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Vitamin D receptor and calcium-sensing receptor polymorphisms and colorectal cancer survival in the Newfoundland population

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Background: Increased serum levels of vitamin D and calcium have been associated with lower risks of colorectal cancer (CRC) incidence and mortality. These inverse associations may be mediated by the vitamin D receptor (VDR) and the calcium-sensing receptor (CASR). We investigated genetic variants in *VDR* and *CASR* for their relevance to CRC prognosis.

Methods: A population-based cohort of 531 CRC patients diagnosed from 1999 to 2003 in Newfoundland and Labrador, Canada, was followed for mortality and cancer recurrence until April 2010. Germline DNA samples were genotyped with the Illumina Omni-Quad 1 Million chip. Multivariate Cox models assessed 41 tag single-nucleotide polymorphisms and relative haplotypes on *VDR* and *CASR* in relation to all-cause mortality (overall survival, OS) and disease-free survival (DFS).

Results: Gene-level associations were observed between *VDR* and the DFS of rectal cancer patients ($P = 0.037$) as well as between *CASR* and the OS of colon cancer patients ($P = 0.014$). Haplotype analysis within linkage blocks of *CASR* revealed the G-G-G-G-G-A-C haplotype (rs10222633-rs10934578-rs3804592-rs17250717-A986S-R990G-rs1802757) to be associated with a decreased OS of colon cancer (HR, 3.15; 95% CI, 1.66–5.96). Potential interactions were seen among prediagnostic dietary calcium intake with the *CASR* R990G ($P_{int} = 0.040$) and the *CASR* G-T-G-G-G-G-C haplotype for rs10222633-rs10934578-rs3804592-rs17250717-A986S-R990G-rs1802757 ($P_{int} = 0.017$), with decreased OS time associated with these variants limited to patients consuming dietary calcium below the median, although the stratified results were not statistically significant after correction for multiple testing.

Conclusions: Polymorphic variations in *VDR* and *CASR* may be associated with survival after a diagnosis of CRC.

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Evidence from both epidemiological (Giovannucci *et al*, 2006; Otani *et al*, 2007) and experimental studies (Harris and Go, 2004; Leyssens *et al*, 2013) support a reduced risk of colorectal cancer (CRC) by higher intake or blood levels of vitamin D. Vitamin D mediates its action through binding to the vitamin D receptor (VDR), a member of the nuclear receptor superfamily that is expressed in various cell types, including colorectal epithelial cells. This binding enables the transactivation of target genes that promote cellular differentiation (Palmer *et al*, 2001), induce apoptosis (Donohue and Demay, 2002), and inhibit angiogenesis and proliferation (Kallay *et al*, 2001). Thus, *VDR* has been implicated in colorectal carcinogenesis. Another gene that influences vitamin D metabolism is the calcium-sensing receptor (*CASR*), which is essential for calcium homeostasis and cellular growth kinetics (Chattopadhyay *et al*, 2000; Jenab *et al*, 2009). In the *CASR* promoter region, vitamin D response elements have been discovered, providing evidence at the molecular level for a potential interaction between vitamin D and calcium in CRC (Canaff and Hendy, 2002; Dong *et al*, 2008). Recent studies have also demonstrated a critical role of *CASR* as a tumour suppressor in the large intestine (Aggarwal *et al*, 2015). Expression of this receptor has been shown to be reduced in colon cancer cells as compared to normal colonic epithelial cells (Sheinin *et al*, 2000).

Current molecular studies have identified numerous single-nucleotide polymorphisms (SNPs) in the human *VDR* and *CASR* genes, but only a handful that are considered potentially functional have been examined in relation to CRC risk, including FokI (rs10735810) (Jenab *et al*, 2009; Sarkissyan *et al*, 2014), BsmI (rs1544410) (Jenab *et al*, 2009; Bai *et al*, 2012), ApaI (rs7975232) (Laczmanska *et al*, 2014; Sarkissyan *et al*, 2014), and TaqI (rs731236) (Laczmanska *et al*, 2014) in *VDR*, and A986S (rs1801725) (Dong *et al*, 2008; Mahmoudi *et al*, 2014) and R990G (rs1042636) (Dong *et al*, 2008) in *CASR*. Several studies have linked one or more of these variants to CRC and, particularly, the *VDR* BsmI bb (GG) (Jenab *et al*, 2009; Bai *et al*, 2012) and *CASR* A986S (TT) genotypes (Bacsi *et al*, 2008) were related to an increased risk of CRC. However, very few relevant studies on CRC survival have been published and none of these were from the Canadian population (Bacsi *et al*, 2008; Hubner *et al*, 2008; Egan *et al*, 2010; Fedirko *et al*, 2012; Perna *et al*, 2013). Limited evidence shows no association of polymorphisms in *VDR* and *CASR* with survival after CRC diagnosis, but can be criticised for limited power or incomplete coverage of the variation within the gene (Bacsi *et al*, 2008; Fedirko *et al*, 2012). In addition, little is known regarding how pre- or postdiagnostic dietary factors could interact with *VDR* and *CASR* genotypes to influence CRC prognosis (Fedirko *et al*, 2012). This is important especially because long-term eating habits prior to diagnosis may affect postdiagnostic diet, and because cancer patients may have a strong desire to make positive changes, and may benefit from recommendations on a healthy diet and supplement use as a complement to their therapy (Patterson *et al*, 2003; Fedirko *et al*, 2012).

Therefore, in this analysis, we examined the hypothesis that genetic variations in *VDR* or *CASR* influence survival among CRC patients with possible effect modification by prediagnostic dietary vitamin D and calcium intakes within the context of a population-based cohort study in Newfoundland.

