MolecularMedicine

Vitamin D Receptor Polymorphisms Are Associated with Reduced Esophageal Vitamin D Receptor Expression and Reduced Esophageal Adenocarcinoma Risk

Vincent T Janmaat,[1] Anouk van de Winkel,[1*] Maikel P Peppelenbosch,[1] Manon C W Spaander,[1] André G Uitterlinden,[2] Farzin Pourfarzad,[3] Hugo W Tilanus,[4] Agnieszka M Rygiel,[5] Leon M G Moons,[1] Pascal P Arp,[2] Kausilia K Krishnadath,[5] Ernst J Kuipers,[1,2] and Luc J W van der Laan[4]*

Departments of [1]Gastroenterology and Hepatology, [2]Internal Medicine, Epidemiology and Clinical Chemistry, [3]Cell Biology, [4]Surgery, Erasmus MC-University Medical Center, Rotterdam, the Netherlands; and [5]Center for Experimental Molecular Medicine, Academic Medical Center, Amsterdam, the Netherlands

Epidemiological studies indicate that vitamin D exerts a protective effect on the development of various solid cancers. However, concerns have been raised regarding the potential deleterious role of high vitamin D levels in the development of esophageal adenocarcinoma (EAC). This study investigated genetic variation in the vitamin D receptor (*VDR*) in relation to its expression and risk of Barrett esophagus (BE) and EAC. *VDR* gene regulation was investigated by immunohistochemistry, reverse transcriptase–polymerase chain reaction (RT-PCR) and gel shift assays. Fifteen haplotype tagging single-nucleotide polymorphisms (SNPs) of the *VDR* gene were analyzed in 858 patients with reflux esophagitis (RE), BE or EAC and 202 healthy controls. *VDR* mRNA expression was higher in BE compared with squamous epithelium. VDR protein was located in the nucleus in BE. An rs1989969T/rs2238135G haplotype was identified in the 5′ regulatory region of the *VDR* gene. It was associated with an approximately two-fold reduced risk of RE, BE and EAC. Analysis of a replication cohort was done for BE that confirmed this. The rs1989969T allele causes a GATA-1 transcription factor binding site to appear. The signaling of GATA-1, which is regarded as a negative transcriptional regulator, could explain the findings for rs1989969. The rs2238135G allele was associated with a significantly reduced *VDR* expression in BE; for the rs1989969T allele, a trend in reduced *VDR* expression was observed. We identified a *VDR* haplotype associated with reduced esophageal *VDR* expression and a reduced incidence of RE, BE and EAC. This *VDR* haplotype could be useful in identifying individuals who benefit most from vitamin D chemoprevention.

Online address: http://www.molmed.org
doi: 10.2119/molmed.2012.00336

INTRODUCTION

The incidence of esophageal adenocarcinoma (EAC) in Western Europe and North America has shown an upward trend for many decades. Despite advances made with respect to its treatment, EAC remains to have a poor prognosis. EAC often arises within Barrett esophagus (BE) (1,2), a metaplastic condition of the distal esophagus, in which through longstanding gastroesophageal reflux esophagitis (RE), the normal squamous epithelium is replaced by columnar epithelium. In Western countries, the prevalence of BE has increased dramatically since the 1970s (3), which explains the increasing incidence of EAC. BE is likely caused by a combination of genetic and environmental factors (4), but few studies have examined the exact association with dietary components.

Vitamins and antioxidants are believed to be key dietary components, some of which pose anticarcinogenic action (5). Vitamin D is a micronutrient and is the precursor to the steroid hormone calcitriol. It is obtained from dietary sources, but can also be produced endogenously under the influence of solar ultraviolet-B radiation (6). Its main action lies in normal development and mineralization of a healthy skeleton. Despite this, the vitamin D receptor (*VDR*) is expressed in a variety of tissues that are not involved in

**VTJ and AvdW contributed equally to this work.*

***Address correspondence to** Luc J W van der Laan, Department of Surgery and Laboratory of Experimental Transplantation and Intestinal Surgery (LETIS), Erasmus MC-University Medical Center, P.O. Box 2040, Room L-459, 3000 CA, Rotterdam, the Netherlands. Phone: +31-(0)10-703-7557; Fax: +31-(0)10-703-2793; E-mail: L.vanderlaan@erasmusmc.nl.*

Submitted November 27, 2012; Accepted for publication April 21, 2015; Published Online (www.molmed.org) April 21, 2015.

Table 1. Patient characteristics.

	HC	RE	BE	Repl BE	EAC
N	202	307	260	150	141
Mean age, years (range)	57 (18–90)	55 (19–88)	61 (33–95)	59 (30–87)	63 (38–87)
Male, %	57	56	69	89	82

HC, healthy controls; Repl BE, BE replication cohort.

calcium or phosphate metabolism. Considerable evidence exists in scientific literature, citing an inverse epidemiological relationship between vitamin D and the incidence of several cancers (5,7).

The protective properties of vitamin D have been most extensively studied for colorectal cancer. Several metaanalyses reported consistent protective effects of high vitamin D status for colorectal neoplasia (8–11). This could be explained by the finding that the VDR controls nuclear β-catenin levels in colon cancer cells and can thus attenuate the effect of mutations that activate the Wnt/β-catenin pathway (12). Additionally, Hummel *et al.* (13) showed that increasing dietary vitamin D intake prevents chemically induced preneoplastic lesions in mice colon. However, a large-scale randomized controlled trial, the Women's Health Initiative, found that calcium and vitamin D supplementation had no effect on the incidence of colorectal cancer compared with the placebo arm after a 7-year follow-up; this follow-up period is possibly insufficient for detecting the effect (14). A recent overview of the influence of vitamin D on cause-specific death shows an inverse association with cardiovascular, oncological and other causes of death (15). However, there have been notable exceptions to this protective character, as reported for serum 25-hydroxy vitamin D levels, exposure to vitamin D or increased exposure to sunlight and upper gastrointestinal cancer (16–19). In addition, a significant direct association was observed between the highest tertile versus the lowest tertile of vitamin D intake and EAC, even after adjustment for confounders (20). Similar observations have been made for pancreatic cancer risks (21). The research of Trowbridge *et al.* (22), although preliminary, suggests that EACs, which do not respond to neoadjuvant therapy, have greater expression of VDR than tumors that do respond completely. A recent review from this group states that no association can be identified with the current epidemiologic data, but that sun exposure is consistently reported to be inversely associated with EAC (23).

