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ORIGINAL RESEARCH

Polymorphisms of Genes Related to Function and Metabolism of Vitamin D in Esophageal Adenocarcinoma

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Abstract

Purpose The vitamin D receptor (VDR) endocrine system has emerged as an endogenous pleiotropic biological cell regulator with anti-neoplastic effects on breast, colorectal, and prostatic adenocarcinomas. We studied the association of gene expression, polymorphisms of VDR, CYP27B1, and CYP24A1 genes and serum vitamin D levels as surrogate markers of disease progression in patients with acid reflux, Barrett's esophagus (BE), or esophageal adenocarcinoma (EAC).

Methods We analyzed blood and tissue samples from patients with biopsy-confirmed BE or EAC for vitamin D levels, gene expressions, and polymorphisms in VDR (*Fok*I [F/f], *Bsm*I [B/b], *Apa*I [A/a], and *Taq*I [T/t]), CYP27B1 (*Hinf*I [H/h]), and CYP24A1 (*Hpy*1881 [Y/y]). Percentages of homozygous dominant/recessive or heterozygous traits were assessed for each polymorphism in all patient subgroups.

Results Genomic Bb and FF polymorphisms were highly prevalent in EAC patients, whereas BE patients had a high prevalence of wild-type *Hpy*1881 (YY polymorphism). Some polymorphisms (Yy for CYP24A1, bb for VDR) were noted only in EAC patients. Yy and bb forms were both uniquely present in some EAC patients without associated Barrett's lesions, but not in patients with concomitant BE. AA and bb polymorphisms were associated with decreased response to neoadjuvant therapy. A high level of VDR and CYP24A1 mRNA expression was observed in EAC tissue of non-responders. Serum vitamin D deficiency was common in EAC patients.

Conclusions Specific polymorphisms in vitamin D metabolism-related genes are associated with the likelihood of reflux–BE–EAC progression. Identifying such polymorphisms may aid in development of better surveillance and diagnostic and therapeutic protocols.

Keywords Barrett's esophagus · Esophageal adenocarcinoma · Gastroesophageal reflux · Single-nucleotide polymorphisms · Vitamin D receptor

Abbreviations

AU	Arbitrary unit
BE	Barrett's esophagus
CYP	Cytochrome P450
EAC	Esophageal adenocarcinoma
GERD	Gastroesophageal reflux disease
IHC	Immunohistochemistry
PCR	Polymerase chain reaction
RFLP	Restriction fragment length polymorphism
VDR	Vitamin D receptor
VRTC	Vital residual tumor cells

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Introduction

The incidence of esophageal adenocarcinoma (EAC) is rapidly rising in the Western world with increasing obesity and lifestyle changes, and is now more prevalent than squamous cell carcinoma [1–3]. EAC accounts for as many as 60% of all esophageal carcinoma cases in the USA, with approximately 11,000 new cases diagnosed in 2014 [4]. EAC carries a poor prognosis, with a 5-year survival of just 30% [5, 6]. Barrett's

esophagus (BE) is a known risk factor for EAC, and individuals with BE are 30–125 times more likely to develop EAC than individuals without BE [4, 7]. Most EAC is preceded by BE; however, the majority of patients with BE do not develop EAC [8]. For this reason, current surveillance protocols have not been shown to be cost-effective [3]. It is therefore important to gain insight into the factors that influence the progression in a metaplasia–dysplasia–carcinoma sequence and that affect the occurrence of EAC. Noninvasive blood-based markers (e.g., serology) could serve as useful addition to the existing surveillance protocols, giving clinicians an edge by helping them identify high-risk patients earlier.

Several immunohistochemical panels have been associated with EAC; however, most are based on nonspecific markers, such as loci within or near BARX1, CRTC1, FOXP1, ALDH1A2, and MUC5AC [9, 10]. Over the past decade, Feldman et al. [11], Holick et al. [12], and many others have highlighted the role of vitamin D and the vitamin D receptor (VDR) endocrine system in autocrine regulation of cell cycles, and in the potential anticarcinogenic effects of the VDR system. Researchers have implicated polymorphisms in the VDR gene as being associated with cancers of brain, bone, breast, prostate, and skin [13–15]. Furthermore, higher serum levels of vitamin D seem to increase squamous dysplasia and squamous cancer of the esophagus, while vitamin D deficiency has been associated with EAC [16–18]. Most notably, cytochrome P450 (CYP) enzymes associated with metabolism of the bioactive form of vitamin D are CYP24A1 (1,25-dihydroxyvitamin D3 24-hydroxylase or calcitriol 24-hydroxylase) and CYP27B1 (25-Hydroxyvitamin D3 1-alpha-hydroxylase or 1-alpha-hydroxylase). Polymorphisms of genes associated with these enzymes may play a meaningful role in carcinogenesis.

In this study, we looked at the association of VDR, CYP27B1, and CYP24A1 gene polymorphisms in both genomic and tissue DNA and circulatory vitamin D levels as markers of the development or progression of the reflux–Barrett's–adenocarcinoma sequence. The response of EAC to current chemotherapy modalities is highly unpredictable, with major response rates of 20–30% to neoadjuvant chemotherapy [19]. We also investigated whether these biomarkers play a role in identifying responders and non-responders to neoadjuvant chemotherapy.