MATERIALS AND METHODS

Study population. The study was performed as part of the Newfoundland Familial Colorectal Cancer Study (NFCCS) effort to investigate environmental and genetic influences on CRC risk and survival outcome. The detailed rationale and methodology of the NFCCS has been described elsewhere (Green *et al*, 2007; Woods *et al*, 2010; Zhu *et al*, 2014). Briefly, histologically confirmed cases of CRC diagnosed under age 75 between 1997 and 2003 were recruited in the province of Newfoundland & Labrador. The 531 patients (201 women and 330 men) included in the current study represented a subset of patients enrolled in the NFCCS ($n = 737$) who had both the disease-outcome data and a germline DNA sample available. Informed consent was obtained for all participants, and the study was carried out with the approval by the Health Research Ethics Authority of Memorial University of Newfoundland in accordance with the tenets of the declaration of Helsinki.

Diet assessment and baseline information collection. Detailed information about demographics, race and ethnicity, individual behaviors, medical history, detailed cancer family history, bowel screening history, and use of alcohol and tobacco was gathered via self-administered Family History Questionnaires (FHQ) and Personal History Questionnaires (PHQ). Participants also completed a 169-item Food Frequency Questionnaire (FFQ) at the time of recruitment that addressed their dietary intake in the one year prior to their diagnosis. The FFQ was self-administered and semi-quantitative that had previously been validated in the Newfoundland population (Liu *et al*, 2013). The nutrient intakes from diet were calculated by multiplying the frequency of consumption of each food item by the nutrient content per average unit (Sun *et al*, 2011).

Study outcomes. Study participants were followed for recurrence and mortality from the date of cancer diagnosis until April 2010, with a combination of active follow-up (periodic follow-up questionnaires, for example, FHQ) and record linkage to death certificates, pathology reports, autopsy records, physicians' notes, and surgical reports. The additional data were obtained from the Dr H. Bliss Murphy Cancer Care Foundation. The main outcomes used for this study were overall survival (OS) and disease-free survival (DFS). The end-point event for the OS analysis was death from all-causes and for the DFS analysis was death from any cause, CRC recurrence, or metastasis, whichever came first.

Genotyping and SNP selection. Genotyping of peripheral blood DNA samples was performed using the Illumina Human Omni-Quad Bead chip that contains about 1.1 million SNPs at Centrillion Biosciences (USA). For quality control purposes, genomic DNA from 200 duplicate samples were sent to the Laboratory of Dr Stephen Gruber (Director, USC Norris Comprehensive Cancer Center, Los Angeles) for genotyping using the Affymetrix Axiom myDesign GW Array Plate, which contains 1.3 million probes. SNPs with genotype concordance $<97\%$ between the two platforms were dropped from all analyses.

We used an aggressive tagging approach to limit the number of SNPs examined to the most relevant. Tagging SNPs capturing most of the common variation in the candidate gene regions were identified using Plink v1.07 based on the following criteria: the minor allele frequency of the SNP $\geqslant 5\%$; pairwise $r^2 > 0.9$; and at least 50 base pairs from any adjacent SNPs (de Bakker *et al*, 2005). The regions analysed included about 103 kb of the *CASR* gene and 65 kb of the *VDR* gene. This process identified 24 SNPs for *VDR* and 14 SNPs for *CASR*. Additionally, *a priori*, we also selected 4 high interest SNPs reported in previous CRC studies, including *VDR* BsmI, *CASR* R990G, *CASR* rs1802757, and *CASR* A986S. For all genotypes, the call frequency was $>99.5\%$ except for one SNP (*VDR* rs2238135, 99.2%). The distribution of the genotypes of all SNPs examined in this study fitted Hardy-Weinberg proportions, with the exception of *VDR* rs3847987 ($P<0.001$), which was excluded from analysis.

Our protocol for MSI testing and mutation detection on *BRAF* V600E in tumour DNA has been described elsewhere (Woods *et al*, 2010). MSI status was determined using 5 to 10 microsatellite

markers. Mutant alleles in the *BRAF* gene were detected using the allele-specific polymerase chain reaction (AS-PCR) technique (Woods *et al*, 2010).

Statistical analysis. The log-rank test compared the survival distributions across groups of baseline factors. We utilised a principal component (PC) analysis that accounts for linkage disequilibrium (LD) between multiple SNPs to test for an overall association of a gene with survival of CRC patients (Gauderman *et al*, 2007). Briefly, this approach computed uncorrelated linear combinations of the original SNPs, grouped as PCs, that explain the greatest amount of variance across the gene. Then, PCs that cumulatively explain at least 80% of the variance were retained and included in a Cox proportional hazards regression analysis with CRC survival as the outcome. Using a likelihood ratio test, we calculated a *P*-value for the global gene-outcome association by comparing models with and without selected PCs with the number of degrees of freedom equal to the number of PCs. Overall survival and DFS were the main outcomes, each stratified by anatomical site (colon and rectum).