Factors that govern the outcome of vitamin D–mediated chemoprevention may lie in differential expression and/or activity of enzymes responsible for local activation and degradation of vitamin D, or variations in the expression or signaling of the VDR itself. The *VDR* gene encompasses about 64 kb, consisting of a 5′ region containing noncoding exon 1a to 1f, the coding exons 2–9 and a 3′ UTR (Supplementary Figure S1A) (24–27). It is known that VDR protein is expressed in BE and normal stomach mucosa (28). Additionally, it is present in the normal colon, where it is more abundantly expressed in premalignant and cancerous lesions (29).

At present, little is known about the role of the *VDR* gene and its single-nucleotide polymorphisms (SNPs) in BE and EAC. Therefore, the aims of this study were to, first, analyze *VDR* RNA and protein levels in BE. Second, this study aims to analyze 15 haplotype tagging SNPs (htSNPs) with respect to the risk of RE, BE and EAC development. HtSNPs are representative SNPs in a region of the genome with high linkage disequilibrium; each htSNP represents a group of SNPs (that is, a haplotype). The 15 htSNPs chosen are sufficient to cover the common genetic diversity across the *VDR* gene (24). Third, our study aims to analyze htSNPs that have a different frequency in patients versus controls for *VDR* expression level. Here, we report two SNPs: 1453C>T (rs1989969) and 1633G>C (rs2238135). The presence of the rs1989969 T/rs2238135 G haplotype was associated with a reduced risk for BE; this finding was confirmed with a replication cohort. The same was found for RE and EAC in a single cohort for each condition. The rs1989969 T allele was found to create a GATA-1 binding site and is associated with a two-fold ($p = 0.11$) decrease in *VDR* expression in BE. The G allele of rs2238135 is associated with a 2.5-fold ($p = 0.01$) decrease in *VDR* expression in BE as well. Potential implications for vitamin D–based chemoprevention will be discussed.

MATERIALS AND METHODS

Human Patients and Healthy Controls

The association between *VDR* alleles and esophageal disease was analyzed in a group of 708 patients with RE, BE or EAC who visited the endoscopy unit of the Erasmus Medical Center Rotterdam or the IJsselland Hospital in Capelle aan den IJssel between November 2002 and February 2005 (30). Additionally, subjects visiting a general practitioner during this period for symptoms unrelated to and without any previous symptoms of gastroesophageal reflux disease (GERD) were asked to participate and served as healthy controls (n = 202). Patient characteristics are given in Table 1. We attempted to contact all patients and subjects included in the study to collect data regarding their genetic background. In roughly half of the cases, information on ethnic background was obtained. Less than 1% of successfully contacted study participants were of non-Caucasian descent. Subjects included in the RE population had endoscopically confirmed RE (n = 307), which was graded according to the Los Angeles (LA) classification (31). Patients were diagnosed endoscopically with BE (n = 260) if they had a columnar-lined segment in the esophagus of >2 cm in length with histological signs of specialized intestinal metaplasia. The length of the columnar-lined segment was determined endoscopically by measuring the distance between the squamocolumnar

junction (the location at which the light-pink mucosa of the squamous-lined esophagus joined the red mucosa of the columnar-lined epithelium) and the gastroesophageal junction. Endoscopic diagnosis of EAC (n = 141) was confirmed by pathologic assessment of the histology of biopsies. An independent BE replication cohort (n = 150) was collected from the Academic Medical Center, located in Amsterdam. BE was identified endoscopically by an expert gastroscopist. Random biopsy specimens were taken from each quadrant of the BE at every 2 cm according to standard protocol. The biopsy specimens used in this study were taken from the middle of the BE segment. The random biopsies therefore surround the study biopsies. All random biopsy specimens were analyzed by an expert gastrointestinal pathologist, and the study biopsy specimens were used only when they contained Barrett epithelium. Squamous epithelial biopsies were taken 5 cm above the squamous Barrett junction. This study was approved by the institutional ethics review committees, and all patients gave informed consent before participating in the study.

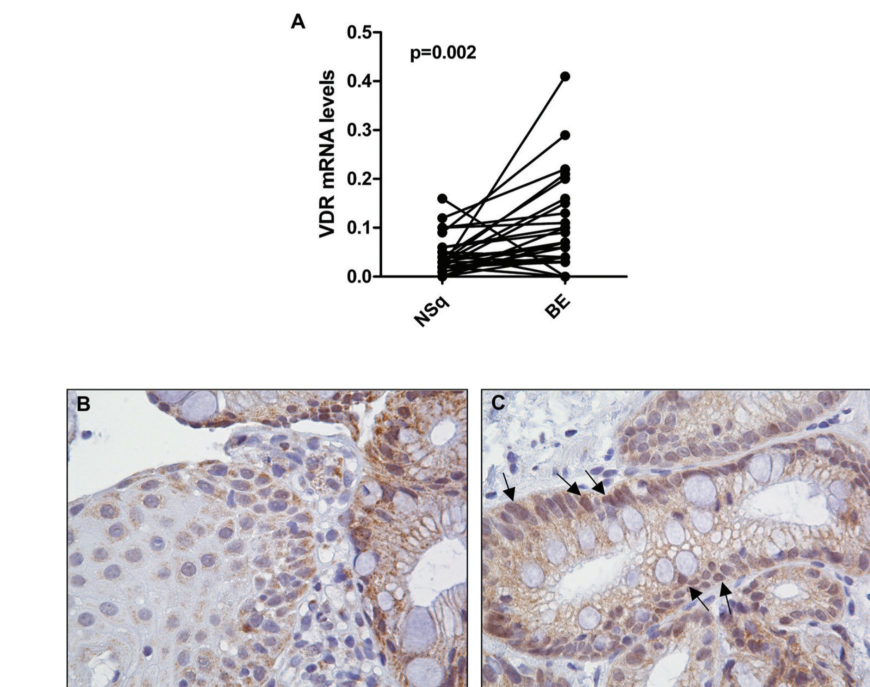

Figure 1. Esophageal expression of *VDR* in BE patients. (A) Relative mRNA levels of *VDR* in paired squamous and columnar epithelium samples of BE patients. In 20 of 25 patients, *VDR* mRNA was highest in columnar epithelium samples. Immunohistochemical staining of *VDR* in squamous (B) and paired columnar epithelium (C) clearly shows a number of *VDR*-positive nuclei in BE and its mere presence in the cytoplasm of epithelial cells. Arrows indicate some positively stained nuclei.

Genotyping

Genomic DNA was extracted from 5 mL whole blood by a wizard genomic DNA purification kit (Promega, Madison, WI, USA). Fifteen htSNPs across the *VDR* gene were genotyped with the use of the high-throughput TaqMan allelic discrimination assays. A random 5% of samples were independently repeated to confirm genotyping results.