Methods

Patient Recruitment

The research protocol of this study was approved by the Institutional Review Board. After obtaining written and informed consent, we recruited patients presenting to our institution between July 2014 and June 2016 with a diagnosis of either BE or EAC. Recruited subjects conferred to the following inclusion criteria: white males, 50 years or older in age, with biopsy-proven cases of either EAC presenting for surgical management or non-dysplastic BE without progression to dysplasia or malignancy (as documented by endoscopic surveillance of at least 10 years from original diagnosis). We adhered to AGA guidelines in classifying BE; that is, endoscopically visible salmon-colored mucosa in the distal esophagus proximal to the gastroesophageal junction, which on biopsy was found to have intestinal metaplasia with goblet cells [20]. Patients may or may not have received neoadjuvant chemo-radiation, and were further grouped as either responders or non-responders to neoadjuvant therapy (described below). Blood samples from healthy volunteers with no history of reflux were collected for controlled comparison. We narrowed inclusion criteria to minimize known confounders (e.g., age, gender, race). The recruitment of patients was based on the power analysis and available time period. Patients with past diagnosis of other esophageal pathologies or history of foregut surgery were excluded from the study.

Sample Collection

Patients were de-identified and clinical data were exported to a Microsoft Excel spreadsheet (Microsoft, Redmond, WA). Then, 20 mL of venous blood was collected in ethylene-diamine-tetra-acetic acid tubes, transported in an ice box, and processed within 30 min of sample collection. Ammonium chloride tris buffer was added (2:1 whole blood) and incubated at room temperature for 20 min, followed by centrifugation with 1% phosphate-buffered saline (1:1 whole blood) at 2000 rpm for 10 min at room temperature. The buffy coat band was extracted and stored at − 80 °C for DNA extraction. We also extracted genomic DNA from blood leukocytes as a surrogate for embryonic genotype.

Endoscopic biopsy specimens were collected from patients with BE using standard biopsy forceps (Radial JawTM 4, large 2.8 mm, Boston Scientific, Marlborough, MA) and 2–3 samples were collected from each patient in addition to routine pathology. Tissue samples were collected from the surgical resection specimen of patients with EAC. For patients with EAC, esophageal resection specimens were opened on the back table. Representative samples from the normal proximal esophagus, BE segment (if present), tumor, and stomach were taken in direct consultation with a pathologist. Tissue specimens were placed in phosphate-buffered saline and RNA*later* buffer (Thermo Fisher Scientific Inc., Waltham, MA) and 10% neutral-buffered formalin. All tissue samples were transported in an ice box and processed within 30 min. Tissue samples in RNA*later* buffer were homogenized (1 mL

TRIzol reagent [Thermo Fisher Scientific Inc.] per 100 mg tissue) on ice, followed by incubation at room temperature for 5 min, and centrifugation at 12,000 rpm at 4 °C for 15 min. The clear supernatant was stored at − 80 °C for RNA extraction. Tissue samples in formalin were processed to obtain formalin-fixed paraffin-embedded sections for immunohistochemistry (IHC).

Laboratory Methodology

The true mode of inheritance of all polymorphisms tested has not been established; we therefore deemed an additive, dominant, or recessive genetic model to be appropriate. We performed molecular analysis for six polymorphisms. These included four polymorphisms in the VDR gene: *Fok*I, *Bsm*I, *Apa*I, and *Taq*I, named after the restriction enzymes originally used. The remaining two polymorphisms analyzed were one each for the enzymes CYP27B1- *Hinf*I and CYP24A1- *Hpy*1881. Mono- and biallelic polymorphisms were detected and reported as present or absent for each allele.

Polymorphisms in Blood Samples

DNA was extracted from peripheral blood leukocytes using GenElute Mammalian Genomic DNA Miniprep Kit G1N70 (Sigma-Aldrich Co. LLC, St. Louis, MO) and quantified with NanoDrop spectrophotometer (Thermo Fisher Scientific Inc.). Specific regions within VDR, CYP27B1, and CYP24A1 genes were amplified by polymerase chain reaction (PCR). PCR was carried out with reaction volume of 50 μL for each sample (master mix) consisting of 10 μL 5× PCR buffer (Mg-free), 2 μL dNTP (10 mmol/L), 1.5 μL DMSO, 0.5 μL Taq Polymerase (Thermo Fisher Scientific Inc.), 1.5 μL of forward primer, 1.5 μL of reverse primer, template DNA (300 ng, volume calculated after quantification), and nuclease-free water (to achieve volume of 50 μL). The primers (ESM I) used have been previously described by Toptas et al. [15], Donath et al. [21], Gunes et al. [22], and Bailey et al. [23], and were standardized prior to commencement of this study. The following PCR cycle program was used: 95 °C for 3 min, 30 cycles of 95 °C for 1 min, annealing for 1 min (at 62 °C for primer pair 1 [*Bsm*I], 60 °C for primer pairs 11 [*Apa*I and *Taq*I] and 18 [*Fok*I], 54 °C for primer pair B1 [*Hinf*I], and 53 °C for primer pair R1 [*Hpy*1881]), and 68 °C for 5 min. PCR amplicons were digested with 1 μL restriction endonuclease and CutSmart buffer (New England Biolabs Inc., Ipswich, MA) for 90 min (at 65 °C for primer pair 1 and 11t [*Taq*I], room temperature for 11a [*Apa*I], and 37 °C for 18, B1, and R1), and the products formed were resolved by 2% agarose gel electrophoresis with GelStar dye (Lonza Group, Basel, Switzerland; 10% of volume). Variations in lengths of restriction fragments were analyzed and sequenced to accurately detect single-nucleotide polymorphisms. RNA extraction was carried out from blood samples with the TRIzol LS kit (Thermo Fisher Scientific Inc.) for the purpose of mRNA fold expression.

