taq*I RFLP is a silent (synonomous) base change not changing the amino acid encoded by this codon. PFK: Phosphofructokinase; VATPase: vacuolar ATPase; HDAC7: histone deacetylase 7.

The interpretation of polymorphic variations in the VDR gene is severely hindered by the fact that until now many of the polymorphisms currently analysed, are anonymous restriction fragment length polymorphisms (RFLP). One expects them to be linked to truly functional polymorphisms elsewhere or in nearby gene(s) which can then explain the associations observed. Thus, to understand the mechanisms underlying the associations one has to analyse the genomic organisation of the VDR locus, to identify which genes are present in the area, categorize all relevant VDR polymorphisms, and finally determine their relationship with the RFLP markers currently used.

The analysis of the genomic organisation of the VDR locus at chromosome 12q13.1 has shown that the VDR gene itself is quite large (about 100 kb; see Fig. 1; van Meurs et al., unpublished) and has an extensive promotor region capable of generating multiple tissue-specific transcripts [4,5]. In view of the genome-wide observed frequency of single nucleotide polymorphisms [6–10], one can expect >100 polymorphisms to be present in the VDR region alone, including in areas that are functionally relevant, such as the promoter region. We have indeed recently conducted such as study and found numerous polymorphisms in the VDR gene (Fang et al., manuscript in preparation). Below studies on different polymorphisms in the VDR gene are briefly discussed, focussed on the mostly anonymous RFLPs. Historically speak-

ing, studies of VDR polymorphisms in relation to bone endpoints, including osteoporosis, have received most attention sofar while the analysis of other diseases has reached the literature somewhat later on. This allows studies on associations with bone endpoints to be compared and to illustrate some of the difficulties in interpreting the results while this is much less possible for VDR polymorphism studies in relation to other disease endpoints, although similar interpretation problems exist.

2. Association studies using the BsmI, ApaI and TaqI RFLPs

The three adjacent RFLPs for *Bsm*I, *Apa*I and *Taq*I, respectively, in intron 8/exon 9 at the 3′ end of the Vitamin D receptor gene, have been most frequently studied sofar. Morrison et al. reported that the *Bsm*I RFLP in the last intron of the VDR gene was related to serum osteocalcin concentration [11] and was subsequently found to be associated with differences in BMD in a twin study and in postmenopausal women [12]. Although the initial observations on the twin study have been withdrawn [13], in the following years dozens of papers were published analysing the same RFLP in relation to BMD. Some of these confirmed the observation, while others could not find an association or found

another allele associated. In the largest single cohort study published sofar and which analysed 1,782 Dutch elderly men and women, no effect of single RFLPs on BMD was observed but a small effect was detected employing haplotypes constructed of the three adjacent 3′ RFLPs [14]. In line with this, a meta-analysis of 29 studies (excluding the Dutch cohort) on the relationship of VDR genotype with BMD [15] concluded that VDR genotype is associated with BMD in elderly subjects but with only 1–2% difference between extreme genotypes. In addition, Gong and colleagues analysed 75 articles and abstracts on VDR genotype and BMD [16] and concluded that BMD is associated with VDR genotype, especially in females before the menopause, and that genetic heterogeneity and non-genetic factors play a role in finding the associations. This notion is supported by other studies that found evidence to suggest an influence on peak BMD in younger subjects (aged 7–29 years) [17,18], found a relation of VDR genotype with bone loss [19,20], and observed influence of dietary calcium intake on the strength of the associations with bone density [21,22]. Interestingly, a study of American Caucasian women [23], of Danish Caucasian women [24], and of Dutch Caucasian women [25] suggested VDR genotype to be associated with increased fracture risk. This effect was mostly independent of (the small) genotype-related differences in BMD [25]. However, because other groups have not seen a relationship between fracture risk and VDR genotype [26,27] this relationship remains uncertain and needs further scrutiny.

Of particular interest in this respect is the fact that not always the same risk allele is being found associated with bone parameters (but also with other endpoints; see below) preventing the straightforward interpretation of these associations. While the initial studies by Morrison et al. [11–13] suggested the "B" allele of the BsmI RFLP-site to be the risk allele associated with low BMD, other studies either could confirm this, did not find any effect, or found the (opposite) "b" allele to be the risk allele associated with low BMD. Such conflicting findings, which are not exclusive for the field of genetic association analysis of osteoporosis could have several reasons.