The data were further explored using a single-SNP analysis, followed by the haplotype analysis. For every individual SNP, the associations with overall and DFS in CRC patients were estimated by hazard ratios (HRs) and 95% confidence intervals (CIs), while assuming an additive model by Cox regression analysis, adjusted for sex, race, age at diagnosis, disease stage at diagnosis, reported screening procedure, marital status, MSI status, and *BRAF* mutation status when applicable. These covariates were retained in the final model because they either entered the model at $P<0.1$ or altered the parameter estimates by $>10\%$. The proportional hazards assumption was verified by testing the statistical significance of time-by-covariate interactions for each covariate in the Cox model. To control type I error inflation, *P*-values were then adjusted for multiple comparisons using the approach specifically created for correlated tests due to LD by Conneely and Boehnke, 2007. For the haplotype analyses, LD plots were generated using the Haploview version 4.2 to evaluate haplotype block structure based on the criteria of Gabriel *et al*, 2002. Haplotype frequencies were estimated using the expectation maximisation algorithm accounting for ambiguous linkage phase, and the association between individual haplotype and CRC survival was assessed by modelling all haplotypes simultaneously with the most frequent haplotype as the reference. Bonferroni correction for multiple testing was performed for thirty-six haplotypes yielding an adjusted *P*-value of 0.0014. A global *P* value for each haplotype block was obtained with a Wald test. Haplotype analyses were performed using SimHap GUI version 1.0.2 (Carter *et al*, 2008). Gene-environment ($G \times E$) interactions were tested through stratified analysis and verified with the Wald method by introducing a multiplicative interaction term into the model and assessing its significance. The $G \times E$ analyses, *a priori*, were not adjusted for multiple comparisons. All tests were two-sided. Other data management and analyses were performed with SAS software version 9.4 (SAS Institute, Cary, NC, USA).

RESULTS

Patient characteristics and clinical predictors. The study sample consisted of 330 men and 201 women (Table 1). The mean age of the study population was 60.7 ± 9.2 yrs, with 96.9% of the participants being white, 11.5% reporting a bowel screening history, and 66.0% having had tumours at the colon subsite. Information on MSI status was available for a total of 503 patients, with 11.5% classified as MSI-H and 88.5% as MSS/MSI-L. Salient characteristics were largely comparable between the NFCCS patients included and those excluded from the current study due to lack of genotype/disease-outcome data. At the end of our study (median follow-up time, 6.4 years), 183 (34.5%) of the 531 patients had died. In the univariate analysis, male gender, other ethnicity, advanced stage at diagnosis (III/IV), chemoradiotherapy, and MSS/MSI-L tumours were significantly associated with reduced OS time, whereas bowel screening procedure, tumour location, and *BRAF* mutation status were not associated with OS among the 531 CRC patients included in this study.

Association of *VDR* and *CASR* with survival of CRC patients. PC analysis was conducted to assess whether there was an overall gene-level association between *VDR* or *CASR* and CRC survival (Table 2). At the gene level, we observed no meaningful relationships for *VDR* or *CASR* and CRC survival. However, after stratification by colorectal subsite, the *VDR* gene exhibited a marginally significant association with the DFS among patients with rectal cancer (Global $P=0.037$), while the *CASR* gene was related to OS in colon cancer patients at a significance level of 0.05 (Global $P=0.014$).

In analyses of individual SNPs within each gene, a total of four SNPs in *VDR* and two SNPs in *CASR* were related to OS under an additive model (Supplementary Tables 1 and 2). However, multiple testing adjustment revealed one association of marginally statistical significance for *VDR* (BsmI polymorphism, rs1544410) and the OS of all CRC ($P_{\text{unadjusted}}=0.002$, $P_{\text{adjusted}}=0.058$). Specifically, the G-allele was related to worse OS as compared with the A-allele (HR per G allele, 1.15; 95% CI, 1.17–1.94) (Figure 1). For DFS, no SNPs approached statistical significance after adjusting for multiple comparisons. Given that SNP prevalence varies across populations, analyses were repeated among those with European ancestry ('white') alone, which produced similar findings.

Haplotypes and survival of CRC patients. To evaluate potential epistatic or combined effects of SNPs, haplotype analysis was conducted to derive haplotype groups within linkage equilibrium blocks of each gene. We identified five major blocks on *VDR* and four blocks on *CASR*, respectively (Supplementary Figure 1, Table 3). For the *VDR* gene, the haplotype A-T-G in LD block 1 (rs11574143-TaqI-BsmI), which contained the borderline significant BsmI risk G allele from previous single SNP analysis, was associated with a reduced OS of rectal cancer in comparison to the most common haplotype (HR, 2.53; 95% CI, 1.20–5.34; block global $P=0.027$), although this association was no longer significant when Bonferroni's correction was applied. For *CASR*, a less frequent (4.2%) haplotype designated as G-G-G-G-G-A-C in block 4 of *CASR* (rs10222633-rs10934578-rs3804592-rs17250717-rs1801725-rs1042636-rs1802757) was associated with a marked increase in odds of all-cause mortality among patients with colon cancers (HR, 3.15; 95% CI, 1.66–5.96; block global $P=0.001$). This association remained significant after Bonferroni correction ($P<0.0014$). Results were similar for DFS.

Gene-diet interactions. We evaluated relationships between *VDR* or *CASR* variations and OS among CRC patients after stratification by dietary vitamin D and calcium intakes (Supplementary Table 3). We saw HRs >1 for *CASR* SNP rs1042636 (R990G) (HR, 2.21; 95% CI, 1.37–3.56; $P_{\text{int}}=0.040$) and the haplotype G-T-G-G-G-G-C in *CASR* LD block 4 (HR, 2.21; 95% CI, 1.36–3.58; $P_{\text{int}}=0.017$) in the stratum with calcium intake below the median, although the associations lost significance after adjustment for multiple tests. No evidence for modification by dietary vitamin D intake was observed.