Polymorphisms in Tissue Samples

RNA extraction was carried out from tissue samples using RNeasy Plus Mini Kit (QIAGEN Sciences, Germantown, MD) and gene expression analysis was performed with SYBR Green Supermix (Thermo Fisher Scientific Inc.) for real-time PCR quantification. We compared genetic polymorphisms in blood leukocytes (surrogates for embryonic genotypes), tumor tissue specimens, and tissue from visually normal esophagus and stomach from the same patient. Because tumor tissue may have additional polymorphisms due to high rate of somatic mutations, we used genomic DNA from leukocytes for comparison.

Gene Expression and Immunohistochemistry

Expressions of CYP27B1 and CYP24A1 were evaluated in formalin-fixed paraffin-embedded sections by IHC using rabbit anti-CYP27B1 and anti-CYP24A1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at a dilution of 1:75. Indirect radio-immunofluorescence staining was done as an adjunct to IHC. A BX51 microscope was used to visualize all slides, and tissue photograph were taken using an Olympus DP71 camera using the same exposure time for each slide. The pictures were analyzed using ImageJ software (National Institutes of Health) and converted to an RGB stack. The image threshold was individually set to minimize the background fluorescence that would be integrated into the intensity measurement. IHC was evaluated semi-quantitatively from 0 to 3 arbitrary units (AUs) with none (0), weak (1), moderate (2), and strong (3) by an observer blinded to biopsy outcomes. Moderate and strong (AU ≥ 2) were considered positive for expression of CYP27B1 or CYP24A1.

Serum 25-Hydroxy Vitamin D Levels

Circulating 25-hydroxyvitamin D levels were measured using a fluorescein-labeled monoclonal antibody-based immunoassay (ADVIA Centaur Vitamin D Total assay [Siemens Healthcare Diagnostics Inc., NY]), standardized with high-performance liquid chromatography–tandem mass spectrometry. Vitamin D deficiency was defined as serum level below 30 ng/mL.

Response Assessment

Response to neoadjuvant chemotherapy in patients with EAC was assessed by histomorphologic regression in surgical gross specimens, based on the estimated percentage of vital residual

tumor cells (VRTC) as described by Schneider et al. [24] [$T/(D + T)$; D = therapy-induced changes characterizing downstaging; T = residual tumor]. Based on response, subjects were classified into one of four grades: grade I: > 50% VRTCs; grade II: 10–50% VRTCs; grade III: nearly complete response with < 10% VRTCs; and grade IV: complete response. Subjects with regression grade II or higher were considered responders, whereas patients with grade I were considered non-responders. A response of grade III or grade IV was considered a major response.

Statistical Analysis

Fisher's exact test was used to compare the significance of difference in the presence of specific polymorphisms. Chi-square was applied when comparing more than two groups. Independent sample t test was applied for comparison of means. Bootstrapping was performed when parametric inference was in doubt due to insufficient sample size to make straightforward statistical inference. Bonferroni correction was applied to multiple comparisons from the same correlation matrix to prevent risk of type-I error. Values were considered significant at $p < 0.05$.

Results

Patients

Of the 52 patients recruited for study, 36 had EAC and 16 had BE. The clinical profiles of the study participants are summarized in Table 1. No significant difference existed between patients with BE and patients with EAC with respect to age, BMI, and comorbidities. Patients with EAC were more likely to be smokers (67%) than patients with BE (19%). Of the 36 patients with esophageal tumors, 31 (86%) had tumors located in the distal esophagus (Table 2). Nearly one-third of tumors were stage III, and more than 80% of tumors showed either moderate or poor differentiation. Microscopic examination revealed concomitant BE with EAC in 9/36 patients with EAC (25%). Surgical margins were negative in all but two patients, both of whom had stage III tumors that were nonresponsive to neoadjuvant therapy. The mean number of lymph nodes retrieved and positive for metastatic deposits in the EAC group were 25 and 3, respectively.

Polymorphisms in Blood

The mRNA expressions for VDR, CYP24A1, and CYP27B1 are shown in Fig. 1a. Patients with EAC showed significant differences in several polymorphisms compared with patients in the BE group. Monoallelic mutation of *Bsm*I (Bb) and homozygous wild form of *Fok*I (FF) were significantly more common in patients with EAC than BE (Fisher's exact test, $p < 0.05$; Table 3). Several polymorphisms absent in patients with BE were detected in a subset of patients with EAC, including heterozygous mutated allele of *Hpy*1881 (Yy) (61%, $p < 0.001$), biallelic wild form of *Apa*I (AA) (14%, $p = 0.145$), and biallelic mutated form of *Bsm*I (bb) (11%, $p = 0.254$) (Fisher's exact test). Patients with BE strongly expressed wild-type *Hpy*1881 (YY) compared with patients with EAC (100%, Fisher's exact test, $p < 0.001$).

Heterozygous *Fok*I (Ff) was absent and biallelic wild form of *Apa*I (AA) was present exclusively in patients with EAC who also had BE. mRNA expression of VDR and CYP27B1 genes were significantly higher in patients with BE than in those with EAC (Fig. 1a).

Polymorphisms in Tissues

Tissue specimens from the normal esophagus, tumor, and normal stomach were available for 26/36 patients with EAC. In addition, eight samples of patients with BE were available (four BE alone, four BE with EAC). Polymorphisms and mRNA expression in the normal esophagus were similar to that of the stomach; however, significant differences were noted in the polymorphisms of four genes (i.e., *Bsm*I, *Apa*I, *Hinf*I, and *Hpy1881*) among polymorphisms of these genes in the tumor, normal esophagus, and stomach (Chi-square test, $p < 0.05$; Fig. 2 and Table 4). mRNA expression was similar between normal esophagus, BE, and EAC tissues (Student's t test, $p > 0.05$) except lower fold expression for CYP27B1 noted in the normal esophageal tissue than in EAC tissue (Student's t test, $p = 0.05$; Fig. 2).

