Vitamin D receptor polymorphisms relate to risk of adenomatous polyps in a sex specific manner.

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Short Title: VDR genotypes and risk of adenomatous polyps

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Abstract

Background/Aims: Vitamin D receptor (VDR) gene polymorphisms may influence risk for adenomatous polyps, a benign precursor to colon cancer, via modulation of vitamin D sensitive pathways, including cell proliferation and differentiation. However, results have been mixed and any association remains contentious. Failure to clinically exclude the presence of adenomatous polyps in control cohorts may contribute to the lack of consensus. Therefore, we assessed the role of the FokI, BsmI, ApaI and TaqI *VDR* polymorphisms in modifying risk for adenomatous polyps, adjusting for a range of dietary and lifestyle variables. **Methods:** Blood was collected from colonoscopy patients (n=258) and *VDR* polymorphisms assessed by RFLP. Dietary habits were estimated from food frequency questionnaires. Odds ratios for adenomatous polyps were calculated by genotype, stratified by sex, and adjusted for age, lifestyle and dietary factors. **Results:** FokI was associated with modified risk for AP in males, whilst the BsmI/ApaI/TaqI haplotype was associated with modified risk in females. No interaction was found between *VDR* variants and vitamin D intake. **Conclusion:** This study offers novel insight into the potential for *VDR* genetics to contribute to risk for adenomatous polyps, and is the first to demonstrate a sex-specific relationship between these polymorphisms and risk for adenomatous polyps.

Introduction

Adenomatous polyps (AP) are the benign precursor to colorectal cancer (CRC), a cancer with multiple known modifiable dietary risk factors (1-3). Vitamin D may contribute to the aetiology of AP (4-6) via modulation of multiple vitamin D sensitive pathways that can influence tumourigenesis, including angiogenesis, cell proliferation and differentiation, immunomodulation, and protection from oxidative stress (7-9). This may occur indirectly via regulation of calcium homeostasis, or via direct interaction with the vitamin D receptor (VDR) (7). *VDR* polymorphisms may influence these pathways and modify risk of disease.

Numerous polymorphisms have been identified in the *VDR* gene. Of these only the FokI (rs2228570, previously rs10735810) polymorphism is known to generate a modified protein (10). The "F' allele has a later start codon than the "f" alle, generating a shorter more transcriptionally active protein (10-12). Three well studied polymorphisms are located at the 3' end of the gene; BsmI (rs1544410) and ApaI (rs7975232) (13-15), and TaqI (rs731236) (16). These polymorphisms are in high linkage disequilibrium forming a haplotype block (13, 16-18). These variants do not result in changes to the final VDR protein, but they have been linked to altered bone density (19) and risk of multiple diseases (20-22). This may occur via altered mRNA stability (11, 16) or these may be silent marker polymorphisms for other highly linked functional polymorphisms (23), such as a variable length of the poly (A) tail. The "baT" haplotype, containing the ancestral version of each allele, is linked to the long poly (A) variant , while the polymorphic "BAt" haplotype is linked to the short variant (16, 24).

Several studies have investigated the role of common *VDR* polymorphisms in the risk of CRC, however results have varied and the association remains contentious. Some studies have found no association between FokI status and CRC (25, 26), while others have found varying associations. Carriage of the "f" allele reportedly increased risk in a Chinese-Singaporean population, (27) and in those with high energy intake, low physical activity or obesity (28). Conversely, others have reported that carriage of the "F" allele increases CRC risk (4, 29, 30). This has been demonstrated in those with the "baT" haplotype (29), and regardless of BsmI genotype and poly (A) tail length, (30) suggesting that the FokI

haplotype may exert a stronger influence than some other polymorphisms. Consideration of the BsmI polymorphism alone has also yielded mixed results; both an increased (25, 31) and reduced risk (32, 33) in the presence the "B" allele, and no association with CRC (28, 34-36) have been reported. A 2014 meta-analyis found reduced risk in the presence of the "b" allele (37).

These discrepancies may be explained by failure to clinically exclude the presence of AP in control subjects. Controls for CRC studies are often recruited from licence and insurance registers and colonoscopies are not commonly performed (25, 27, 28, 30, 34). Only limited studies have examined the influence of *VDR* polymorphisms on AP risk in colonoscopy verified cases and controls; therefore it is difficult to draw firm conclusions. Some have found no relationship for multiple *VDR* polymorphisms (38, 39), BsmI (40), TaqI (41) or FokI (42). While Ingles *et al.* found that BsmI and FokI did not influence risk of AP alone, presence of the "f" allele was associated with increased risk of large (>1cm) polyps (18). A small 2011 meta-analysis found no association between FokI or BsmI genotype and AP risk (6). These discrepancies may be explained by differences in other dietary and lifestyle risk factors included in the analyses, or interactions between genotypes and nutritional status in the absence of direct association with genotype alone (43, 44).

Combined analysis of the BsmI/ApaI/TaqI haplotype and FokI are yet to be conducted in a single cohort using colonoscopy confirmed AP as cases and confirmed absence of polyps as controls. Therefore, we assessed the role of these four common *VDR* polymorphisms in modifying the risk for AP in a colonoscopy confirmed cohort, applying adjustment for a range of dietary and lifestyle factors.