The most obvious explanation is that, given the small effect, e.g., on BMD, very often the statistical power of the individual studies is much too low and no reliable conclusions on presence or absence of an effect can in fact be drawn.

Another explanation is due to the fact that the 3′ Bsm–Apa–Taq RFLPs are not functional themselves, as far as we know. The BsmI and ApaI RFLPs are located in intron 8 and are not affecting any splicing site and/or transcription factor binding site. The TaqI RFLP is a "synonomous" polymorphisms meaning that it is present in the coding sequence (i.e., exon 9) but that it is not changing the amino acid sequence of the encoded protein. When polymorphisms are (supposedly) non-functional they are nevertheless still useful in association studies because they can be used as markers. When association is found with a marker allele, the association is then believed to be caused by a truly functional allele which is linked to the marker allele and which is located elsewhere but usually nearby in the same gene. However, such linkage between marker allele and functional allele depends on the extent and strength of linkage disequilibrium across that area of the chromosome. Consequently, differences in LD between the marker allele and the truly functional allele can lead to varying associations.

Finally, interactions among genes and interactions with environmental factors play a role in the action of this steroid hormone receptor transcription factor. For example, dietary Ca-intake is known to differ substantially between countries and populations while circulating serum Vitamin D levels, which are determined by several metabolizing enzymes, also differ between populations. Consequently, gene–gene and gene-environment interactions can then differ between different populations. In addition, pleiotropic effects of this gene can play a role in influencing an association, but these will be discussed below.

3. Other VDR polymorphisms

The alleles of the BsmI, ApaI and TaqI polymorphisms in intron 8 and exon 9 are closely linked and haplotypes can be constructed over this 2.2 kb region [11,14]. The linkage disequilibrium of these RFLPs extends into the 3′ untranslated region (UTR) which is a 3.2 kb sequence immediately adjacent to exon 9 [11,28,29]. More than 10 different sequence variations in the 3′UTR have been described including a poly(A) repeat polymorphism. Analysis of the LD over this 5.5 kb region at the 3′UTR of the VDR gene in different ethnic population groups indicated that the LD differed among populations [28]. A single RFLP, such as the BsmI RFLP which is the most frequently used in association studies of the VDR gene, is therefore not a good marker for the LD with other sequence variations and, thus, the use of the BsmI RFLP might contribute to heterogeneity among association studies.

This notion is strongly supported by the recent comprehensive sequence analyses of other genes such as the LPL gene [6,7], and the description of haplotype structures in the genes [30,31]. These studies showed that there are islands of LD across a gene in which blocks of dozens of SNPs are linked together and form haplotype alleles. A practical advantage is that only a few SNPs have to be genotyped to identify the haplotype allele. A disadvantage is that when association is observed with such a haplotype allele, functional studies have to be performed to identify the "causative" allele.