Genetic Polymorphisms and Response to Neoadjuvant Therapy

Of the 30 patients who underwent neoadjuvant chemotherapy, 16 (53%) were responders. A "major response" to neoadjuvant therapy (i.e., VRTC < 10%) was noted in 5/30 (17%) patients. Genomic polymorphisms of VDR, CYP24A1, and CYP27B1 in blood were compared among responders and non-responders (Table 3). Biallelic mutation of *Bsm*I (bb) and homozygous wild form of *Apa*I genes (AA) were only present in non-responders (Fisher's exact test, $p < 0.05$). A homozygous mutated form of the *Apa*I gene (aa) was exclusive to responders (Fisher's exact test, $p < 0.001$). No significant difference was noted in any other polymorphism. The mRNA fold expressions in blood for VDR and CYP24A1 were significantly higher for responders than non-responders (Student's t test, $p < 0.05$ for both; Fig. 1b). Among the non-responders, normal esophageal tissue had a lower mRNA fold expression for VDR and CYP27B1 as compared to the EAC tissue (Student's t test, $p \leq 0.05$; Fig. 2).

Table 1 Clinical profiles of 52 recruited study participants

Variable	Blood samples			Tissue samples		
	BE ($n=16$)	EAC ($n=36$)	p value	BE ($n=4$)	EAC ($n=26$)	p value
Mean age, years	59.3	62.6	0.4469	53.2	58.7	0.2985
Mean BMI, kg/m^2	32.2	26.7	0.0801	27.8	26.3	0.5618
History of other cancers	1	1	0.5479	0	0	–
Charlson Comorbidity Index, n (%)						
< 2	5 (31.3%)	0	0.6013	2 (50%)	0	–
2–5	9 (56.3%)	23 (63.9%)		2 (50%)	18 (69.2%)	
> 5	2 (12.5%)	13 (36.1%)		0	8 (30.8%)	
Number of patients with reflux, n (%)	16	27	*0.0279*	4	18	0.1951
Median duration of reflux before diagnosis, years	11.5	8	0.6918	6.8	7.6	0.4874
History of smoking n (%)	3 (18.8%)	24 (66.7%)	*0.0014*	1 (25%)	19 (73.1%)	0.0576
Mean serum vitamin D levels (ng/mL)[a]	34.67	27.77[b]	*0.0322*	N/A	N/A	–
Subjects with vitamin D deficiency, n (%)[a]	9 (56%)	22 (76%)[b]	0.7416	N/A	N/A	–

BE Barrett's esophagus, *EAC* esophageal adenocarcinoma

[a] Compared with mean serum Vit-D level in 7 controls—59.0 ng/mL and 1/7 (14%) deficient

[b] $n=29$ for serum vitamin D levels in EAC patients

Serum 25 Hydroxy Vitamin D Levels

Serum vitamin D deficiency was more common in patients with EAC than in patients with BE but there was no statistically significant difference between mean serum vitamin D levels between the two groups (Student's t test, p-NS; Table 1). In total, 45 samples were processed for serum vitamin D estimation (from 16 patients with BE and 29 patients with EAC). No significant difference was observed in serum vitamin D levels in patients with low (< 18 kg/m^2), normal (18–25 kg/m^2), and high (> 25 kg/m^2) BMI (59, 63, and 58%, respectively; Student's t test, p-NS). No difference was seen in proportion of responders and non-responders in terms of vitamin D deficiency (75 and 77%, respectively; Fisher's exact test, p-NS).

Table 2 Tumor characteristics in patients with EAC

Variable	EAC patients n(%) $n=36$
Neoadjuvant therapy	30 (83.3%)
Non-responders (Grade I)	14 (46.7%)
Responders	16 (53.3%)
Grade II	11 (36.7%)
Grade III	4 (13.3%)
Grade IV	1 (3.3%)
Location of tumor—distal esophagus: EGJ	31(86%): 5(14%)
Barrett's segment present	9 (25%)
Differentiation—Well: Moderate: Poor	7(19%): 20(56%): 9(25%)
R0 resection	34 (94.4%)
Mean number of lymph nodes retrieved	24.7
Mean number of lymph nodes positive	2.7

EAC esophageal adenocarcinoma, *EGJ* esophagogastric junction

Immunohistochemical Analysis

Expression of CYP24A1 positivity (AU ≥ 2) was detected in 69% of EAC tissue samples compared to 41% CYP27B1 positivity (Fig. 3). No significant difference existed between the two groups for any tissue type (Fisher's exact test, $p > 0.05$). Radio immunofluorescence revealed a higher expression of CYP24A1 in tumor tissues and a lower expression of CYP27B1 compared with normal esophageal tissue and stomach tissue (Fisher's exact test, $p > 0.05$).

