RESEARCH ARTICLE

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Association of Single Nucleotide Polymorphism in VDR, GC Globulin and *CYP2R1* with the Risk of Esophageal Cancer

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Abstract

Background: The proactive role of vitamin D has been well determined in different cancers. The protein that encodes the components of the vitamin D metabolism could appear to play a pivotal role in vitamin D stability and its maintenance. A polymorphism in vitamin-D-receptor (VDR), carrier globulin/binding protein (GC) and cytochrome P-450 family 2, subfamily R, polypeptide 1 (*CYP2R1*) genes has been predicted to be associated with the development of cancer. This study was designed to detect the association of VDR, GC Globulin and *CYP2R1* gene polymorphism with the risk of esophageal cancer in the North-east Indian population. **Methods:** To carry out the study, a total of 100 patients diagnosed with esophageal cancer and 101 healthy controls were enrolled. In a case-control manner, all samples were subjected to do genotype testing for known SNPs on the VDR (rs1544410), GC (rs4588), and *CYP2R1* (rs10741657) genes using Restriction-fragment length polymorphism (RFLP) followed by Sanger sequencing. The collected demographic and clinical data were analysed using the statistical software package SPSS v22.0. **Results:** The VDR haplotype heterozygous TC was found strongly associated with the carcinoma group (OR:1.09, 95%CI:0.67-1.75). The risk factors analysis using the GC globulin rs4588 phenotype, found a positive correlation in terms of mutant AA's harmful influence on the cancer cohort (OR = 1.125, OR=1.125, 95% CI, 0.573-2.206). The influence of the *CYP2R1* rs10741657 polymorphism on the malignant cohort revealed that the GG mutant had a significant negative influence on the carcinoma, has an influential role in disease severity (OR:1.736, at 95% CI; 0.368-8.180). **Conclusion:** In conclusion, this study revealed the potential association of VDR gene polymorphism in the progression and development of esophageal cancer in north east Indian population cohort.

Keywords: SNP- VDR- GC- *CYP2R1*- RFLP- Sanger Sequencing

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Introduction

Cancer is profoundly recognized as a genetic disorder which is anonymously associated with numerous physiological and lifestyle factors. Indeed, cancer is one of the leading causes of death in recent times and appears to be a major contributor to the disease burden. Among all, Esophageal cancer is the 8th most common cancer worldwide and its incidence rate is increasing tremendously with time (Pennathur et al., 2013; Simard et al., 2012). The two common histologic sorts of Esophageal cancer, esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma (EAC) appears to be equally severe with incredibly poor survival rate (Lagergren et al., 2017). Vitamin D is a steroid hormone and is one of the key modulators of the immune system (Chiang et al., 2011). Vitamin D interacts with the vitamin D receptor (VDR) to interfere with its action in cells. It is involved in many biological processes, including regulation of cell proliferation and differentiation in normal tissue, apoptosis and cell adhesion in tumor cells (Rai et al., 2017). The VDR is a type II nuclear receptor that communicates with the promoters of vitamin D-responsive genes. VDR is found to be differentially communicated in numerous kinds of cancer (Friedrich et al., 2003).

Vitamin D binding protein (VDBP), originally called "group specific component" (GC)-globulin is a serum α2-globulin of molecular weight 52–59 kDa. The encoding