In our previous studies [14] we have identified at least three major haplotype alleles to exist across the 3′UTR region. Functional studies are currently ongoing to identify what the causative polymorphisms in this region are. This will be imperative to understand the associations found using VDR polymorphisms derived from this region of the gene. However, when we evaluate a particular candidate gene of
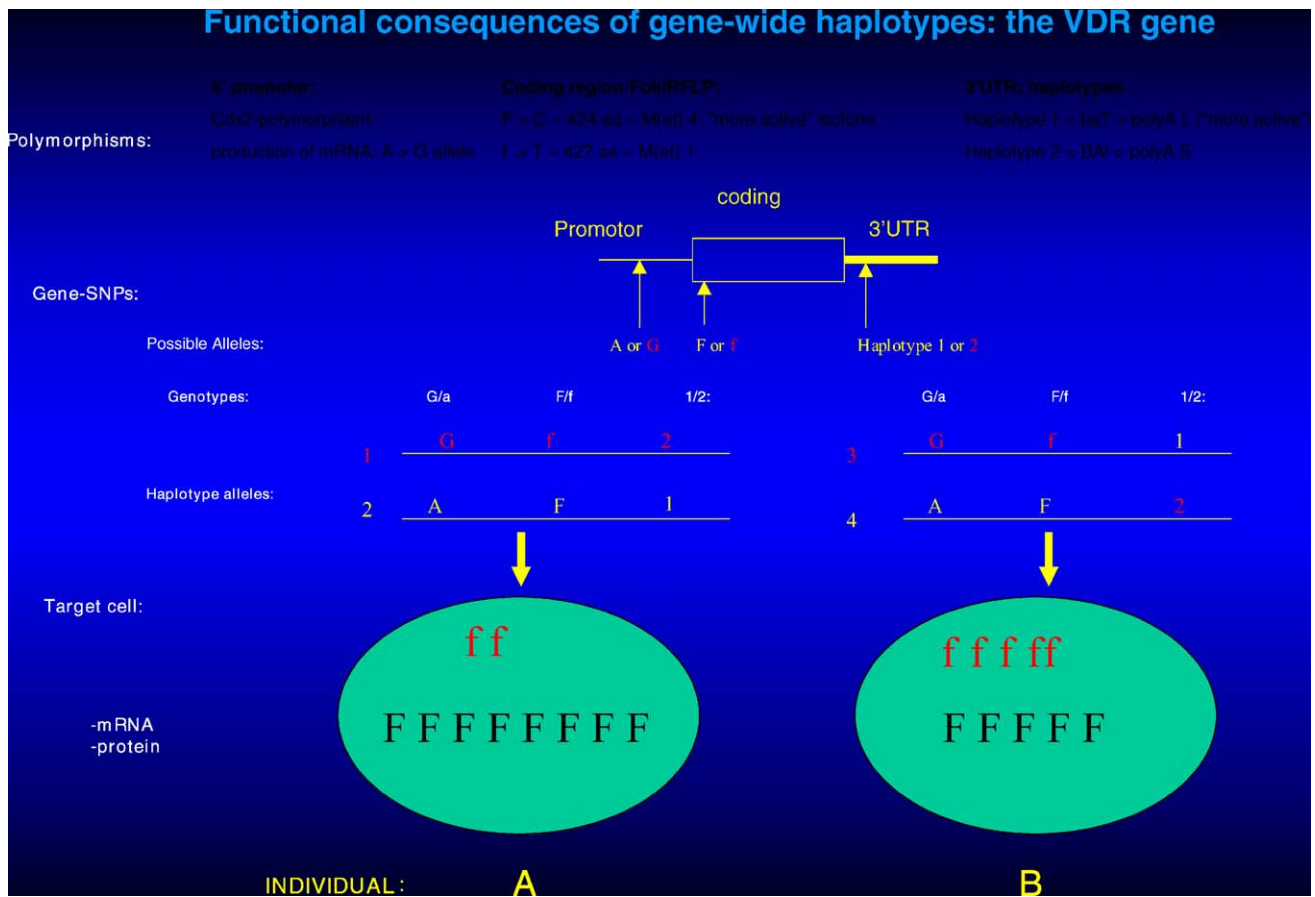
Fig. 2. The importance of gene-wide haplotypes. Three adjacent SNPs in different parts of the VDR gene are shown for two individuals (A and B indicated at the bottom) with identical genotypes (they are both heterozygous for all three SNPs) but with different haplotypes (1,2 for subject A and 3,4 for subject B). The promoter area regulates production of mRNA while the 3′UTR is involved in stability/degradation of mRNA and their interaction/combined effects regulates the net availability of the mRNA for translation into the protein. The protein can occur in two variants: little f (less active, M1, 427 aa) and big F (more active, M4, 423 aa) and both A and B are heterozygous for this polymorphism. The result of the particular haplotype combinations is that Individual A has less of the "risk" VDR protein (i.e., the little f variant, M1, 427 aa long) than Individual B in the target cell. This could not have been predicted by analysing single SNPs and/or only looking at genotypes of individual SNPs, but is only evident upon analysis of the gene-wide haplotypes.

interest, such as the VDR gene, it is important to take into account all polymorphisms which could influence expression and activity of the mRNA and protein. This is illustrated in Fig. 2 for a hypothetical gene carrying functional SNPs in the promoter, coding, and 3′UTR region. Because the promoter and 3′UTR both influence mRNA levels (production and stability, respectively), their interaction determines net availability of mRNA for translation into protein. In Fig. 2, it is shown that only particular combinations of risk alleles on a haplotype across the complete gene, result in high levels of mRNA encoding a defective protein. Thus, although subjects might have identical genotypes for a number of SNPs across a gene, different bio-response effects might be observed because the individuals differ with respect to the haplotype.