Discussion

The incidence of EAC has been steadily rising and, despite advances in adjuvant therapies, and surgical outcomes, it continues to have a poor prognosis with a median survival of about 12 months and an overall 5-year survival rate of 30% [5, 6, 25]. EAC develops via a well-described inflammation–metaplasia–dysplasia–carcinoma sequence secondary to chronic gastroesophageal reflux disease (GERD). BE is associated with the greatest risk of EAC development (30–125 times), but less than 5% of patients with BE go on to develop EAC [7]. An annual incidence of 0.33% EAC in patients with non-dysplastic BE has been described [26]. Although many extrinsic factors have been associated with GERD, BE, and EAC (e.g., heavy smoking, strong alcoholic beverages, and intake of spiced food), hereditary influence accounts for about

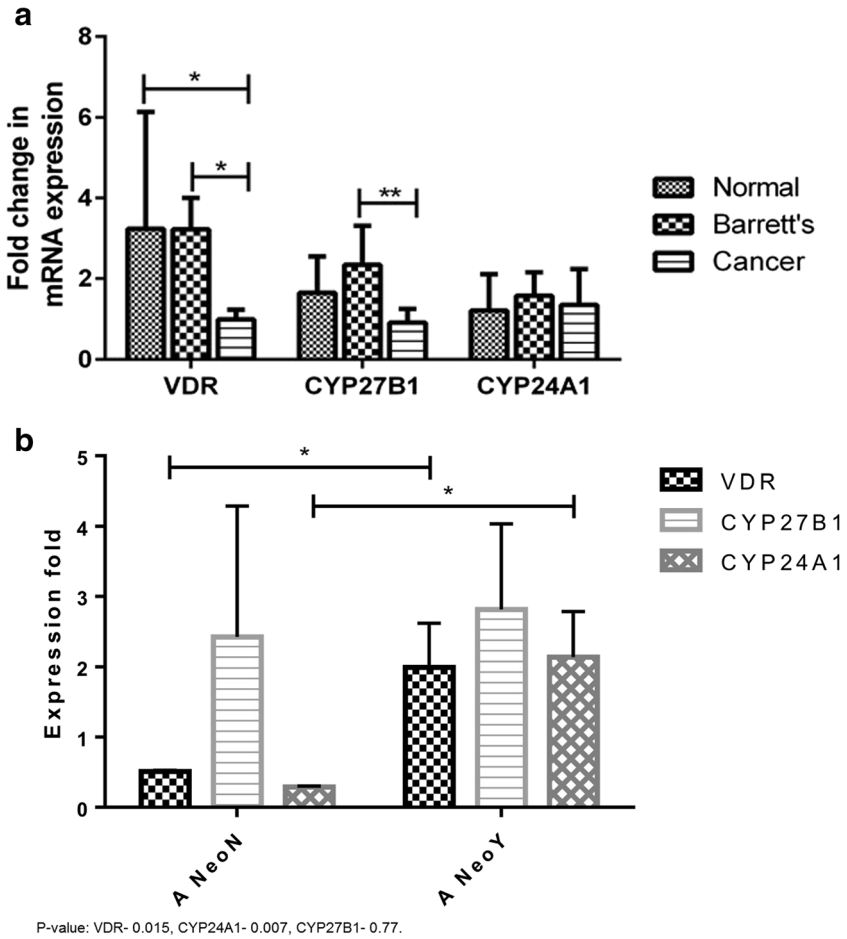

Fig. 1 **a** mRNA expression in blood samples from patients with Barrett's esophagus or cancer compared to 8 healthy control volunteers. **b** mRNA expression in blood samples of patients with cancer who were responders (Neo Y) or non-responders (Neo N) to neoadjuvant chemoradiotherapy

30% of GERD cases and a genetic influence is highly likely in individuals with chronic reflux who develop EAC [27].

The pathogenesis of GERD has been postulated to have a polygenic basis and shares significant overlap in genetic makeup with the pathogenesis of BE and EAC [28]. A recent genome-wide meta-analysis identified several high-risk loci in addition to the already-known loci associated with EAC (either alone or with BE), stressing the role of BE lesions as precursors to EAC [10]. Moreover, the influence of micronutrients such as vitamin D is highly likely; however, evidence of a direct relationship between vitamin D levels and risk of esophageal cancer is inconsistent. Higher serum levels of vitamin D were seen in patients with squamous dysplasia and squamous cancer of the esophagus, while lower serum levels of vitamin D have been associated with EAC; however, this has not been conclusively implicated with progression of BE lesions to EAC [16–18, 29]. No well-designed study has investigated the relationship between vitamin D levels and the development of BE or EAC. The role of vitamin D in the reflux–BE–EAC sequence has not yet been studied. Ours is a prospective controlled study in which we exhaustively included polymorphisms related to vitamin D endocrine system and, to our best knowledge, ours is the first study to have studied all these polymorphisms in relation to BE and EAC.

The vitamin D endocrine system is primarily associated with calcium homeostasis. However, vitamin D has been shown to play roles in several other metabolic and cell cycle pathways independent of its effect on calcium absorption and distribution [12]. More than 470 single-nucleotide polymorphisms have been identified in VDR (gene 12q13.11), and several more in the enzymes responsible for the metabolism of vitamin D [30]. Some cytochrome P450 (CYP) enzymes catalyze the formation and degradation of the bioactive form of vitamin D; most notably, CYP24A1 (gene 20q13) and CYP27B1 (gene 12q14.1). CYP24A1, also known as 1,25-dihydroxyvitamin D3 24-hydroxylase or calcitriol 24-hydroxylase, is a mitochondrial enzyme in the kidneys that initiates degradation of 1,25-dihydroxy Vit D3 (calcitriol), the physiologically active form, to calcitroic acid (1α-hydroxy-23-carboxy-24,25,26,27-tetranorvitamin D3) by hydroxylation of the side chain. CYP27B1 (25-Hydroxyvitamin D3 1-alpha-hydroxylase or 1-alpha-hydroxylase), on the other hand, is present in the kidneys and converts inactive vitamin D to active vitamin D, thereby catalyzing calcitriol formation by hydroxylation of calcifediol (also known as calcidiol, 25-hydroxycholecalciferol, or 25-hydroxyvitamin D).