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VDBP has 35kb of DNA and consists of 13 exons and 12 introns out of which exon 11 has a single nucleotide polymorphism at two different loci rs7041 and rs4588 (Abbas et al., 2008; Haldar et al., 2018). The GC has a high degree of variability at these two loci, and has been shown to be associated with altered levels of serum vitamin D. The rs7041 and rs4588 polymorphisms of GC have been previously connected with several cancer risks in different populations and show great inter-ethnic variability (Zhou et al., 2012). The *CYP2R1* encodes the critical enzyme responsible for vitamin D conversion to 25(OH)D in the liver (Berry and Hyppönen, 2011). It is a five-exon gene located on chromosome 11p15.2 and active in the 25-hydroxylation of both vitamin D2 and D3. It is highly predicted that SNPs in this gene have an effect on 25(OH)D synthesis and thus serum concentration. GC encodes the vitamin D binding protein (DBP) that binds to and transports vitamin D and its metabolites throughout the body (Falleti et al., 2013). The *CYP2R1* rs10741657 polymorphism, located 2 kb upstream of the gene, is one of the major polymorphisms associated with circulating 25(OH) vitamin D levels (Wang et al., 2010). It is a promotor polymorphism that results in decreased *CYP2R1* synthesis in the variant G-allele (Ramos-Lopez et al., 2007), presumably resulting in a decreased rate of cholecalciferol conversion to 25(OH)D. In several populations, *CYP2R1*/rs10741657 has been consistently associated with serum 25(OH)D concentrations (Bu et al., 2010; Lasky-Su et al., 2012; NissenRasmussen et al., 2014; NissenVogel et al., 2014; Ramos-Lopez et al., 2007; Wang et al., 2010; Zhang et al., 2013), with carriers of the G-allele having the lowest serum 25(OH)D concentrations. Past studies revealed the overexpression of the *CYP2R1* gene indirectly responsible for the imbalance of vitamin D metabolites in Renal Cell Carcinoma, thereby contributing to its pathogenesis (Urbschat et al., 2013). The serum 25(OH) D level has also been associated with susceptibility in breast cancer (O'Brien et al., 2017), gastric cancer (Kwak and Paik, 2020), thyroid cancer (Hu et al., 2020), prostate cancer (Gao et al., 2018), and colorectal cancer (Zhang et al., 2019).

This study aimed to investigate three single nucleotide polymorphisms (SNPs), rs1544410, rs4588 and rs10741657 of VDR, GC-Globulin and *CYP2R1* genes, respectively, in esophageal cancer patients to assess their association with the risk of cancer development and progression. The associations between the genetic variations in these genes engaged in mediating the biological effects of vitamin D and the occurrence of esophageal cancer may give more grounded proof of a causal connection between vitamin D and the occurrence of such diseases than intermittent measurements of serum vitamin D levels.

Materials and Methods

Patients

To carry out the study, 100 esophageal cancer patients and 101 healthy controls were recruited from Gauhati Medical College Hospital, Guwahati and North-East Cancer Hospital, Jorabat. Assam. 5ml of whole blood was collected from each individual with proper consent. The detailed information about food habits, physiological factors, medical history and lifestyle habits was also collected. The study was approved by the institutional ethical committee, Gauhati University/GMC.No. MC/190/2007/pt-11/39, date24/08/2018.

Genomic DNA Preparation and Genotyping

Genomic DNA was extracted from whole blood using the conventional phenol-chloroform method, dissolved in 50µL of nuclease-free water and stored at -80°C until use. The SNPs of VDR (rs1544410), GC Globulin (rs4588) and *CYP2R1*(rs10741657) were assessed using polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP). New primers were designed using the Primer3 software mentioned below.

PCR and Restriction digestion of VDR, GC globulin and CYP2R1

The PCR was carried out distinctly for three genes i.e. VDR, GC globulin and *CYP2R1* using separate newly designed forward and reverse primers (Primers are listed in Table 5). A reaction mixture of total volume of 20 µL was prepared by 200ng genomic DNA, 0.8 U of Taq polymerase (Thermo Fisher Scientific), 1xbuffer, 6 pmol of each primer, 1.6mM of MgCl2 and 0.4 mM deoxyribonucleotide triphosphate (dNTP) (Thermo Fisher Scientific). Genomic DNA was denatured at 95°C for 5 minutes, followed by 35 cycles of amplification with denaturation at 95°C for 30 seconds, annealing at 56.4°C for 30 seconds for VDR and 57.3 C for GC, and extension at 72°C for 45 seconds. A final extension was carried out at 72°C for 2 min.

Restriction digestion was carried out with separate restriction enzymes, BsmI, StyI and MnI1 for three genes, VDR, GC globulin and *CYP2R1* respectively. Amplified fragments were digested with endonuclease by incubating overnight. BsmI digestion of the VDR gene resulted in 190 bp and 109 bp fragments with the presence of restriction sites. The presence of the mutant A genotype at the GC globulin polymorphic site was detected using the Sty1 restriction enzyme, which produced two fragments of 278 bp and 124 bp in size. whereas the MnlI restriction enzyme was used for *CYP2R1* to determine the wild G genotype that obtained two fragments of 128 and 105 bp. The restriction digestion was carried out following the manufacturer's protocol with slight modification.