DISCUSSION

In this study, *VDR* and *CASR* genes were associated with DFS and OS of CRC, respectively, at the gene level. Particularly, *VDR* BsmI

Table 1. Demographical and clinicopathological characteristics of patients in the Newfoundland Familial Colorectal Cancer Study (NFCCS)

	Subjects included in this study ($N = 531$)				Subjects without genotype/ disease-outcome information ($N = 206$)
Characteristic	**No. patients (%)**	**No. deaths (%)**	**MST (y)[a]**	**$P_{log\text{-}rank}$[a]**	**No. patients (%)**
Age at diagnosis (y)[b]	60.7 ± 9.2	61.3 ± 9.7	–	–	62.1 ± 9.6
Sex					
Female	201 (37.9)	56 (27.9)	6.5		83 (40.3)
Male	330 (62.1)	127 (38.5)	6.3	0.005	123 (59.7)
Race					
White	439 (96.9)	133 (30.3)	6.4		27 (93.1)
Other	14 (3.1)	8 (57.1)	4.7	0.009	2 (6.9)
Reported screening procedure					
Yes	52 (11.5)	10 (19.2)	6.6		7 (24.1)
No	401 (88.5)	131 (32.7)	6.4	0.059	22 (75.9)
Tumour location					
Colon	341 (66.0)	110 (32.3)	6.4		124 (72.1)
Rectum	176 (34.0)	65 (36.9)	6.3	0.444	48 (27.9)
Stage at diagnosis					
I/II	302 (56.9)	76 (25.2)	6.6		56 (27.2)
III/IV	229 (43.1)	107 (46.7)	6.0	<0.001	150 (72.8)
Surgery					
Yes	516 (97.2)	177 (34.3)	6.4		204 (99.0)
No	15 (2.8)	6 (40.0)	6.8	0.790	2 (1.0)
Chemoradiotherapy					
Yes	106 (20.5)	33 (41.51)	6.0		33 (19.2)
No	411 (79.5)	131 (31.87)	6.4	0.036	139 (80.8)
MSI status					
MSS/MSI-L	445 (88.5)	168 (37.8)	6.3		190 (92.2)
MSI-H	58 (11.5)	6 (10.3)	6.7	<0.001	16 (7.7)
BRAF mutation status					
Wild type	432 (89.8)	153 (35.4)	6.4		165 (84.2)
BRAF mutant	49 (10.2)	15 (30.6)	6.3	0.370	31 (15.8)
Dietary vitamin D intake (μg/d)[b]	6.3 ± 3.5	7.0 ± 4.2	–	–	5.5 ± 3.0
Dietary calcium intake (mg/d)[b]	965.6 ± 460.7	1024.8 ± 504.2	–	–	888.3 ± 427.8

Abbreviations: BMI=body mass index; MSI=microsatellite instability; MST=median overall survival time; MSI-H=microsatellite instability-high; MSS/MSI-L= microsatellite stable/microsatellite instability-low.
[a]The values reported were calculated over the number of subjects with valid information; the numbers of subjects with missing values for each variable are as follows: race (78), reported screening procedure (78), tumour location (14), chemoradiotherapy (14), dietary vitamin D intake (78), dietary calcium intake (78), MSI status (28), *BRAF* mutation status (50).
[b]Continuous variables presented as mean ± s.d. (standard deviation).

Table 2. Association between *VDR* and *CASR* genes and colorectal cancer overall and disease-free survival ($n = 531$)

	Overall survival HR (95% CI)[a]			Disease-free survival HR (95% CI)[a]		
	All CRC	**Colon cancer**	**Rectum cancer**	**All CRC**	**Colon cancer**	**Rectum cancer**
VDR						
PC1	0.80 (0.67–0.95)	0.76 (0.61–0.95)	0.90 (0.64–1.25)	0.83 (0.71–0.98)	0.82 (0.66–1.00)	0.86 (0.64–1.15)
PC2	0.99 (0.83–1.17)	0.85 (0.68–1.07)	1.41 (1.01–1.98)	1.00 (0.84–1.19)	0.84 (0.68–1.04)	1.78 (1.27–2.50)
PC3	0.87 (0.74–1.03)	0.90 (0.73–1.11)	0.81 (0.58–1.11)	0.91 (0.77–1.07)	0.95 (0.77–1.17)	0.74 (0.55–1.00)
PC4	1.03 (0.87–1.21)	1.07 (0.87–1.12)	0.91 (0.66–1.26)	1.05 (0.90–1.23)	1.12 (0.91–1.38)	0.83 (0.62–1.11)
PC5	0.90 (0.75–1.08)	0.89 (0.69–1.14)	0.97 (0.73–1.30)	0.91 (0.77–1.08)	0.92 (0.73–1.15)	1.02 (0.79–1.32)
PC6	0.96 (0.81–1.13)	1.01 (0.82–1.25)	0.84 (0.63–1.12)	1.07 (0.91–1.26)	1.06 (0.85–1.30)	1.10 (0.83–1.45)
PC7	1.05 (0.88–1.24)	1.10 (0.87–1.38)	1.11 (0.83–1.47)	1.09 (0.93–1.28)	1.10 (0.89–1.37)	1.09 (0.85–1.39)
PC8	0.98 (0.83–1.17)	0.90 (0.73–1.11)	1.26 (0.90–1.77)	1.02 (0.86–1.20)	0.87 (0.71–1.07)	1.60 (1.17–2.17)
Global P[b]	0.201	0.193	0.206	0.320	0.241	0.037
CASR						
PC1	1.09 (0.91–1.29)	1.04 (0.84–1.30)	1.25 (0.90–1.74)	1.10 (0.93–1.29)	1.02 (0.83–1.26)	1.29 (0.95–1.75)
PC2	0.94 (0.80–1.10)	0.84 (0.68–1.04)	1.20 (0.90–1.61)	0.96 (0.83–1.12)	0.92 (0.76–1.12)	1.15 (0.89–1.49)
PC3	1.12 (0.95–1.31)	1.23 (1.01–1.50)	1.07 (0.81–1.41)	1.13 (0.98–1.30)	1.15 (0.95–1.39)	1.15 (0.91–1.44)
PC4	0.95 (0.80–1.13)	1.02 (0.83–1.26)	0.91 (0.66–1.25)	1.03 (0.89–1.20)	1.07 (0.90–1.28)	1.03 (0.79–1.34)
PC5	0.94 (0.78–1.13)	0.85 (0.68–1.07)	1.19 (0.81–1.76)	0.94 (0.80–1.11)	0.87 (0.71–1.06)	1.15 (0.82–1.60)
PC6	1.10 (0.91–1.32)	1.26 (0.98–1.61)	0.82 (0.60–1.12)	1.12 (0.95–1.33)	1.27 (1.01–1.59)	0.89 (0.69–1.15)
PC7	0.80 (0.65–0.98)	0.72 (0.55–0.94)	0.95 (0.68–1.33)	0.88 (0.73–1.05)	0.81 (0.64–1.03)	0.96 (0.71–1.29)
Global P[b]	0.209	0.014	0.486	0.258	0.075	0.492