Vitamin D and the VDR endocrine system are endogenous pleiotropic biological cell regulators with clear anti-neoplastic

Table 3 Genomic polymorphisms in blood for VDR, CYP24A1, and CYP27B1 genes

	Control[a] ($n = 7$)	Barrett's ($n = 16$)	All EAC ($n = 36$)	EAC post neoadjuvant therapy ($n = 30$)	
				Responders ($n = 16$)	Non-responders ($n = 14$)
BB	71%	81%*	11%	19%	0
Bb	29%	19%*	78%	81%	71%
bb	0	0	11%	0†	29%
AA	29%	0	14%	0†	36%
Aa	71%	69%	42%	38%	64%
aa	0	31%	44%	63%†	0
FF	29%	25%*	86%	88%	86%
Ff	71%	75%*	14%	13%	14%
ff	0	0	0	0	0
TT	14%	6%	19%	19%	21%
Tt	71%	81%*	28%	25%	43%
tt	14%	13%*	53%	56%	35%
HH	71%	81%	58%	63%	71%
Hh	29%	19%	42%	38%	29%
hh	0	0	0	0	0
YY	100%	100%*	39%	63%	29%
Yy	0	0*	61%	38%	71%
yy	0	0	0	0	0

B/b: *Bsm*I, A/a: *Apa*I, F/f: *Fok*I, T/t: *Taq*I, H/h: *Hinf*I, Y/y: *Hpy*1881 (upper case—dominant/wild allele, lower case—recessive/mutated allele)

*$p < 0.05$ vs EAC. †$p < 0.05$ vs Non-responders

[a] Controls were used for comparison. No statistical analysis done due to small number

effects [31, 32]. Vitamin D has shown promise as a pro-differentiating agent—unsurprisingly, considering that vitamin D is consanguineous to ATRA (a carboxylic acid form of vitamin A) [33]. Indeed, strong evidence supports the beneficial effects of vitamin D on survival in lung, brain, breast, and colorectal cancers [13, 34, 35]. Recent research on the molecular mechanisms of Barrett's carcinogenesis has unraveled numerous tumor-specific genetic and epigenetic alterations, as well as highlighted the role of inflammation and stromal-luminal-microbiota interactions in the pathogenesis of Barrett's carcinogenesis. Cancers of self-renewing epithelial systems (e.g., the gastrointestinal mucosa) are considered to result from loss of the intricate balance of the processes of continuous division, differentiation, and programmed cell death. Vitamin D and its receptor have been shown to guard the vulnerable multiplying epithelial cells by their anti-proliferative and pro-differentiating effect [36]. The possible role of vitamin D in the pathogenesis of EAC is inherently appealing in view of its key involvement in host response to acid- and bile reflux-induced injury and epithelial healing [29]. Bile acid, a lithocholic acid, is a potent endogenous VDR ligand [37]. Changes in the gastrointestinal microbiota with altered distal esophageal pH in patients with GERD have been shown to release endogenous histone deacetylators such as sodium butyrate, which may regulate epigenetic silencing of VDR response genes [38]. Deficiency in vitamin D_3 upregulated protein 1 (VDURP1) has been shown to promote *Helicobacter pylori*-induced gastric carcinogenesis in mice [39], and some have attributed the EAC epidemic to the eradication of *H. pylori* [40]. Furthermore, vitamin D reduces inducible COX2 levels, which have been found to facilitate Barrett's metaplasia [29, 41]. VDR levels are upregulated in the mucosa of patients with BE, and these levels decrease with disease progression, indicating a neoplastic escape from the vitamin D control [42]. Vitamin D has also been found to induce production of the peptide cathelicidin, which was recently shown to have a tumor-suppressive role in gastrointestinal cancers [43]. Trowbridge et al. found that normal VDR expression is only seen in the columnar mucosa and that Barrett's mucosa showed a strong VDR expression, suggesting an endogenous effect of vitamin D in this tissue [44]. VDR expression was also higher in tumors resistant to neoadjuvant therapy [42].

Mimori et al. found that overexpression of CYP24A1 was inversely correlated to VDR expression in 42 patients with EAC [45]. They further reported that overall survival was significantly affected by CYP24A1 expression status. Researchers have found multiple membrane-based morphogenetic signaling pathways that regulate homeobox genes and interact intricately with vitamin D, including Wnt, NOTCH,

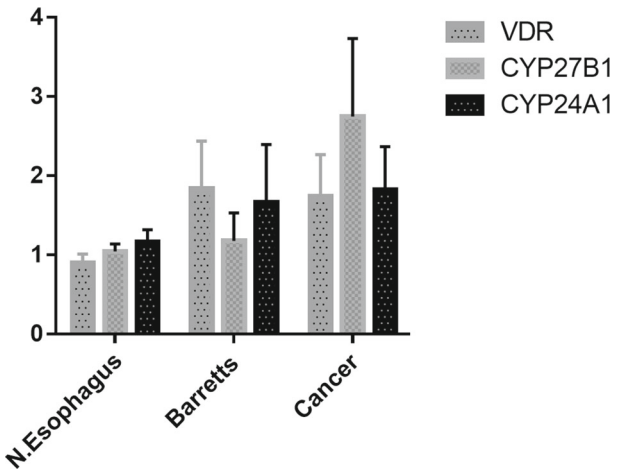

Fig. 2 mRNA expression in tissues of all cancer patients

		p-values		
		VDR	**CYP27B1**	**CYP24A1**
All patients	N. Esophagus vs Cancer	0.24	**0.05**	0.24
	Barrett's vs Cancer	0.21	0.10	0.89
	N. Esophagus vs Barrett's	0.06	0.54	0.60
Patients with residual tumor	N. Esophagus vs Cancer	**0.05**	**0.04**	0.10
	Barrett's vs Cancer	0.84	0.06	0.76
	N. Esophagus vs Barrett's	**0.05**	0.56	0.30

and Sonic Hedgehog pathways [46]. These pathways can potentially trigger embryonic reprogramming of esophageal squamous cells to columnar cells [47].