Sanger Sequencing and Validation

The representative samples of each cohort were subjected to Sanger sequencing. Standard double stranded sequencing was performed using 50ng-100ng of purified PCR product and 4 pM primer, 4µl of BigDye terminator ready reaction kit (Perkin Elmer), in 10µl volume adjusted with nuclease free water. Cycle sequencing was carried out in a GeneAmp9600 thermal cycler (Perkin Elmer) employing 30 cycles at 96°C for 10 s, 49°C to 50°C for 5 s, and 60°for 4 min. Extended products were purified by alcohol precipitation. Purified samples were dissolved in ten liters of 50% Hi-Di formamide before being analyzed on an ABI3700 automated DNA analyzer.

Statistical analysis

The statistical analysis of the generated data was performed using SPSS software version 22.0 (IBM, New York City, NY). Categorical variables and qualitative data were presented as numbers and percentages, and continuous variables were presented as means – standard deviations. Genotypes and allele frequencies of the SNPs were compared by using Pearson's chi-square test or Fisher's exact test. Distributions of continuous variables were analysed by a one-way analysis of variance test or Mann–Whitney U test. To assess the influence of each factor on the risk of developing esophageal cancer, the potential factors different from $p<0.1$ in the univariate analysis were included in the multivariate analysis based on a stepwise logistic regression model. This software was used for haplotype analyses. The assumption of Hardy–Weinberg equilibrium was assessed for all SNPs using the chi-square test. For all statistical studies, the level of significance was set at $p<0.05$.

Results

Characteristics of the clinicopathological and physiological parameter

The demographic, clinical and biochemical characteristics of the recruited individuals (case and control) were summarized in Table 1. The median age of the patients was found greater than that of the control groups. In contrast, the male-to-female ratio in patients (1.77) is lower than in the control sample (2.09). For analyzing food habit and lifestyle habit the cases were split into three groups based on their etiology: smokers, drinkers and tobacco consumers. Out of which 46 patients were smokers,29 were light drinkers and 17 were non-smokers. While, 42 were occasional drinkers and 29 were strong drinkers. The results suggest 74 of the patients chewed tobacco occasionally, whereas 22 chewed heavily (Table 1). In food habit, only one percent was found to be purely vegetarian, whereas 99% of the subjects consume non-vegetarian diets. Apart from that, 59% of the patients found to consume smoked foods (Table 1).

Genotypes distribution of VDR, GC globulin and CYP2R1

The haplotype analysis of VDR, GC Globulin, and *CYP2R1* genes in case and control observed some significant results. The heterozygous TC was found significantly common in VDR genotype in the patients group as compared to control group($p=0.515$). The wild type TT was found in 29% cases while only 5% healthy controls express the wild type genotype. On the other hand, homozygous CC mutant represent almost equally, found 36% and 37% in case and control group respectively (Table 2). The results of the genotypic frequencies of the GC globulin (rs4588) observed 40% occurrence of AA homozygous mutant and 60% wild type CC mutant in control group. Whereas, the GG homozygous genotype were significantly found most common in *CYP2R1* (rs10741657) gene compared to AA wild type genotype incases ($p=0.66$), indicating that the SNP has a significant influence on the carcinoma group. On the other hand, the wild AA type was observed to be constant in both the patient and control groups (Table 3). The agarose gel electrophoresis of PCR-RFLP results and sanger sequencing results of selective sample are shown in Figure 1(a), 1(b) and Figure 2 respectively.

Factors associated with the carcinoma development/ progression by logistic regression analysis

Univariate analysis was performed to identify association of independent factors (SNP; VDR, GC

Table 1. Baseline Characteristics of the Study Population

	Characteristic	Patient group	Control group
Age	Age (in years.)	56.914 (±10.245)	54.686(±12.223)
Sex	Male	64 (64%)	67.307
	Female	36 (34%)	32.692
Etiology	Smoking habit		
	Smoker	46	NA
	Non-smoker	17	NA
	Alcohol intake		
	Limited consumer	29	NA
	Occasionally consumer	42	NA
	Heavy consumer	29	NA
	Tobacco intake		
	Light chewer	74	NA
	Heavy chewer	22	NA
Diet	Smoked food intake		
	Yes	59	NA
	No	41	NA
	Non-Veg food intake		
	Yes	99	NA
	No	1	NA