Abbreviations: CRC = colorectal cancer; HR = hazard ratio; PC = principal component.
[a]Cox proportional hazard model adjusted for age at diagnosis, sex, race, stage at diagnosis, reported screening procedure, marital status, MSI status, and *BRAF* mutation status where applicable; subjects with missing information on tumour location ($n = 14$) were excluded from the stratified analysis.
[b]Global P for association is from a likelihood ratio test with degrees of freedom equal to the number of PCs.

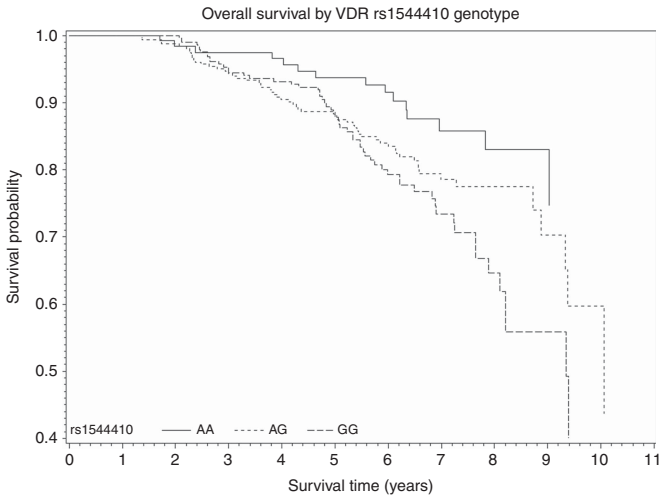

Figure 1. Overall survival curves by *VDR* rs1544410 genotype. Adjusted for age at diagnosis, sex, race, stage at diagnosis, reported screening procedure, marital status, and MSI status.

polymorphism exhibited marginally significant association with the OS of CRC patients after adjustment for multiple comparisons. Haplotype analyses showed that the *CASR* block 4 haplotype G-G-G-G-G-A-C, defined by rs10222633-rs10934578-rs3804592-rs17250717-rs1801725-rs1042636-rs1802757, was associated with a reduced OS among colon cancer patients.

Experimental work indicates that vitamin D has pleiotropic biological activities with complex anticancer properties, which include inhibition of cell proliferation, invasion, induction of apoptosis, cell cycle arrest, as well as simulation of differentiation (Palmer *et al*, 2001). The *VDR* has been proposed as a potential mediator that could modulate the effects of vitamin D. For example, *VDR* interacts with β-catenin and inhibits β-catenin signalling that is deregulated in most CRCs (Palmer *et al*, 2001; Egan *et al*, 2010). Through the nuclear VDR, $1,25(OH)_2D_3$ induces E-cadherin, increases β-catenin nuclear export, and inhibits β-catenin gene regulatory activity to hinder proliferation and loss of differentiation in the early stage of carcinogenesis (Palmer *et al*, 2001). *VDR* is highly polymorphic with over 100 known allelic variants. Some polymorphisms may have functional importance and thus have been evaluated in previous association studies on CRC. Specifically, the FokI translational start codon polymorphism alters the *VDR* structurally, with the F-variant *VDR* being three amino acids shorter and functionally more effective than the protein produced from the f allele (Miyamoto *et al*, 1997; Wong *et al*, 2003). BsmI (intron 8), ApaI (intron 8) and TaqI (exon 9) polymorphisms have been reported to influence *VDR* expression and thus serum levels of $1,25(OH)_2D_3$, but they are speculated to affect *VDR* function through linkage disequilibrium with other mutations in the 3′-UTR region that alter mRNA transcriptional activity and stability (Ingles *et al*, 1997; Kim *et al*, 2013). Although not a universal finding, the B allele of the *VDR* BsmI polymorphism has been found to be linked with a reduced risk of CRC (Jenab *et al*, 2009; Bai *et al*, 2012). Carriage of the variant FokI allele revealed either null (Peters *et al*, 2001; Slatter *et al*, 2001) or conflicting associations (Ingles *et al*, 2001; Wong *et al*, 2003; Park *et al*, 2006). With regard to survival after cancer diagnosis, several SNPs and haplotypes in *VDR* gene that are related to lower *VDR* function or expression (Cdx2, FokI and G-T-C for Cdx2-FokI-BsmI) have been linked with poorer survival for a variety of cancers such as non-small cell lung (Zhou *et al*, 2006; Heist *et al*, 2008) and epithelial ovarian (Tamez *et al*, 2009) cancers. Similar to the results for CRC incidence, in our study, the variant B (A) allele of the *VDR* BsmI appeared to be associated with a reduction in risk of all-cause mortality among CRC patients; yet no association was detected for *VDR* FokI polymorphism. Few studies have been published on *VDR* polymorphisms and CRC survival (Hubner *et al*, 2008; Egan *et al*, 2010; Fedirko *et al*, 2012; Perna *et al*, 2013; Zgaga *et al*, 2014). Nevertheless, these studies did not observe any significant associations of *VDR* variants with CRC survival. In our study, the variant B (A) allele of the *VDR* BsmI appeared to be associated with a reduction in risk of all-cause mortality among CRC patients; yet no association was detected for *VDR* FokI polymorphism. The inconsistencies in findings with previous studies may be explained by differences in population, other environmental factors (e.g., diet and lifestyle), and incomplete coverage of the gene in some studies (Hubner *et al*, 2008; Fedirko *et al*, 2012; Perna *et al*, 2013; Zgaga *et al*, 2014). While we do not presume to know how the *VDR* BsmI SNP influences survival for CRC, the pathway may be involved in the biological response to treatments. As such, the BsmI polymorphism warrants further investigation in terms of its role in treatment response and patient prognosis in CRC.