Real-Time PCR mRNA Quantification from Human Esophagus Samples

Total RNA was extracted from tissue biopsies by using TriReagent (Sigma-Aldrich, St. Louis, MO, USA) and purified by using an RNeasy micro column kit (Qiagen, Hilden, CA, USA). One-fortieth of a 1-μg cDNA synthesis reaction (iScript cDNA Synthesis Kit; Bio-Rad, Hercules, CA, USA) was used in a 25-μL quantitative reverse transcriptase–polymerase chain reaction (RT-PCR) using SYBR GreenER (Invitrogen [Thermo Fisher Scientific Inc., Waltham, MA, USA]). The following primers were used for *VDR* gene amplification: 5′-CCGCATCACCAAGGACAAC-3′ and 5′- GCTCCCTCCACCATCATTCAC-3′. Duplicate samples were run in independent PCR runs, and the average level of *VDR* gene expression was normalized to RNA polymerase II and GAPDH gene expression by using the Pfaffl method (32).

Immunohistochemical Staining

From the formalin-fixed, paraffin-embedded tissue, 5-μm tissue sections were sliced and mounted on adhesive slides (Starfrost, Berlin, Germany). After deparaffinization in xylene and dehydration in alcohol, endogenous peroxidase was inactivated by incubation with 0.5% hydrogen peroxidase in methanol for 15 min. Antigen retrieval was performed by boiling the sections for 10 min in 10 mmol/L citric acid monohydrate buffer (pH 6.0). Sections were blocked with 5% bovine serum albumin. Anti-VDR monoclonal antibody (1:200; clone 9A7, Affinity Bioreagents, Golden, CO, USA) was incubated for 1 h at room temperature, followed by polyclonal biotin–labeled goat anti-rat (1:500; Dako, Denmark). After 45 min of incubation with streptavidin–horseradish peroxidase (HRP) (1:300; Dako, Glostrup, Denmark), VDR was visualized by using 3-amino-9-ethylcarbazole as a substrate and hematoxylin counterstaining. Sections were evaluated by using light microscopy (Axioskop 20; Zeiss, Oberkochen, Germany), and pictures were taken and analyzed by using Nikon software (NisElements 2008; Tokyo, Japan).

Electrophoretic Mobility Shift Assay

Oligonucleotides used in electrophoretic mobility shift assay (EMSA) and supershift assays were 5′-CCAGG

Table 2. Genotype distribution of *VDR* SNPs rs2238135 and rs1989969 in healthy controls and patients.

		Genotype, N (frequency)				
SNP	Genotype	HC (N = 202)	RE (N = 307)[a]	BE (N = 260)[a]	Repl BE (N = 150)[a]	EAC (N = 141)
rs2238135						
	G/G	127 (0.648)	159 (0.530)	157 (0.625)	72 (0.507)	76 (0.551)
	G/C	58 (0.296)	121 (0.403)	81 (0.323)	65 (0.458)	54 (0.391)
	C/C	11 (0.056)	20 (0.067)	13 (0.052)	5 (0.035)	8 (0.058)
p		1	*0.03*	0.83	*0.01*	0.18
rs1989969						
	C/C	53 (0.273)	107 (0.357)	85 (0.343)	49 (0.340)	49 (0.360)
	C/T	94 (0.485)	147 (0.490)	128 (0.516)	76 (0.528)	65 (0.478)
	T/T	47 (0.242)	46 (0.153)	35 (0.141)	19 (0.132)	22 (0.162)
p		1	*0.02*	*0.02*	*0.03*	0.1

HC, healthy controls; Repl BE, BE replication cohort.
[a]Values in italic are significant at 95% CI.

GTGGTTGTCTA**C**CTGGATGTCACCT CTGACCTCTG-3′ (wild-type, representing the rs1989969 C allele) and 5′-CCAGG GTGGTTGTCTA**T**CTGGATGTCACCT CTGACCTCTG-3′ (mutant represents the rs1989969 T allele; underlined section represents the GATA binding site, and the bold nucleotide represents the position of the rs1989969 SNP). Probes were 5′-end labeled with [γ-^{32}P]ATP. Nuclear extracts were prepared from murine erythroleukemia (MEL) cells according to the methods used by Wall *et al.* (33). For EMSA experiments, 2.5 μg nuclear extract prepared from MEL cells was incubated for 30 min at 37°C with 1-ng ^{32}P-labeled or 25-ng unlabeled VDR oligonucleotide probe in a binding buffer consisting of 50 mmol/L Tris, pH 8.0, 250 mmol/L NaCl, 5 mmol/L dithiothreitol, 5 mmol/L ethylenediaminetetraacetic acid and 10% Ficoll in a total volume of 10 μL. In competition assays, 25-fold molar excess of unlabeled competitor was included in the binding reaction. For supershift assays, 2 μg GATA-1 (N6)X mouse monoclonal antibody (sc-265 X; Santa Cruz Biotechnology, Santa Cruz, CA, USA) was added to reaction mixtures 30 min before addition of the nuclear extract. The protein-DNA complexes were separated from free probes by electrophoresis through a 4% nondenaturing polyacrylamide gel and visualized on a orthochromatic film (Super HR-U30; Fuji Film, Tokyo, Japan) and developed by using a Fuji medical film processor (Model FPM 100A).

Statistical Analyses

Genotype distribution was tested for Hardy-Weinberg equilibrium. The study was powered (80%) to allow detection of a 10% difference in genotype distribution of the *VDR* SNPs or haplotypes between the groups by performing Pearson χ^2 analysis. Odds ratio (OR) and 95% confidence interval (CI) were calculated by risk estimate analysis. Logistic regression analysis was applied to establish allele dose effects. Statistical analyses were conducted by using SPSS v11.0 (SPSS [IBM, Armonk, NY, USA]), and two-tailed significance was $p < 0.05$. We did not adjust for multiple testing. However, a replication cohort was included for BE. One-tailed *t* tests were performed to test the hypothesis that GG versus CC and TT versus CC genotypes were associated with lower VDR levels.

In Silico Sequence Analysis

The human genomic DNA sequence of the intronic region near the noncoding exon 1c ~1.5 kb upstream of the translation start site was downloaded from the National Center for Biotechnology Information database (http://www.ncbi.nlm.nih.gov). The reference sequence used was NC_018923.2. Transcription factor binding sites were predicted by PROMO (version 3.0.2), which is a virtual laboratory for the identification of putative transcription factor binding sites. PROMO uses the 8.3 version of TRANSFAC (34,35). The dissimilarity threshold was set at 5%.