Methods

Subjects and sample collection

Patients (n=263) undergoing routine screening for colonic pathology at a gastroenterology clinic (Gosford Hospital, NSW, Australia), aged 18-89 were recruited. 258 participants (43.80% male) who completed the required food frequency questionnaire, gave blood samples for DNA isolation, and received a definitive diagnosis as to the presence or absence of AP were included in this analysis.

Informed consent was obtained prior to participation under University of Newcastle Human Research Ethics Committee approval number H-429-0407.

Food frequency questionnaires

Daily intake of macro and micronutrients were estimated using a validated interviewer administered food frequency questionnaire, covering 225 food items and all food groups. Subjects also provided a list of all supplements and their frequency of intake. The food frequency questionnaires were analysed using Foodworks^{TM} (Version 2.10.146, Xyris Software, Brisbane, QLD, Australia) (45, 46).

Genotyping

Genomic DNA was isolated from whole blood and amplified using PCR. Restriction fragment length polymorphism (RFLP) assays and gel electrophoresis were used to genotype four common polymorphisms of the *VDR* gene; BsmI, ApaI, TaqI and FokI (45). The presence of restriction sites were coded with lower case letters (BsmI- 'b'; ApaI- 'a'; TaqI- 't'; FokI- 'f'), and the absence of restriction sites as the same letters in upper-case (BsmI- 'B'; ApaI- 'A'; TaqI- 'T'; FokI- 'F'). See supplementary methods for additional details. Haplotypes were reconstructed using PHASE v2.1(47, 48).

Statistics

Statistical analyses were performed using JMP (Version 11, SAS Institute Inc., Cary, NC, USA). Relationships were analysed by standard least squares or nominal logistic regression analysis, or Chi-squared tests, as appropriate. Analyses were stratified by sex and adjusted at a minimum for age, smoking history and reported alcohol intake. Additional models were performed adjusting for intake of energy, and a range of micro- and macro-nutrients previously linked to AP or CRC risk (total energy, dietary fibre, fat, folate, vitamin D, calcium, and iron intakes) and adjusted odds ratios (OR) with 95% confidence intervals (CI) were calculated. Outcomes were considered to be statistically significant at $p \leq 0.05$. Models were conducted with crossed terms to investigate interactions between variables where

appropriate. Descriptive statistics (mean±SEM, range) were calculated by sex and presented as required. Due to the restricted sample size, genotypes were combined to allow analysis by presence vs. absence of the ancestral or polymorphic alleles, as appropriate. Hardy-Weinberg equilibrium was tested for each polymorphism using a χ^2 test.

Results

There were several significant differences between cases and controls, and male and female cohorts (Table 1). In females, cases were significantly older than female controls (65.50±1.71 vs. 59.25±1.08 years, p=0.003). In males, cases consumed significantly less calcium (844.64±63.39 vs. 1112.21±73.36 mg/day, p=0.007) than controls. Somewhat surprisingly, male controls were more likely to have a history of smoking than cases (χ^2= 4.4, p=0.04; Table 1). Males (both cases and controls) consumed significantly more alcohol (2.00±0.50 vs. 0.69±0.25 g/day, p=0.02; 1.89±0.25 vs. 0.57±0.10 g/day, p=0.001, respectively), and more kilojoules (10167±459 vs. 8693±568 kj/day, p=0.05; 10670.60±441.30 vs. 9013.28±340.61 kj/day, p=0.003, respectively) than female cases. Male cases consumed significantly less calcium (844.64±63.39 vs. 1304.88±213.80 g/day, p=0.05), and significantly more iron (15.97±0.97 vs. 12.76±8.91 g/day, p=0.01), than female cases. Male controls were significantly older (63.82±1.45 vs. 59.25±1.08 years, p=0.01), were more likely to be smokers (χ^2= 4.6, p=0.03), and consumed significantly more fat (96.49±5.38 vs. 80.84±3.11 g/day, p=0.01) relative to female controls (Table 1).

The allele and genotype frequencies for the tested polymorphisms are given in Table 2. The genotype frequencies did not deviate from Hardy-Weinberg equilibrium expectations (FokI p=0.28; BsmI p=0.63; ApaI p=0.91; TaqI p=0.56). As expected, BsmI, ApaI and TaqI demonstrated high linkage disequilibrium in this cohort (Supplementary Figure 1), all with D' statistics over 0.95 (Supplementary Table 1). These results are comparable to those obtained in the HapMap (http://hapmap.ncbi.nlm.nih.gov/, (49)) and 1000 Genomes (http://www.1000genomes.org/data, (50)) CEU populations (Centre d'Etude du Polymorphisme Humain (CEPH) Utah residents with ancestry

from northern and western Europe), which have D' values of 0.98-1.0. There was no evidence of linkage disequilibrium between FokI and any of the other polymorphic sites (Supplementary table1).