Another gene analysed in the present study is the *CASR*. The *CASR* is crucial for the maintenance of extracellular calcium homeostasis by affecting parathyroid hormone secretion and calcium reabsorption (Chattopadhyay *et al*, 2000; Jenab *et al*, 2009). It may also influence vitamin D metabolism. *CASR* has been implicated in breast and prostate cancers (Jeong *et al*, 2016). Indeed, several commonly studied *CASR* polymorphisms, including rs1801725 (Speer *et al*, 2002), rs1042636 (Hibler *et al*, 2012), rs10934578, rs12485716, rs2270916 and rs4678174 (Dong *et al*, 2008), have been related to the risk of CRC in some studies, but not others (Jenab *et al*, 2009; Jacobs *et al*, 2010; Mahmoudi *et al*, 2014). In the work by Kim *et al*, 2013, these SNPs were significant only under low calcium intake. Despite the many studies investigating *CASR* and CRC risk, we found only two published papers (Bacsi *et al*, 2008; Fedirko *et al*, 2012) that evaluated the association of *CASR* and CRC survival and observed no relationship, though only one SNP (rs1801725) was included in both studies. In the current study, none of these high-interest SNPs in the literature (rs10934578, rs1801725 and rs1042636) were significantly related to CRC survival. But, there was a suggestion that an intronic SNP in *CASR*, rs1354162, was associated with more favorable survival in colon cancer patients. The possible mechanisms explaining this association remain undetermined, particularly since rs1354162 is within an intron. Recent studies suggest that intronic SNPs have the potential to influence alternative splicing of RNA (Webb *et al*, 2003; ElSharawy *et al*, 2006). Interestingly, we found that the wild-type haplotype, G-G-G-G-G-A-C, in block 4 of *CASR* was associated with worse OS of colon cancer patients compared with the most common haplotype. Several SNPs defining this haplotype are nonsynonymous coding SNPs (that is, rs1801725 and rs1042636) or intron variants shown to be robustly associated with serum levels of calcium in recent GWAS studies (i.e., rs10222633 and rs10934578) (O'Seaghdha *et al*, 2013). We speculate that the region where these SNPs located may harbor a site of causative variants that in conjunction with each other impact on disease outcome; and these seven SNPs should be considered as candidate tag SNPs within the *CASR* gene for future association studies. Our results confirm with the theoretical expectation that haplotype-based approaches may have greater power than single-locus tests (Clark, 2004).

While it is unclear why *CASR* variation is related to survival only among colon cancer patients, and not rectal cancer patients, the difference in structure and cellular composition of the surface epithelium between colon (ciliated columnar epithelium) and lower rectum (squamous epithelium) may partially account. Alternatively, bile acids, formed in the liver and absorbed from the intestine has been shown to enhance intestinal proliferation and tumour yield (McMichael and Potter, 1985). Calcium could

Table 3. Haplotypes on *VDR* and *casr* genes and associations with overall and disease-free survival among colorectal cancer patients ($n = 531$)