All supplementary materials are available online at www.molmed.org.

RESULTS

BE Epithelium Has a High Expression of VDR Compared with Squamous Epithelium

We investigated *VDR* expression in normal squamous epithelium compared with BE tissue in individual BE patients. In the majority of patients (20 of 25, 80%), presence of BE correlated with a two-fold increase in *VDR* mRNA expression (Figure 1A; $p = 0.002$). This result was associated with higher levels of VDR protein in the BE segment, especially in the epithelial compartment, as evident from immunohistochemical staining of squamous and BE biopsies from the same patient (Figure 1B). As shown in Figure 1C, in most BE tissue, the VDR protein had a nuclear localization, suggesting activation of the receptor. Thus, *VDR* mRNA expression is upregulated and VDR protein is activated in BE compared with squamous epithelium.

The rs1989969 T/rs2238135 G Haplotype Is Associated with Reduced Risk for Neoplasm-Associated Esophageal Disease

Genomic DNA was obtained from a group of 708 patients with RE, BE or EAC and compared with a group of 202 healthy controls without any symptoms of esophageal disease. Patient characteristics are given in Table 1. As shown in Table 2, rs1989969 and rs2238135, the two htSNPs in the exon 1c region, were found to be differently distributed in

Table 3. Haplotype-risk analysis of RE, BE and EAC compared with controls.[a]

		OR (95% CI)[b]			
Haplotype	Total alleles (N)	RE versus HC	BE versus HC	BE Repl versus HC	EAC versus HC
0 GT copies	345	1	1	1	1
1 GT copies	507	0.76 (0.50–1.15)	0.84 (0.54–1.30)	0.88 (0.54–1.43)	0.74 (0.45–1.22)
2 GT copies	169	0.48 (0.28–0.81)	0.46 (0.26–0.80)	0.44 (0.23–0.85)	0.50 (0.27–0.96)
0 GC copies	442	1	1	1	1
1 GC copies	444	1.37 (0.93–2.02)	*1.53 (1.02–2.30)*	*1.64 (1.03–2.61)*	1.08 (0.67–1.74)
2 GC copies	125	1.05 (0.57–1.93)	*1.84 (1.02–3.32)*	0.95 (0.44–2.05)	1.40 (0.71–2.77)
0 CC copies	590	1	1	1	1
1 CC copies	376	*1.65 (1.12–2.44)*	1.12 (0.74–1.68)	*1.98 (1.25–3.12)*	1.56 (0.98–2.48)
2 CC copies	57	1.24 (0.68–3.16)	0.96 (0.41–2.21)	0.80 (0.27–2.40)	1.22 (0.47–3.16)

HC, healthy controls; BE Repl, BE replication cohort; EAC, esophageal adenocarcinoma.
[a]The CT haplotype contained too few alleles for a meaningful comparison.
[b]Values in italic are significant at 95% CI.

healthy controls versus patients with RE, BE and EAC in this study. Thirteen other VDR htSNPs were found to not be significantly associated with the presence of esophageal disease (data not shown). As shown in Table 3, allele dose analysis revealed that individuals carrying the rs1989969 T/rs2238135 G haplotype were two-fold less susceptible to RE (OR 0.48, 95% CI 0.28–0.81), BE (OR 0.46, 95% CI 0.26–0.80) and EAC (OR 0.50, 95% CI 0.27–0.96). A BE replication cohort closely mimicked the observations for the rs1989969 T/rs2238135 G haplotype (OR 0.44, 95% CI 0.23–0.85).

Identification of an rs1989969-Dependent GATA-1 Binding Site in the *VDR* Intronic Region Near the Noncoding Exon 1c

With the finding established that carriers of the T allele of rs1989969 and the G allele of rs2238135 are two-fold less susceptible to neoplasm-associated disease, we further analyzed these SNPs. The availability of a multispecies genomic sequence allowed us to examine the sequence conservation across the transcriptional unit and indicated various highly pan-vertebrate conserved regions, especially around the location of rs1989969 and rs2238135, near the noncoding exon 1c ~1.5 kb upstream of the translation start site (Figure 2). The strong evolutionary conservation in this region might well be consistent with a role in transcriptional regulation. Interestingly, rs1989969 was found to convert the transcriptionally inert majority allele into a canonical GATA-1 binding site (that is, T/A GATA A/G) (36) (Figure 3, Supplementary Figure S1). rs1989969 could thus be expected to alter *VDR* expression in cell types expressing GATA-1 transcription factor. *GATA1* expression has been shown in the human stomach and duodenum but not in the small intestine, appendix and colon (37). *GATA1/2/3* ortholog expression has been reported in the esophagus of the polychaete annelid, Capitella (38). Additionally, *GATA4* and *GATA6* mRNA was found to be expressed highly in the proximal gastrointestinal tract (39,40). The various GATA transcription factors have closely related and sometimes overlapping binding sites; therefore, GATA-4 and GATA-6 could have a similar function as GATA-1 (36).

In EMSA, the binding of the GATA-1 transcription factor was tested by using oligonucleotides bearing the C and T allele of rs1989969, together with nuclear extracts and antibodies against GATA-1 (Figure 3A). Supershift experiments indicated that GATA-1 transcription factor binds to the oligonucleotide with the rs1989969 T allele, whereas the oligonucleotide bearing the common rs1989969 C allele does not show detectable binding (Figure 3B). Thus, the rs1989969 SNP results in a differential binding of GATA-1 to the *VDR* gene.

The rs1989969 and rs2238135 SNPs Influence Esophageal *VDR* Expression

To study the functional consequences of the rs1989969 and rs2238135 SNPs on *VDR* expression, esophageal biopsies were taken from BE of patients, and *VDR* expression levels were determined by using quantitative RT-PCR. The expression of *VDR* in the esophagus was on average 2.5-fold lower in BE patients carrying two copies of the rs2238135 G allele versus subjects carrying two copies of the rs2238135 C allele (p = 0.01). *VDR* expression was two-fold lower in BE patients carrying two copies of the rs1989969 T allele versus subjects carrying two copies of the rs1989969 C allele. However, this association did not reach statistical significance (p = 0.11; Figure 4). Thus, the rs2238135 C allele results in 2.5-fold higher esophageal *VDR* expression. The C allele of rs1989969, in which the GATA-1 binding site is absent, shows a trend in higher esophageal *VDR* expression.