NA, Non-Significant

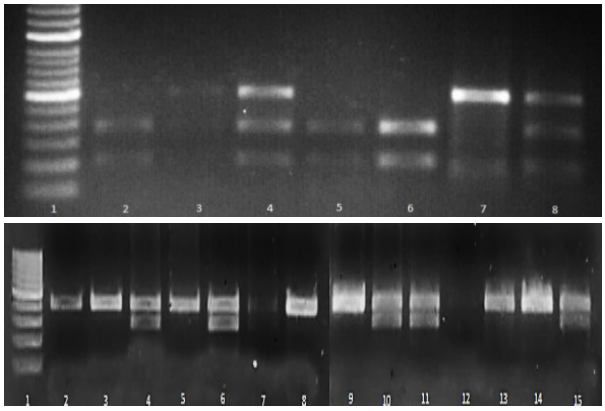

Figure 1. (a). PCR-restriction length Polymorphism assay for analyzing VDR rs1544410 polymorphism. Lane 1 shows 100bp marker, Lane 2, 5 and 6 shows rs1544410 CC genotype and lane 3, 4, 7, 8 shows rs1544410 TC genotype. (b). PCR-restriction length Polymorphism assay for analyzing GC Globulin rs4588 polymorphism. Lane 1 shows 100bp marker, Lane 4, 6, 7, 10, 11, 15 shows rs4588 AA genotype and lanes 2, 3, 5, 8, 9, 13, 14 shows rs4588 CC genotype.

Table 2. Genotype Distributions of VDR, GC Globulin, and *CYP2R1* Polymorphisms

Gene polymorphism profiles			Fishers exact test	
VDR Bsm rs 1544410 polymorphism				
	Patients	Control		p-value
CC (Mutant/Homozygous)	36 (36%)	37 (74%)		0.515772051
Heterozygous TC	35 (35%)	10 (20%)	NS	1
Wild Type	29 %(29%)	5 (5%)		
GC Globulin Sty1 rs4588 Polymorphism				
AA (Homozygous/Mutant)	40 (40%)	10 (20%)	NS	1
Wild type CC	60 (60%)	25 (50%)		0.88
CYP2R1 Mn1 rs 10741657 Polymorphism				
AA(Wild Type)	37 (37%)	17 (34%)	NS	
GG (Mutant/Homozygous)	63 (63%)	35% (70%)		0.666091795

NA, Non-Significant

and *CYP2R1*) with carcinoma development (Table 4). The heterozygous VDR haplotype (TC) was strongly associated with the carcinoma group (OR: 1.09, 95% CI: 0.67-1.75). Stepwise logistic regression analysis revealed that the CC mutant was likely linked to cancer, (OR: 0.876 at 95% CI 0.673-.114). The computed risk

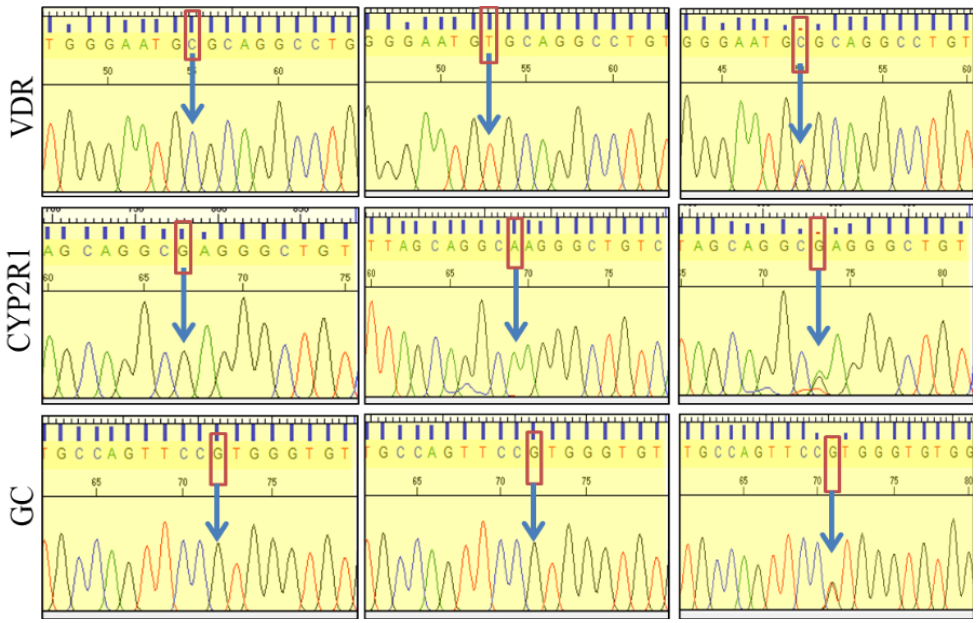

Figure 2. Sequencing Results of Genotype Distribution of Three Genes in Representative Samples.