		Overall survival HR (95% CI)[a]			Disease-free survival HR (95% CI)[a]		
Haplotypes	**Frequency[b]**	**All CRC**	**Colon cancer**	**Rectum cancer**	**All CRC**	**Colon cancer**	**Rectum cancer**
***VDR*, block 1[c]**							
GTG	0.5010	1.00	1.00	1.00	1.00	1.00	1.00
GCA	0.4060	0.67 (0.51–0.87)	0.65 (0.47–0.90)	0.66 (0.40–1.08)	0.72 (0.57–0.92)	0.77 (0.57–1.04)	0.66 (0.43–1.02)
ATG	0.0846	0.96 (0.61–1.50)	0.64 (0.46–1.16)	2.53 (1.20–5.34)	0.87 (0.58–1.31)	0.81 (0.48–1.36)	1.31 (0.65–2.64)
Global *P*[d]		0.236	0.790	0.027	0.293	0.773	0.229
***VDR*, block 2[e]**							
CTC	0.4473	1.00	1.00	1.00	1.00	1.00	1.00
ACC	0.1992	1.39 (1.01–1.92)	1.37 (0.91–2.07)	1.30 (0.73–2.32)	1.36 (1.00–1.84)	1.18 (0.81–1.72)	1.71 (0.98–2.99)
ATC	0.1749	0.94 (0.65–1.35)	0.89 (0.56–1.40)	1.12 (0.58–2.14)	0.99 (0.72–1.38)	0.95 (0.62–1.45)	1.03 (0.59–1.81)
CTT	0.1729	0.90 (0.62–1.29)	0.92 (0.58–1.44)	0.71 (0.36–1.41)	1.02 (0.74–1.42)	1.05 (0.69–1.60)	0.86 (0.48–1.54)
Global *P*[d]		0.325	0.559	0.470	0.923	0.967	0.880
***VDR*, block 3[f]**							
GG	0.7469	1.00	1.00	1.00	1.00	1.00	1.00
AC	0.2178	1.13 (0.84–1.51)	1.04 (0.70–1.55)	1.30 (0.83–2.040)	1.09 (0.83–1.43)	0.99 (0.69–1.43)	1.16 (0.76–1.78)
AG	0.0238	1.38 (0.62–3.04)	1.21 (0.46–3.16)	3.07 (0.62–14.59)	1.31 (0.63–2.71)	1.39 (0.58–3.30)	1.30 (0.28–5.81)
GC	0.0115	0.80 (0.25–2.52)	0.59 (0.14–2.41)	2.18 (0.21–18.83)	0.76 (0.24–2.36)	0.67 (0.16–2.69)	1.35 (0.17–9.76)
Global *P*[d]		0.638	0.897	0.228	0.550	0.678	0.456
***VDR*, block 4[g]**							
CC	0.4176	1.00	1.00	1.00	1.00	1.00	1.00
TT	0.4091	1.06 (0.82–1.38)	0.99 (0.72–1.38)	1.26 (0.80–2.00)	1.10 (0.86–1.39)	1.05 (0.77–1.42)	1.17 (0.78–1.77)
TC	0.1698	1.09 (0.77–1.53)	0.95 (0.60–1.51)	1.36 (0.80–2.33)	1.09 (0.80–1.50)	0.98 (0.65–1.48)	1.19 (0.72–1.97)
Global *P*[d]		0.762	0.926	0.447	0.171	0.438	0.289
***VDR*, block 5[h]**							
GG	0.4044	1.00	1.00	1.00	1.00	1.00	1.00
TT	0.3264	0.97 (0.73–1.30)	1.25 (0.86–1.83)	0.63 (0.38–1.04)	0.96 (0.74–1.26)	1.29 (0.92–1.82)	0.59 (0.37–0.97)
TG	0.2667	1.03 (0.77–1.38)	1.19 (0.81–1.75)	0.87 (0.55–1.38)	1.08 (0.82–1.41)	1.26 (0.88–1.80)	0.82 (0.53–1.27)
Global *P*[d]		0.707	0.383	0.119	0.283	0.313	0.038
***CASR*, block 1[i]**							
AG	0.6175	1.00	1.00	1.00	1.00	1.00	1.00
AA	0.1983	1.04 (0.77–1.42)	1.37 (0.95–1.99)	0.67 (0.38–1.19)	1.08 (0.81–1.42)	1.27 (0.89–1.81)	0.85 (0.53–1.37)
GA	0.1842	0.90 (0.65–1.24)	1.06 (0.72–1.57)	0.65 (0.37–1.14)	0.87 (0.64–1.17)	1.03 (0.70–1.50)	0.66 (0.39–1.11)
Global *P*[d]		0.841	0.125	0.123	0.792	0.627	0.357
***CASR*, block 2[j]**							
GGCC	0.3272	1.00	1.00	1.00	1.00	1.00	1.00
AGCT	0.2284	0.74 (0.54–1.02)	0.70 (0.46–1.06)	0.91(0.55–1.50)	0.76 (0.57–1.02)	0.72 (0.49–1.05)	0.91 (0.59–1.41)
AGCC	0.1860	0.89 (0.63–1.25)	1.13 (0.73–1.74)	0.62(0.34–1.13)	0.94 (0.69–1.28)	1.11 (0.75–1.66)	0.70(0.41–1.18)
GACC	0.1617	0.65 (0.45–0.96)	0.74 (0.47–1.19)	0.50 (0.24–1.05)	0.67 (0.47–0.96)	0.80 (0.51–1.23)	0.49(0.25–0.95)
AGAC	0.0911	0.49 (0.28–0.85)	0.29 (0.13–0.68)	0.92 (0.39–2.12)	0.65 (0.41–1.04)	0.50 (0.26–0.94)	0.95(0.45–2.02)
Global *P*[d]		0.070	0.047	0.477	0.300	0.113	0.490
***CASR*, block 3[k]**							
CT	0.8618	1.00	1.00	1.00	1.00	1.00	1.00
CC	0.0846	1.02 (0.66–1.57)	1.19 (0.70–2.05)	0.86 (0.40–1.84)	1.19 (0.81–1.76)	1.13 (0.68–1.89)	1.37 (0.77–2.46)
TC	0.0536	1.19 (0.74–1.92)	1.37 (0.78–2.39)	0.82 (0.32–2.09)	1.33 (0.89–2.01)	1.49 (0.91–2.43)	1.08 (0.48–2.45)
Global *P*[d]		0.910	0.741	0.819	0.677	0.617	0.908
***CASR*, block 4[l]**							
AGGGGAC	0.3440	1.00	1.00	1.00	1.00	1.00	1.00
AGGGGAT	0.1598	1.54 (1.03–2.31)	1.55 (0.93–2.59)	1.55 (0.78–3.07)	1.46 (1.01–2.10)	1.54 (0.95–2.50)	1.48 (0.82–2.66)
GTGGTAC	0.1363	1.10 (0.73–1.65)	1.36 (0.81–2.28)	0.68 (0.32–1.42)	1.19 (0.83–1.70)	1.39 (0.88–2.20)	0.86 (0.47–1.58)
GGAGGAC	0.1231	1.42 (0.91–2.22)	1.62 (0.96–2.76)	0.75 (0.30–1.85)	1.40 (0.94–2.09)	1.59 (0.99–2.56)	0.88 (0.39–1.99)
GTGTGAC	0.0968	1.11 (0.66–1.88)	0.84 (0.42–1.68)	1.66 (0.70–3.93)	1.03 (0.65–1.64)	0.82 (0.44–1.54)	1.38 (0.64–2.96)
GTGGGGC	0.0808	1.83 (1.20–2.79)	2.34 (1.38–3.97)	1.18 (0.54–2.58)	1.56 (1.07–2.28)	1.73 (1.06–2.82)	1.21 (0.64–2.28)
GGGGGAC	0.0423	2.30 (1.33–3.98)	**3.15 (1.66–5.96)**	0.75 (0.21–2.69)	2.15 (1.31–3.52)	**3.25 (1.80–5.85)**	0.73 (0.24–2.24)
GTGGGAC	0.0151	1.69 (0.60–4.68)	3.05 (0.85–10.63)	0.59 (0.07–4.39)	1.16 (0.39–3.31)	1.89 (0.51–6.64)	0.45 (0.06–3.21)
Global *P*[d]		0.015	0.001	0.423	0.014	0.001	0.944