DISCUSSION

The incidence of EAC rises to date despite surveillance strategies. In many cases, the development of EAC is related to BE. This premalignant stage provides the opportunity to prevent the

```
HOM: CTAGCTGTSGGACCCTGGGTAAGTCACTCAGAGT-GCTCT----GAAATTTGGCTTTGCTAC-----AAGTA-----GGACTGCTCCCTGC--CTCA--CAGAACTGTTGTGAG 100%
PAN: CTAGCTGTGGGACCCTGGGCAAGTCACTCAGAGT-GCTCT----GAAATTTGGCTTTGCTAC-----AAGTA-----GGACTGCTCCCTGC--CTCA--CAGAACTGTTGTGAG  99%
MAC: CTGGCTGTGGGACCCTGGGCAAGTCACTCAGAGT-GCTCT----GAACTTTGGCTTTGCTACCTGTAAAGTA-----GGACTGCTCCCTGC--GTCA--CAGAACTGTTGTGAG  93%
EQU: ------TGGGACCCTGCGCAAGTCACTCAAAGT-GCTCT----GGACCTCGGTTTTGCCCTCTGTGAAGTG-----GGACTGCTCCTTGC--CTCA--CAGGACTGTTGTGAG  80%
CAN: CGGGCCATGGCACCCTGGGCAAGTCACTCAAAGT-AGTCT----GGACCTCGGCTTTGCCGTCTGGAAAGTG-----GGACTGCTCCCCAC--CTCA--CAGGATTGCCATGAG  74%
BOS: CTGGCCGTGGGACCCTGGGCAAGTC--TCAAAGT-GCCCTACCTGGAGTTTGGTTTTTCCAT-----CTGTAAAATGGGACTGCTC--TGC--ATCTTGCGGGACGGTTAGGAG  73%
MUS: ACCGGTGAAAGACGCTCTGAACTTTGGTTTGGGTATCTAT----AAAATGAGAGTT--CTCC-----CCTTG-----CCCCCCCTCCCCCCAGCTCA--AAG-ACTACTGTGAG  66%

HOM: GGCTAAAT-GAAATAATGTATGCAGAGCTTAGCAGGCCTGGCATGTAGTAAAT--ACTCCGGAAACAttttttt-----tAAGTTCCAGGGTGGTTGTCTAYCTGGATGTCACC 100%
PAN: GGCTAAAT-GAAATAATGTATGCAGAGCTTAGCAGGCCTGGCATGTAGTAAAT--ACTCCGGAAACATTTTTTT-----TAAGTTCCAGGGTGGTTGTCTACCTGGATGTCACC  99%
MAC: GGCTAAAT-GAAATAATATATGCAGAGCTTAGCAGGCCTGGCATGTAGTAAAT--ACTCCAGAAACATTTTTT------TAAGTTCCAGGGTGGTGGTCTACCTGGATGTCACC  93%
EQU: -----------ATGCTGTATGTGGAGCTCAGCAGGCT--GCACACAGTCAGT--GCTCCATACAC-CATTTTT-----GAAGCTCACAGGTGGTGGTCTACCTGGATCTCAAG  80%
CAN: GCTTCTGTCGGCGGTACGTAT--AGCGCCTGGC-GGCCTGGCACATGGTAGAT--ACTCCGGACACGCTTTTCT-----TAAGCTCAAGGGTGGCAGTCTACCTGGATCTCCAG  74%
BOS: GT-TACAT-GAGATCCCAGATGGAAGCTTAGCAGTC-TGGCGCATGGTAGAT--ACGC---ACACACTTTTCTC--TGTAAGTTCAGGGATGGCGAGCTACTTGGATCTCAGA  73%
MUS: GATTAAACTAAAT-AAAGTATGTATCGCTTGGCAGTTCTAGAATGTGTTAGATAGACTCCAGACTTTTTTTTTTCCTTTTAAGTTCAAGGGTGATGGTCTACCTGAATG--AAC  66%
```

Abbreviated name for all mammalian species used:

Hom:	*Homo sapiens*	(human)
Pan:	*Pan troglodytes*	(chimpanzee)
Mac:	*Macaca mulatta*	(rhesus macaque)
Equ:	*Equus caballus*	(domestic horse)
Can:	*Canis familiaris*	(domestic dog)
Bos:	*Bos taurus*	(domestic cow)
Mus:	*Mus musculus*	(house mouse)

Figure 2. Comparative sequence analysis of multi-species alignment of the *VDR* exon 1c noncoding regulatory region. This region lies near the noncoding exon 1c ~1.5 kb upstream of the translation start site. The regions containing the rs2238135 and rs1989969 SNPs (in boxes) are highly conserved in the human genome and six other mammalian species. The SNPs rs2238135 and rs1989969 are indicated by an "S" and a "Y." The "S" represents a G or a C, and the "Y" represents a C or a T according to the International Union of Pure and Applied Chemistry (IUPAC) nucleotide base code.

development of BE-related adenocarcinoma by stratifying BE patients at risk for neoplastic progression. Additionally, it provides the opportunity to identify BE patients who are likely to respond negatively to vitamin D supplementation. Vitamin D supplementation is likely to convey a level of chemopreventive properties against oncogenic transformation in other tissues. Identifying genetic, tissue-specific markers that distinguish individuals on their responsiveness to vitamin D would represent a rationale for detecting groups of patients that could benefit most from vitamin D–based chemoprevention. Obtaining DNA from blood would provide an easy and cheap way to identify patients that have the highest benefit. Therefore, the aim of this study was to investigate the role of *VDR* in BE; study the consequences of SNPs in the *VDR* gene on the development of RE, BE and EAC; and elucidate the mechanisms by which these SNPs exert their effect.

We found that BE epithelium has a two-fold higher expression of *VDR* mRNA compared with squamous epithelium. This result was concomitant with a higher expression of VDR protein detected by immunohistochemistry. In addition, VDR protein was found to predominantly have a nuclear localization, suggesting activation of the receptor. Subsequently, 15 htSNPs of the *VDR* gene were analyzed with respect to the risk of RE, BE and EAC development. The T allele of rs1989969 was associated with a reduced risk for BE. The same was found for RE in a single cohort. A similar trend was observed for EAC, also in a single cohort. The G allele of rs2238135 was associated with a reduced risk for RE in a single cohort. A similar trend was observed for BE and EAC. The presence of the rs1989969 T/rs2238135 G haplotype was associated with a reduced risk for BE; this finding was confirmed with a replication cohort. The same was found for RE and EAC in a single cohort. The two SNPs were further analyzed to elucidate the mechanisms by which they exert their effect. The rs1989969 minor T allele resulted in a GATA-1 binding site in the *VDR* intronic region near the noncoding exon 1c. This result was identified *in silico* and was verified by EMSA. Here, oligos containing the T allele of rs1989969 displayed strong GATA-1 transcription factor binding, whereas oligos derived from the major C allele were not capable of doing so. Furthermore, the *VDR* mRNA level in BE tissue of rs2238135 G allele carriers was found to be lower than the mRNA

level in rs2238135 C allele carriers. A trend in the same direction was found for the rs1989969 T versus C allele. This finding resulted in possible vitamin D sensitivity in this organ. This result provides a mechanism that could explain why the rs1989969 T/rs2238135 G haplotype is associated with a reduced risk for neoplasm-associated esophageal disease.