Different alleles were related to an altered risk of AP in males and females (Table 3). In females, presence of the ancestral alleles for BsmI ("b") and TaqI ("T") significantly reduced the risk for AP (BsmI unadjusted OR= 0.23, 95% CI= 0.09-0.63, p=0.005; TaqI unadjusted OR= 0.25, 95% CI= 0.09-0.69, p=0.008). In males, presence of the polymorphic allele for FokI ("F") significantly increased risk for AP (unadjusted OR= 5.65, 95% CI= 1.07-104.95, p=0.043). Adjustment for age, smoking history and alcohol consumption (Model 1; Table 3) and further adjustment for vitamin D and calcium intake (Model 2; Table 3), energy, dietary fibre, fat, folate, and iron (Model 3; Table 3) did not alter the significance of these relationships. No associations were found between AP risk and ApaI alleles in either sex (Table 3).

As BsmI/ApaI/TaqI form a haplotype block (13, 16-18), analysis was repeated with reconstructed haplotypes. Haplotype frequencies are shown in Table 4. The most common haplotype, "baT" (45.16%), contains the ancestral version of each allele (restriction sites for BsmI/ApaI, no restriction site for TaqI). The second most common haplotype "BAt" (30.62%), contains the polymorphic version of each allele (no restriction sites for BsmI/ApaI, restriction site for TaqI). Analysis was repeated for risk of AP by presence of these two common haplotypes. Consistent with the single allele analysis, presence of the "baT" haplotype reduced risk for AP in females (unadjusted OR=0.27, 95% CI=0.10-0.74, p= 0.01; Table 5). Results remained significant following adjustments (Table 5). No association was found in males.

Combining analyses for both the BsmI/ApaI/TaqI haplotype and the FokI polymorphism revealed that risk was decreased in females with the baTf combination after adjustment for age, smoking history and alcohol consumption (Model 1 OR=0.35, 95% CI=0.11-0.99, p=0.05; Table 6), but increased in females with the BAtf combination (Model 1 OR=2.78, 95% CI=1.08-8.19, p=0.05; Table 6), suggesting that the haplotype block is a bigger factor in deciding risk than the FokI allele. Again, results remained significant following adjustment, with the exception of model 3 in the presence of the BAtf

combination. No statistically significant results were found in males (Table 6). No significant interaction was found between reported vitamin D or calcium intake and any allele or haplotype studied ($p>0.05$).

Discussion

In this Australian cohort the *VDR* polymorphism significantly influenced the risk of AP in a sex specific manner. The start codon FokI polymorphism significantly altered risk in males, whilst the BsmI/ApaI/TaqI haplotype block significantly altered risk in females. Factors contributing to the sex specific associations may include differences in the hormonal milieu and in overall incidence and age of incidence between the sexes. Information on BMI and energy excess are not available in this population and these are likely to differ between males and females. These factors may interact with genetic and hormonal differences to alter risk.

The data presented here supports previous data from Boyapti *et al*. (43) who, in a similar sized study, suggested that high blood calcium levels may reduce risk of AP, particularly in those with at least one "b" allele. However, it is in conflict with the data of Kim *et al.* (40) who found no influence of BsmI genotype, except in those with low serum vitamin D and/or calcium intake. This study is limited in that vitamin D status only represents reported dietary and supplementary intake, and not serum vitamin D levels. Calcium and vitamin D homeostasis may be modulated by *VDR* polymorphisms, and this may be a potential mechanism for altered risk (51, 52). Additional factors may influence systemic levels of vitamin D, calcium and other micronutrients including sun exposure, skin pigmentation, variance in vitamin D binding protein, and other genetic factors, that influence vitamin D and calcium homeostasis. It is also possible that dietary vitamin D and systemic vitamin D may have different influences on AP risk. No interaction was found between the polymorphisms studied and vitamin D or calcium intake. This may suggest that the mechanism of altered risk occurs via an indirect mechanism, or a larger cohort may be required to adequately identify an interaction.

The data presented here is in conflict with that of Slattery *et al*. (28) and Wong *et al* . (27), who found that the "f" allele increased risk of CRC, and supports the data of Sweeney (30), Ingles (18) and Park

(29), who found that the "F" allele increased risk of CRC. Differences between dietary and lifestyle habits of cohorts from different demographics and socio-cultural backgrounds may explain these discrepant results. However, potential differences in risk factors for AP incidence as opposed to the progression to CRC need to be considered. For example, whilst high folate levels may protect against initial occurrence of neoplasms via its essential role in DNA synthesis and repair processes, high folate levels after the initial occurrence may encourage tumour growth via enhanced opportunity for proliferation (53, 54). Differential roles for vitamin D (from diet and UV exposure) and other micronutrients may also exist in the aetiology of AP compared to its sequela, CRC. Additional studies are needed to determine if this is the case and to determine the biological processes involved. Despite the limitations identified, this study offers novel insight into the nutrigenetic risk factors for AP, and is the first to demonstrate a sex specific relationship between these *VDR* polymorphisms and risk for AP.

Conflict of Interest Declaration

All authors declare that they have no conflicts of interest.

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