More than 25 different polymorphisms are currently known to be present at the VDR locus (see Fig. 1), sofar mostly near the 3′ end of the gene. However, also towards the 5′ end of the gene in and near the promotor region other sequence variations have been reported. For example,

a substitution (T to C) at exon 2 eliminates the first ATG translation inition site and allows a second one 9 bp downstream to be used. Thus, two variant forms of the VDR protein can be translated which differ by three amino acids resulting in proteins of 427 (M1) and 424 (M4) amino acids. The existence of these two different forms of the VDR protein has been demonstrated while the shorter form was found to give greater transcriptional activation [32,33]. The sequence change can be detected as a *Fok*I RFLP and the "f" allele (corresponding to M1, the longer protein) has been found associated with low BMD in several study populations [32,34,35] but this finding is not consistent [36,37]. The RFLP seems not to be in linkage disequilibrium with the 3′ polymorphisms. It is therefore, unlikely to explain the association results of the *Bsm*I, *Apa*I and *Taq*I polymorphisms and, in view of the considerable distance between the two sites (±40 kb) and the different nature of the polymorphisms, should be treated as a different marker. This also holds true for the recently described G to A sequence variation in the Cdx-2 binding element just upstream of exon 1A

[38,39]. Arai and colleagues reported the G allele to have a decreased transactivation capacity and to be associated with 10% decreased lumbar spine BMD in 123 Japanese women. More recently, we have shown this variant to be associated with increased fracture risk in Caucasians [40].

4. Pleiotropic effects

The Vitamin D endocrine system has been shown to be involved in a number of endocrine pathways related to calcium metabolism, immune-modulation, regulation of cell growth and differentiation (of keratinocytes, osteoblasts, cancer cells, T-cells), etc. [1]. Thus, for a pleiotropic "master" gene such as the VDR one can expect to find associations of this gene with multiple traits and disease phenotypes. Indeed, the VDR gene has been found associated with a number of different phenotypes of which, especially, the associations with osteoarthritis, hyperparathyroidism, cancer and infection-susceptibility, sofar are supported by several independent and large studies reporting similar associatons. However, also here different alleles are sometimes reported to be the risk allele and, thus, the same considerations as described above should be taken into account. In addition, the potential confounding effects which arise from this pleiotropy can influence the associations observed. For example, VDR gene variants can influence calcium metabolism through differential absorption in the intestine and, at the same time, influence bone turnover, while also the occurrence of osteophytosis (as a part of osteoarthritis) can be influenced, together resulting in a net effect on BMD measured at a certain site, at a certain age and in a subject with a certain diet.

5. Functional studies

The interpretation of the VDR association studies is severely hindered by the fact that most of the polymorphisms used are anonymous, i.e., have an unknown functional effect. The likely explanation for any observed association is then to assume the presence of a truly functional sequence variation elsewhere in the gene which is to a certain extent in linkage with an allele of the anonymous polymorphism used. As can be understood from the complex organization of the VDR gene (see Fig. 1) the identification of these functional polymorphisms in the VDR gene is a challenging enterprise. While these results are still eagerly awaited, several investigators have nevertheless analysed multiple bio-response parameters using the anonymous polymorphisms, including the FokI, BsmI, and Bsm–Apa–Taq haplotypes, and a polyA tract in the 3′UTR. These studies include in vitro cell biological and molecular biological studies, and in vivo measurements of biochemical markers and response to treatments with Vitamin D, calcium and even HRT or bisphosphonates.

In view of what has been discussed above it is not very surprising that these "functional" studies have not shown one allele being consistently associated with all of the different parameters. Major caveats of these studies are (a) the use of the anonymous rather then functional polymorphisms to group subjects and cells by genotype, and (b) the use of different types of bio-responses and different cell types and cell culture conditions in which the Vitamin D response might not be evident under the conditions of the experiment. Therefore, the identification of a functional polymorphism and the use of different well-defined cell types will help in clarifying the molecular mechanisms underlying the associations observed.