A role of vitamin D in the etiology of BE is expected from the well-known mutual positive interaction between VDR signaling and signaling of bone morphogenetic proteins. Bone morphogenetic protein-4 expressed in RE induces a columnar phenotype in esophageal squamous cells and is thus possibly important for the precancerous process (41). The present study further supports this concept by establishing the upregulation of *VDR* signaling during the metaplastic process. GATA factors are well established, mostly negative transcriptional regulators. Accordingly, the rs1989969 T allele, which results in a GATA-1 binding site, reduces expression of the *VDR*. It was previously shown that IL-4 induces *GATA1* expression, which, subsequently, represses *VDR* expression and enables monocyte-derived dendritic cell differentiation within inflammatory sites (42). This could take place in BE during inflammation, providing additional support for the here-presented findings.

The rs1989969 T/rs2238135 G haplotype was associated with a reduced incidence of RE at a level on par with the reduced incidence in BE and EAC observed in this study. This finding would support the notion that this haplotype exerts its effect on the risk of BE and EAC by reducing the rate of RE instead of reducing the rate of progression from RE to BE and EAC. Both SNPs reported here are haplotype tagging; the association found between the T allele of rs1989969 and a lower incidence of neoplasm-associated esophageal disease and *VDR* expression levels can in part be due to other genetic variation with

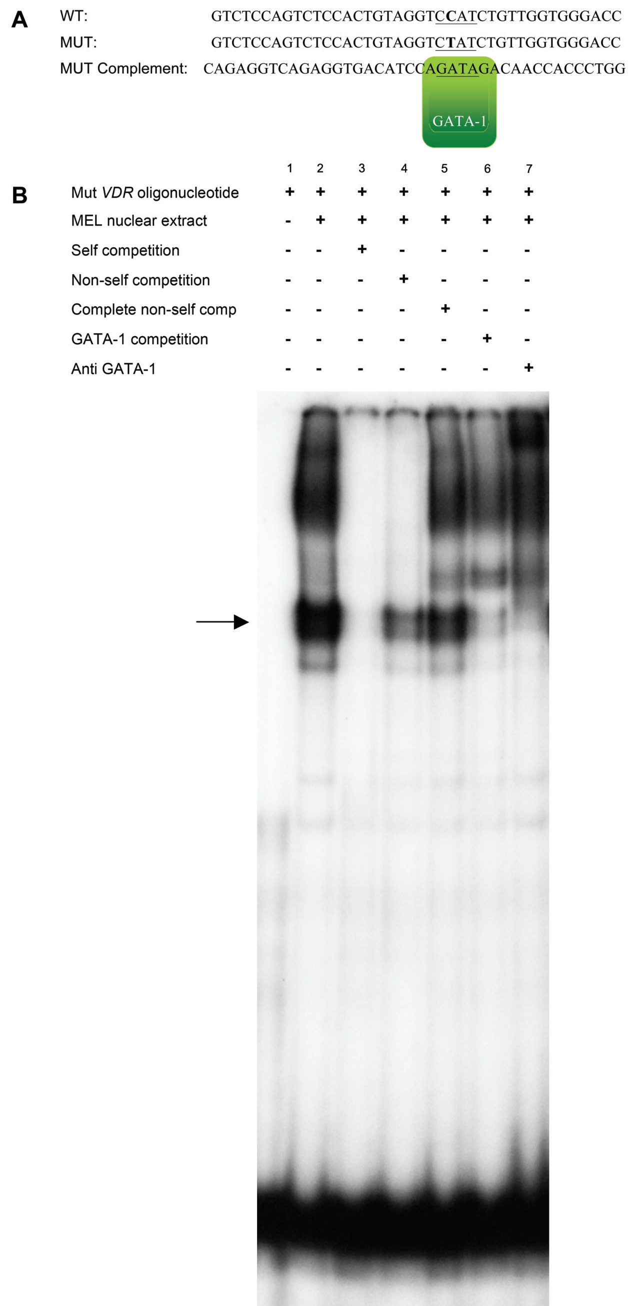

Figure 3. The *VDR* rs1989969 T allele causes a functional GATA-1 binding motif to appear. (A) A rs1989969-dependent GATA-1 binding site is predicted. (B) Gel shift assay using radioactively-labeled oligonucleotides from the rs1989969 region and nuclear extract of MEL cells. The arrow indicates the motility of the oligo–GATA-1 complex. *Lane 1*, Mut oligo without nuclear extract; *lane 2*, Mut oligo with nuclear extract; *lane 3*, with 100× excess of unlabeled Mut oligo (self-competitor); *lane 4*, with 100× excess of unlabeled WT oligo (non–self-competitor); *lane 5*, with complete non–self-competitor; *lane 6*, with 100× excess unlabeled known GATA-1 oligo; *lane 7*, 1 μg anti–GATA-1 monoclonal antibody. The signal found on the Mut oligo (*lane 2*) was almost completely eliminated by a 100-fold excess of unlabeled self-competitor and a known GATA-1 oligonucleotide (*lanes 3* and *6*) but not with WT and complete non–self-oligonucleotides (*lanes 4* and *5*). Labeled WT oligo did not result in GATA-1 binding (data not shown).