Five VDR polymorphisms have been studied with respect to cancer. These include *Fok*I, *tru*I, *Bsm*I, *Apa*I, and *Taq*I, named after the restriction enzymes originally used. Functionally speaking, a Start codon polymorphism in exon II at the 5′ end determined by using the *Fok*I restriction enzyme results in a truncated protein. At the 3′ end of the gene, three polymorphisms have been identified that do not lead to any change in either the transcribed mRNA or the translated protein (Supplementary Fig. 1). The first two sequences generate *Bsm*I and *Apa*I restriction sites and are intronic, lying between exons 8 and 9. The third polymorphism generates a *Taq*I restriction site which lies in exon 9 and leads to a silent change. These three polymorphisms are linked to a further gene variation, a variable length adenosine sequence within the 3′ untranslated region (3'UTR). The length of the poly (A) tail can determine mRNA stability [48, 49].

In breast cancer, the VDR polymorphism demonstrates a significant association with cancer risk [13, 14]. *Bsm*I with *Apa*I polymorphism also predicts the propensity of metastases to bone [50]. Interestingly, combined polymorphisms and serum vitamin D levels have been shown to compound breast cancer risk and disease severity. Despite a small sample size, we saw significant variation in expression of polymorphisms among various groups. Genotypes Bb, FF, and tt seemed to reflect a higher propensity for EAC development. An exclusive presence of bb and Yy in cancer patients may indicate the role of these polymorphisms in the pathogenesis of cancer; however, these polymorphisms were associated with EAC lesions. Additional research with a larger sample size may be helpful in illuminating potential associations. Furthermore, we found that the presence of specific polymorphisms may be associated with degree of response to neoadjuvant therapies.

The polymorphisms of vitamin D metabolism-related genes have not been studied in the tissue of patients with GERD, BE, or EAC. With the hypothesis that tumor tissue may feature additional polymorphisms, we studied all six polymorphisms in normal and tumor tissues from patients with EAC. However, we observed a significant difference in allelic forms of *Bsm*I in DNA of the tumor tissue compared to the normal esophageal or gastric tissue. The B allele of the VDR gene complex is considered to increase transcriptional activity of the VDR gene and, ultimately, prolonged mRNA stability [51]. This could be seen as higher mRNA expression

Table 4 Tissue polymorphisms in EAC patients for VDR, CYP24A1, and CYP27B1 genes

	Normal esophagus ($n = 26$)	Barrett's ($n = 8$)[a]	Tumor tissue ($n = 26$)	Stomach ($n = 26$)
BB*	12%	75%	73%	19%
Bb*	73%	25%	15%	69%
bb	15%	0	12%	12%
AA	12%	0	19%	8%
Aa*	42%	100%	42%	46%
aa	46%	0	38%	46%
FF	77%	63%	58%	69%
Ff	23%	38%	42%	31%
ff	0	0	0	0
TT	19%	13%	19%	23%
Tt	31%	75%	54%	35%
tt	50%	13%	27%	42%
HH*	62%	13%	27%	54%
Hh*	38%	88%	65%	46%
hh	0	0	8%	0
YY*	31%	100%	35%	31%
Yy*	69%	0	65%	69%
yy	0	0	0	0

B/b: *Bsm*I, A/a: *Apa*I, F/f: *Fok*I, T/t: *Taq*I, H/h: *Hinf*I, Y/y: *Hpy*1881 (upper case—dominant/wild allele, lower case—recessive/mutated allele)

*$p < 0.05$

[a] Four of these eight patients had Barrett's alone that had not progressed to EAC. Additionally, four EAC patients also had a Barrett's segment, bringing the total number of available Barrett's tissues to eight

of VDR in patients with esophageal cancer compared to patients with normal esophageal tissue ($p = 0.24$ in all patients, $p = 0.05$ in patients with residual tumor). Although gene expressions in patients with BE differed compared with other tissues, the small sample size limits drawing conclusions.

Ability to predict the response to neoadjuvant therapy in esophageal cancer patients is highly desirable. Response to chemotherapy in esophageal and gastric cancer has been found to be associated with expression of excision repair cross-complementation group1 (ERCC1), glutathione S transferase P1, and thymidylate synthase [52]. While studies have evaluated several molecular markers toward response to neoadjuvant therapy, very few studies have looked at vitamin D endocrine system as the potential marker for response to cancer chemotherapy. Xiong et al. have shown that in patients with non-small cell lung cancer, *Apa*I T>G polymorphisms of VDR with GG genotype was associated with increased response to the neoadjuvant therapy [53]. In esophageal cancer, Trowbridge et al. reported that stronger staining for VDR was associated with poorer response to the neoadjuvant therapy [42]. However, due to small sample size, statistical significance could not be reached. We found a significantly higher mRNA expression for VDR and CYP24A1 in in genomic DNA in patients showing major response to the neoadjuvant therapy. To our knowledge, our study is the first to look at the genomic polymorphisms in relation to such response. The polymorphisms of *Bsm*I and *Apa*I seem to be the potential predictors of the poor response to neoadjuvant therapy in EAC.

