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. So far, three adjacent restriction fragment length polymorphisms (RFLP) for BsmI, ApalI and TaqI, respectively, at the 3’ end of the VDR gene have been the most frequently studied so far. 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.

Keywords: DNA; Genetic; Steroid receptor; Haplotype; Osteoporosis

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-dihydroxyvitamin D resistant rickets, a rare monogenetic disease. More subtle sequence variations (polymorphisms) in the VDR gene occur much more frequently in the population but 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].

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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 (RFLPs). 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 promoter 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 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 speaking, studies of VDR polymorphisms in relation to bone endpoints, including osteoporosis, have received most attention so far 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 BsmI, ApaI and TaqI, respectively, in intron 8/exon 9 at the 3’ end of the Vitamin D receptor gene, have been most frequently studied so far. Morrison et al. reported that the BsmI 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 so far 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 haplo-
types 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 be-
tween 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
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 genotype-related differences in BMD [25]. However,
because other groups have not seen a relationship be-
 tween 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 asso-
ciations. While the initial studies by Morrison et al. [11–13]
suggested the “B” allele of the Bsm I 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 ef-
fect, e.g., on BMD, very often the statistical power of the indi-
nual 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 I RFLPs are not functional themselves, as far
as we know. The Bsm I and Apa I RFLPs are located in intron
8 and are not affecting any splicing site and/or transcription
factor binding site. The Taq I RFLP is a “synonymous”
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 func-
tional 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 func-
tional allele depends on the extent and strength of linkage
disequilibrium across that area of the chromosome. Conse-
quently, 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 coun-
tries and populations while circulating vitamin D level,
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
genome can play a role in influencing an association, but these
will be discussed below.

3. Other VDR polymorphisms

The alleles of the Bsm I, Apa I and Taq I 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 de-
scribed 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 Bsm I 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 Bsm I RFLP might contribute to
heterogeneity among association studies.

This notion is strongly supported by the recent compre-
hensive 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 as-
sociation is observed with such a haplotype allele, functional
studies have to be performed to identify the “causative” al-
lele.

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.

More than 25 different polymorphisms are currently known to be present at the VDR locus (see Fig. 1), so far mostly near the 3' end of the gene. However, also towards the 5' end of the gene in and near the promoter region other sequence variations have been reported. For example, a substitution (T to C) at exon 2 eliminates the first ATG translation initiation site and allows a second one 9bp down stream 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 FokI 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 BsmI, Apal and TaqI polymorphisms and, in view of the considerable distance between the two sites (±40kb) 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.
lotypes, and a polyA tract in 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). Dur- rin 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 polyA-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 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 differ- ing 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 D3-responsive reporter gene. This study provided evidence for so-called high (of the “FL” genotype) and low (of the “IS” genotype) VDR activity. One of the possible explana-

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, so far are supported by several independent and large studies reporting similar associations. However, also here different alleles are sometimes reported to be the risk allele and, thus, the same consider- ations 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 osteoporosis (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 ef- fect. 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. 2) the identification of these functional polymorphisms in the VDR gene is a challeng- ing enterprise. While these results are still eagerly awaited, several investigators have nevertheless analysed multiple bio-response parameters using the anonymous polymor- phisms, including the FokI, BsmI, and Bst–Apa–Tag hap- lotypes, 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 dif- ferent parameters. Major caveats of these studies are (a) the use of the anonymous rather than 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 associ- ations observed.

Part of the initial efforts to identify functional sequence variations have been focused on the 3’ regulatory region because this is close to the anonymous markers used so far in associations studies (see Fig. 1). While the BsmI, Apal and TaqI RFLPs are located near the 3’ end of the gene, the LD extends into the 3’ regulatory region containing the UTR. 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].
tions mentioned included differences in translational activity (rather than mRNA stability) of the different mRNA–3′UTR variations. 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 variants exist in the VDR gene which could each have different types of consequences (as is illustrated in Fig. 2). Thus, 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 haplotypes, allelic heterogeneity is expected to be the case as is illustrated in Fig. 2. Thus, 3′UTR sequence variations can affect the VDR protein levels and/or function, depending on the cell type, developmental stage and activation status.

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