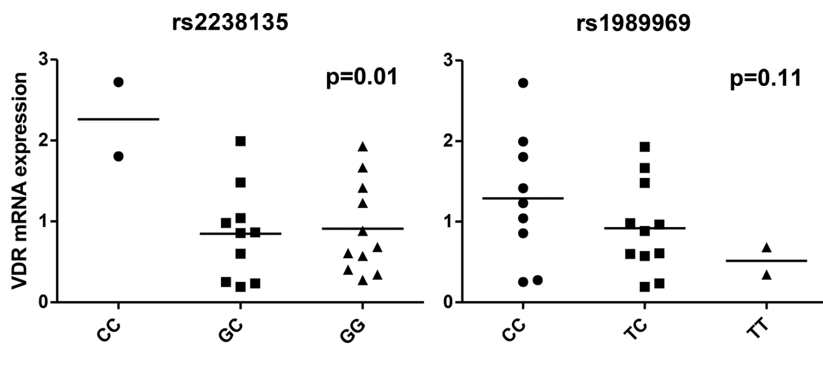

Figure 4. Presence of the rs1989969 T or rs2238135 G allele decreases esophageal *VDR* expression. BE biopsies were analyzed for *VDR* mRNA levels by quantitative RT-PCR. In the first panel, the esophageal expression of *VDR* was on average 2.5-fold lower in BE patients carrying two copies of the rs2238135 G allele versus subjects carrying two copies of the rs2238135 C allele (p = 0.01, one-tailed). In the second panel, *VDR* expression was two-fold lower in BE patients carrying two copies of the T allele of rs1989969 versus subjects carrying two copies of the C allele of rs1989969. However, this association did not reach statistical significance (p = 0.11, one-tailed).

which the rs1989969 T allele is associated. The same holds true for rs2238135. Its action could, in part, even be due to an association with rs1989969 by the linkage disequilibrium that exists between the two SNPs. Further research can improve our understanding of the relative importance of individual genetic variations with which the SNPs we report on are associated.

Chang *et al.* (43) found that rs2238139 (277+2550C>T) and rs2107301 (277+3260C>T) TT homozygotes had a significantly reduced risk of EAC compared with CC homozygotes. Unfortunately, however, we did not analyze the same SNPs and therefore extrapolation is not straightforward. Chang *et al.* also analyzed SNP rs2238135 for an association with EAC and reported negative results. Our study also reported negative findings for this particular association, but we did observe an association between the G allele of rs2238135 and both RE and reduced *VDR* expression in this study. An association was also observed in the BE replication cohort but not in the original cohort. The lack of association between rs2238135 and EAC might therefore be a consequence of our study being underpowered for this particular question. Unfortunately, the same reason has probably also prohibited us from being able to draw strong conclusions with regard to the relation between the rs1989969 T allele and reduced *VDR* mRNA expression in BE. Whereas genetic association studies require replication cohorts, this study did not include replication cohorts for both RE and EAC. Because a BE replication cohort was included and RE, BE and EAC are related conditions, extrapolation of our conclusions with respect to BE to RE and EAC is plausible. However, confirmation of our findings by others, especially with respect to RE and EAC, is necessary. Whereas the ethnic composition determined from roughly half of the subjects suggests no substantial ethnic admixture of our study group, this notion is uncertain.

Currently, a path between *VDR* SNPs, probably via influence of *VDR* expression, and the cancerous process has been established. This association is implied from theoretical (positive interaction with BMP signaling), epidemiological observations (dietary vitamin D intake and UV-B exposure are associated with increased risk for esophageal cancer) as well as the observations made in the present study. GATA transcription factor expression appears to be lower in the distal tract, which has been shown for *GATA1* (37), *GATA4* and *GATA6* (39). In addition, the vitamin D–based chemopreventive effects in the colon might be transferred through mechanisms other than those involving these *VDR* SNPs. Therefore, we propose that individuals carrying more rs1989969 T and rs2238135 G alleles will benefit more from vitamin D–based chemopreventive strategies because they are less likely to be confronted with the negative consequences of these strategies, being malignant esophageal disease. Confirmation of this notion obviously requires reevaluation of previous trials involving dietary vitamin D supplementation, assessing the number of rs1989969 T and rs2238135 G alleles in the participants. If confirmed, the rs1989969 and rs2238135 SNPs would represent the first polymorphisms that stratify individuals for the use of a particular chemopreventive strategy.

CONCLUSION

This study serves as a proof of principle that SNPs in the *VDR* gene can modify the risk of RE, BE and EAC, probably through modification of *VDR* expression. To investigate how serious the impact of vitamin D truly is, additional research is needed into the mechanisms by which vitamin D affects the risk of developing RE, BE and its sequelae EAC. The rs1989969-dependent GATA-1 binding site in the *VDR* intronic region near the noncoding exon 1c, identified *in silico* and tested in EMSAs, provides a starting point for this. At the same time, evidence from epidemiological studies mimicking the true longlife effects of vitamin D are required to endorse the idea of personalized recommendations for vitamin D supplementation.

DISCLOSURE

The authors declare that they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