The level of vitamin D in the serum seems to affect risk of cancer. Almost 70% of our patients with EAC showed deficient levels of serum vitamin D. Epidemiological studies provide strong support of increased EAC incidence at higher latitudes with reduced environmental ultraviolet light B intensity, which is the primary stimulus for vitamin D formation in the body [54]. Additional indirect evidence comes from the fact that IL-4, a cytokine released by t cells and implicated in goblet cell metaplasia of respiratory and gastrointestinal epithelium, has been shown to increase vitamin D catabolism [45, 55]. BMI, a well-established risk factor for EAC, is also linked with lower circulating 25-hydroxy concentrations of vitamin D [56]. However, in our patient cohort, we found no significant difference in serum vitamin D levels between different BMI groups, so we assume that vitamin D deficiency is unrelated to general nutritional status.

Study Limitations

The primary limitation of this study is the relatively small sample size, especially for the controls and BE-only group.

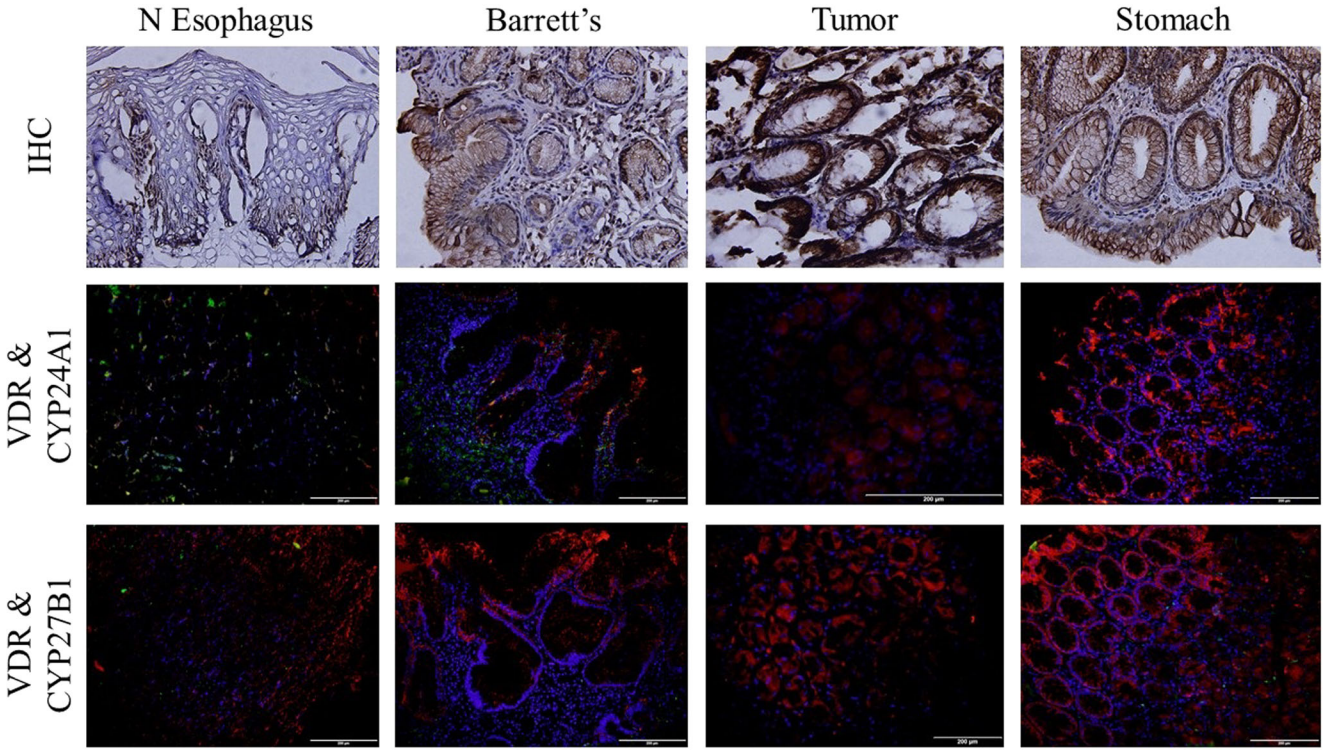

Fig. 3 Immunohistochemistry and immunofluorescence of tissues in cancer patients

This limits our ability to draw definitive conclusions. However, this study undoubtedly shows a significant difference in the vitamin D metabolism-related polymorphism which suggests an association that demands further exploration in a larger sample. The findings of this single-center study would be strengthened by corroboration from other larger studies. The other key enzymes of vitamin D_3 metabolism such as Cytochrome P450 27A1 and 2R1 were not included in the current study and their inclusion in future research may expand the role of vitamin D endocrine system in esophageal carcinogenesis and prognosis.

Conclusions

This prospective study has examined vitamin D metabolism-related gene polymorphisms and their association with BE and EAC. An association has been observed between these polymorphisms in EAC and the response of EAC to neoadjuvant therapy. These results may aid in risk stratification for development of EAC and the response of this aggressive cancer to neoadjuvant chemotherapy.

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Compliance with Ethical Standards

The research protocol of this study was approved by the Institutional Review Board (IRB#10-15855) (in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments).

Conflict of Interest The authors declare that they have no conflict of interest.

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