Abbreviations: CRC = colorectal cancer; HR = hazard ratio. Those with significant *P*-values after Bonferroni correction for thirty-six haplotypes are shown in bold (i.e. the adjusted *P*-value at the 0.05 significance level is 0.0014).

[a]Cox proportional hazard model adjusted for age at diagnosis, sex, race, stage at diagnosis, reported screening procedure, marital status, and MSI status where applicable; subjects with missing information on tumour location ($n = 14$) were excluded from the stratified analysis.

[b]Rare haplotypes with frequencies less than 1% were excluded from analyses.

[c]*VDR*, block 1 includes rs11574143, rs731236, and rs1544410.

[d]Global *P* for association is from a Wald test with degrees of freedom equal to the number of haplotypes.

[e]*VDR*, block 2 includes rs2189480, rs2239186, and rs6580642.

[f]*VDR*, block 3 includes rs2238136 and rs2238135.

[g]*VDR*, block 4 includes rs2853564 and rs4760648.

[h]*VDR*, block 5 includes rs4328262 and rs11168293.

[i]*CASR*, block 1 includes rs34028592 and rs6762782.

[j]*CASR*, block 2 includes rs1814740, rs35274320, rs1354162, and rs7637874.

[k]*CASR*, block 3 includes rs34345120 and rs1463890.

[l]*CASR*, block 4 includes rs10222633, rs10934578, rs3804592, rs17250717, rs1801725, rs1042636, and rs1802757.

bind to secondary bile acids to neutralise mucosal toxicity and reduce cell proliferation. The longer transit time in colon than rectum might simply allow more time for calcium to exert its action. Therefore, the *CASR* variants may be more influential in the progression of colon cancer where calcium may have a stronger protective effect.

A novel aspect of our study is the inclusion of gene-diet interaction. A previous study (Kim *et al*, 2013) on *CASR* polymorphisms (rs10934578, rs12485716, rs2270916, and rs4678174) and CRC risk has linked all four of these SNPs to an elevated risk of CRC only in the lower calcium category, which is consistent with our findings of a stronger effect of *CASR* variants in the low calcium group. Presumably the influence of subtle differences between genotypes was overwhelmed by the protective effect of high-dose calcium (Wong *et al*, 2003). Although the probability that these interactions are false-positive findings is high, this study still contributes to the overall evidence that calcium may act as a potential effect modifier in relation to the relationship between *CASR* genotypes and CRC survival. If the gene-nutrient interaction will be replicated in further studies, then cancer patients, especially those with detrimental genotypes, may benefit from the use of calcium supplements to improve their survival. Such supplementations should be based on well-designed and carefully conducted RCTs.

The strengths of the current study include its relatively large size, long follow-up period (up to 10 years), and detailed information on potential confounders and effect modifiers. Limitations to this study include a lack of the cause of death data for all deceased patients; however, we obtained the cause of death defined by ICD codes for 104 of 183 deceased patients; thereof the majority (90.4%) was due to CRC. In addition, the lack of serum levels of 25-hydroxyvitamin D (25(OH)D) and calcium impeded us to test possible 25(OH)D/calcium-diet/gene interactions and to evaluate the extent to which the gene-CRC outcome association is mediated through serum levels of 25(OH)D/calcium. Besides, we cannot rule out effects of other genes with polymorphisms in the vitamin D and calcium metabolism pathway that may also influence the overall CRC initiation and progression. It is also notable that most SNPs examined in this work are tagging SNPs, which are selected merely as indicators for specific regions of interest; thus, there is a low probability that they are the causal SNPs (Egan *et al*, 2010). Therefore, first the replication of this work in other populations and then in detail examination of the other polymorphisms in the *VDR* and *CASR* genes is necessary to identify truly causal variants.

CONCLUSIONS

Our results suggest that polymorphic variations of *VDR* and *CASR* are associated with survival in patients with CRC. These findings indicate that certain variants of the *VDR* and *CASR* genes may be utilised as novel biomarkers for predicting prognosis in CRC patients.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

PPW, GZ, PSP, and JRM conceived and designed this study. YZ analysed the data and drafted the manuscript. PPW, PTC, JRW, IS, YL, XZ, NY, and BB revised the paper. ED, PSP, GZ, SS, and JRM contributed to sample and data collection. All authors read and approved the final manuscript.

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