REFERENCES

1. Hvid-Jensen F, Pedersen L, Drewes AM, Sorensen HT, Funch-Jensen P. (2011) Incidence of adenocarcinoma among patients with Barrett's esophagus. *N. Engl. J. Med.* 365:1375–83.
2. Sikkema M, de Jonge PJ, Steyerberg EW, Kuipers EJ. (2010) Risk of esophageal adenocarcinoma and mortality in patients with Barrett's esophagus: a systematic review and meta-analysis. *Clin. Gastroenterol. Hepatol.* 8:235–44.
3. Holmes RS, Vaughan TL. (2007) Epidemiology and pathogenesis of esophageal cancer. *Semin. Radiat. Oncol.* 17:2–9.
4. Fitzgerald RC. (2006) Molecular basis of Barrett's oesophagus and oesophageal adenocarcinoma. *Gut.* 55:1810–20.
5. Feldman D, Krishnan AV, Swami S, Giovannucci E, Feldman BJ. (2014) The role of vitamin D in reducing cancer risk and progression. *Nat. Rev. Cancer.* 14:342–57.
6. Bogh MK, Schmedes AV, Philipsen PA, Thieden E, Wulf HC. (2011) Vitamin D production depends on ultraviolet-B dose but not on dose rate: a randomized controlled trial. *Exp. Dermatol.* 20:14–8.
7. Giovannucci E. (2009) Vitamin D and cancer incidence in the Harvard cohorts. *Ann. Epidemiol.* 19:84–8.
8. Gorham ED, *et al.* (2007) Optimal vitamin D status for colorectal cancer prevention: a quantitative meta analysis. *Am. J. Prev. Med.* 32:210–6.
9. Wei MY, Garland CF, Gorham ED, Mohr SB, Giovannucci E. (2008) Vitamin D and prevention of colorectal adenoma: a meta-analysis. *Cancer Epidemiol. Biomarkers Prev.* 17:2958–69.
10. Gandini S, *et al.* (2011) Meta-analysis of observational studies of serum 25-hydroxyvitamin D levels and colorectal, breast and prostate cancer and colorectal adenoma. *Int. J. Cancer.* 128:1414–24.
11. Yin L, *et al.* (2011) Meta-analysis: serum vitamin D and colorectal adenoma risk. *Prev. Med.* 53:10–6.
12. Larriba MJ, *et al.* (2011) Vitamin D receptor deficiency enhances Wnt/beta-catenin signaling and tumor burden in colon cancer. *PLoS One.* 6:e23524.
13. Hummel DM, *et al.* (2013) Prevention of preneoplastic lesions by dietary vitamin D in a mouse model of colorectal carcinogenesis. *J. Steroid. Biochem. Mol. Biol.* 136:284–8.
14. Wactawski-Wende J, *et al.* (2006) Calcium plus vitamin D supplementation and the risk of colorectal cancer. *N. Engl. J. Med.* 354:684–96.
15. Chowdhury R, *et al.* (2014) Vitamin D and risk of cause specific death: systematic review and meta-analysis of observational cohort and randomised intervention studies. *BMJ.* 348:g1903.
16. Abnet CC, *et al.* (2010) Circulating 25-hydroxyvitamin D and risk of esophageal and gastric cancer: Cohort Consortium Vitamin D Pooling Project of Rarer Cancers. *Am. J. Epidemiol.* 172:94–106.
17. Chen W, *et al.* (2007) Prospective study of serum 25(OH)-vitamin D concentration and risk of oesophageal and gastric cancers. *Br. J. Cancer.* 97:123–8.
18. Lipworth L, *et al.* (2009) Dietary vitamin D and cancers of the oral cavity and esophagus. *Ann. Oncol.* 20:1576–81.
19. Toner CD, Davis CD, Milner JA. (2010) The vitamin D and cancer conundrum: aiming at a moving target. *J. Am. Diet. Assoc.* 110:1492–500.
20. Mulholland HG, Murray LJ, Anderson LA, Cantwell MM. (2011) Vitamin D, calcium and dairy intake, and risk of oesophageal adenocarcinoma and its precursor conditions. *Br. J. Nutr.* 106:732–41.
21. Stolzenberg-Solomon RZ, *et al.* (2010) Circulating 25-hydroxyvitamin D and risk of pancreatic cancer: Cohort Consortium Vitamin D Pooling Project of Rarer Cancers. *Am. J. Epidemiol.* 172:81–93.
22. Trowbridge R, Sharma P, Hunter WJ, Agrawal DK. (2012) Vitamin D receptor expression and neoadjuvant therapy in esophageal adenocarcinoma. *Exp. Mol. Pathol.* 93:147–53.
23. Trowbridge R, Mittal SK, Agrawal DK. (2013) Vitamin D and the epidemiology of upper gastrointestinal cancers: a critical analysis of the current evidence. *Cancer Epidemiol. Biomarkers Prev.* 22:1007–14.
24. Fang Y, *et al.* (2005) Promoter and 3′-untranslated-region haplotypes in the vitamin D receptor gene predispose to osteoporotic fracture: the rotterdam study. *Am. J. Hum. Genet.* 77:807–23.
25. Miyamoto K, *et al.* (1997) Structural organization of the human vitamin D receptor chromosomal gene and its promoter. *Mol. Endocrinol.* 11:1165–79.
26. Baker AR, *et al.* (1988) Cloning and expression of full-length cDNA encoding human vitamin D receptor. *Proc. Natl. Acad. Sci. U. S. A.* 85:3294–8.
27. Crofts LA, Hancock MS, Morrison NA, Eisman JA. (1998) Multiple promoters direct the tissue-specific expression of novel N-terminal variant human vitamin D receptor gene transcripts. *Proc. Natl. Acad. Sci. U. S. A.* 95:10529–34.
28. Trowbridge R, Mittal SK, Sharma P, Hunter WJ, Agrawal DK. (2012) Vitamin D receptor expression in the mucosal tissue at the gastroesophageal junction. *Exp. Mol. Pathol.* 93:246–9.
29. Matusiak D, Murillo G, Carroll RE, Mehta RG, Benya RV. (2005) Expression of vitamin D receptor and 25-hydroxyvitamin D3-1α-hydroxylase in normal and malignant human colon. *Cancer Epidemiol. Biomarkers Prev.* 14:2370–6.
30. Moons LM, *et al.* (2007) COX-2 CA-haplotype is a risk factor for the development of esophageal adenocarcinoma. *Am J. Gastroenterol.* 102:2373–9.
31. DiBaise JK. (1999) The LA classification for esophagitis: a call for standardization. *Am J. Gastroenterol.* 94:3403–4.
32. Pfaffl MW. (2001) A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* 29:e45.
33. Wall L, deBoer E, Grosveld F. (1988) The human beta-globin gene 3′ enhancer contains multiple binding sites for an erythroid-specific protein. *Genes Dev.* 2:1089–100.
34. Messeguer X, *et al.* (2002) PROMO: detection of known transcription regulatory elements using species-tailored searches. *Bioinformatics.* 18:333–4.
35. Farre D, *et al.* (2003) Identification of patterns in biological sequences at the ALGGEN server: PROMO and MALGEN. *Nucleic Acids Res.* 31:3651–3.
36. Merika M, Orkin SH. (1993) DNA-binding specificity of GATA family transcription factors. *Mol. Cell. Biol.* 13:3999–4010.
37. Uhlen M, *et al.* (2010) Towards a knowledge-based Human Protein Atlas. *Nat. Biotechnol.* 28:1248–50.
38. Boyle MJ, Seaver EC. (2008) Developmental expression of foxA and gata genes during gut formation in the polychaete annelid, Capitella sp. I. *Evol. Dev.* 10:89–105.
39. Haveri H, *et al.* (2008) Transcription factors GATA-4 and GATA-6 in normal and neoplastic human gastrointestinal mucosa. *BMC Gastroenterol.* 8:9.
40. Hyland PL, *et al.* (2014) Global changes in gene expression of Barrett's esophagus compared to normal squamous esophagus and gastric cardia tissues. *PLoS One.* 9:e93219.
41. Milano F, *et al.* (2007) Bone morphogenetic protein 4 expressed in esophagitis induces a columnar phenotype in esophageal squamous cells. *Gastroenterology.* 132:2412–21.
42. Gobel F, *et al.* (2009) Reciprocal role of GATA-1 and vitamin D receptor in human myeloid dendritic cell differentiation. *Blood.* 114:3813–21.
43. Chang CK, *et al.* (2012) Vitamin D receptor gene variants and esophageal adenocarcinoma risk: a population-based case-control study. *J. Gastrointest. Cancer.* 43:512–7.

Cite this article as: Janmaat VT, *et al*. (2015) Vitamin D receptor polymorphisms are associated with reduced esophageal vitamin D receptor expression and reduced esophageal adenocarcinoma risk. *Mol. Med*. 21:346–54.