Part of the initial efforts to identify functional sequence variations have been focussed on the 3′ regulatory region because this is close to the anonymous markers used sofar in associations studies (see Fig. 1). While the BsmI, ApaI and TaqI RFLPs are located near the 3′ end of the gene, the LD extends into the 3′ regulatory region containing the UTR. Morrison and colleagues already showed the 3′UTR to contain sequence variations that were suggested to explain the observed associations [12] and provided evidence of differential luciferase activity for the two UTR variants that are linked to the two most frequent haplotypes, i.e., "baT" (haplotype 1 according to [14]) and "BAt" (haplotype 2). Durrin and colleagues have shown certain parts of the UTR, so-called destabilizing elements, to be involved in determining stability of the VDR–mRNA [29]. Yet, when eight individuals, selected by their genotype of the poly(A)-stretch in the 3′UTR, were sequenced no polymorphisms were found in the destabilizing elements of the 3′UTR. Furthermore, the UTRs linked to the two most common variants (the "baT" and "BAt" haplotype) were not found to differ with respect to mRNA stability [29]. However, only few individuals were sequenced so variations could have still been missed while also heterologous constructs (human VDR–UTR sequences coupled to a rabbit β-globin gene) and cell types (mouse NIH3T3 cells) were used to test for functionality. Especially, since it is known that 3′UTRs display cell type specific effects on mRNA stability this could be important in demonstrating functionality of sequence variations in the UTR. Although it is assumed that mRNA stability differences might underly the allelic differences, alternative explanations should be considered.

Recently, Whitfield and colleagues demonstrated functional significance of the translation initiation codon polymorphism (detected as FokI RFLP) and the poly(A) stretch in the 3′UTR [41]. In a series of 20 fibroblast cell lines of different VDR genotype, the relative transcription efficiency was measured of the endogenous VDR protein which was differing by the genotype at the FokI RFLP (F and f alleles) and the poly(A) stretch with long (L) and short (S) alleles which is acting as a transcription factor for a 1,25-dihydroxyvitamin D_3-responsive reporter gene. This study provided evidence for so-called high (of the "FL" genotype) and low (of the "fS" genotype) VDR activity. One of the possible explana-

tions mentioned included differences in translational activity (rather than mRNA stability) of the different mRNA–3′UTR variants. However, further research is necessary to prove that assumption. In any case, the study also illustrated the importance of analysing multiple polymorphisms in the VDR gene in relation to each other, as is illustrated in Fig. 2.

6. Conclusions

It is likely that still more polymorphisms, including functional ones, will be discovered in the complex promoter region of the VDR gene and larger population studies will be necessary to document the LD over the region and to evaluate the associations with relevant endpoints such as BMD and fracture risk. In particular, studies should be undertaken in which the VDR gene is systematically scanned for sequence variations such as has been done for other candidate genes [6,7]. Haplotype analyses should be used to identify groups of SNPs linked together and, thus, simplify the association analyses and understand the associations observed. Until clearly functional polymorphisms are identified in the VDR gene, interpretation of meta-analyses to evaluate consistency of associations and estimate effect size of a polymorphism, will be cumbersome. Because of difference in LD and the resulting haplotypes, allelic heterogeneity is expected a priori while for truly functional polymorphisms the same risk allele is expected to display similar effects and associations.

Taken together, it is clear that multiple polymorphic variations exist in the VDR gene which could each have different types of consequences (as is illustrated in Fig. 2). Thus, 5′ promotor variations can affect mRNA expression patterns and levels while 3′UTR sequence variations can affect the mRNA stability and/or protein translation efficiency. In combination these genotypic differences are likely to affect the VDR protein levels and/or function, depending on the cell type, developmental stage and activation status.

In summary, one can conclude that VDR gene variants seem to influence a number of biological endpoints, including those related to osteoporosis. Yet, the associations have different magnitudes with BMD probably being one of the weaker effects. In different study populations, different alleles of the anonymous RFLPs can be found associated with the same endpoint. This probably reflects that linkage disequilibrium, between the anonymous marker alleles and the causative alleles in (or very near) the VDR gene, is likely to be different between populations. Finding functional sequence variants that matter, establishing the phase of alleles across the entire VDR gene, and defining haplotype patterns is therefore required to put the associations observed with VDR gene polymorphisms in biological perspective.

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