Table 3. Distribution Frequencies of Haplotypes in VDR, GC Globulin and *CYP2R1* Genes SNPs

Gene (s)	Patients (%)	Control (%)	Fisher's exact test
VDR Bms rs1544410 polymorphism			
Heterozygus TC	35 (35)	10 (19.6)	NS
Homozygous CC	36(36)	37 (72.549)	NS
Wild Type TT	29(29)	5 (9.803)	NS
GC Globulin Sty1 rs4588 Polymorphism			
Homozygous AA	40 (40)	19 (37.254)	
Wild Type CC	60 (60)	33 (64.705)	
Cyp2r1 Mn rs10741657 polymorphism			
AA (Wild Type)	13 (13)	17 (33.33)	NS
GG (Homo)	47 (47)	35 (68.627)	NS

NA, Non-Significant

factor analysis using the GC globulin (rs4588) phenotype, found a positive correlation in terms of mutant AA's harmful influence on the cancer cohort, (OR= 1.125 ,95% CI, 0.573-2.206). The wild type variant CC was also found to be associated with the group (OR: 0.957, at 95%CI 0.7424-1.234). The influence of the *CYP2R1* (rs10741657) polymorphism on the malignant cohort revealed a significant negative impact of GG mutant on the carcinoma group, suggesting that the gene phenotype has a causal role in disease severity (OR: 1.736, at 95% CI; 0.368-8.180). The wild type AA, on the other hand, may have a protective or no effect on the development of

Table 4. Univariate Analyses and Stepwise Multivariate Analyses of Factors Associated with the Cancer

Gene	patient	control	OR(Odds Ratio, 95% CI)	Lower limit	Upper limit	p-value (Fisher's exact test)
CC	36 (36%)	37 (74%)	0.877	0.673	1.142	0.516
TC	35 (35%)	10 (20%)	1.091	0.677	1.759	
TC	29 (29%)	5 (5%)	0.857	0.349	2.106	1.000
Dysphasia Grade		TC	1.394	0.859	2.264	0.204
		TT	0.680	0.390	1.184	
		CC	0.864	0.732	1.020	0.279
Male/Female		TC	1.333	0.850	2.088	0.281
		TT	0.683	0.350	1.332	
		CC	0.934	0.760	1.149	0.603
GC Globulin sty1 rs4588 polymorphism						
AA	40 (40%)	10 (20%)	1.125	0.574	2.206	0.880
CC	60 (60%)	25 (50%)	0.957	0.742	1.235	
Dysphagia Grade		AA	1.017	0.622	1.664	1.000
		CC	0.988	0.704	1.387	
Male/Female		AA	0.967	0.581	1.607	1.000
		CC	1.024	0.724	1.447	
CYP2R1 Mn11 rs10741657 polymorphism						
AA	37 (37%)	17 (34%)	0.965	0.867	1.074	0.666
GG	63 (63%)	35 (70%)	1.737	0.369	8.181	
Dysphasia Grade		AA	1.043	0.608	1.790	1.000
		GG	0.976	0.717	1.330	
Male/Female		AA	0.690	0.376	1.267	0.269
		GG	1.218	0.902	1.647	

Table 5. List of Primer Used

Name of the gene	Primer sequence	Product size
VDR -forward	5'-GGAGACGTAGCAAAAGGAGAC-3'	299bp
VDR -reverse	5'-GAACCATCTCTCAGGCTCCA-3'	
GC globulin- forward	5'-GACTTCCAATTCAGCAGCGA-3'	402bp
GC globulin - reverse	5'-TGCCATGTTAAGTGGAGGGT-3'	
CYP2R1 - forward	5'-AGGGAAGAGCAATGACATGGA-3'	233bp
CYP2R1 - reverse	5'-TGTGTGGTTTTAAGCCATCAGA-3'	

cancer in the diseased group (OR: 0.964, at 95% CI, 0.867-1.073). In addition, we also investigated if there is any link between gene polymorphism/haplotype distributions and dysphagia grade, albeit based on the Odd's ratio (OR). A positive connection between the mutant TC of the VDR gene and dysphagia grade have been observed (OR 1.09; p=0.203, Table 4). Furthermore, with OR 1.394 and 1.736, haplotypes AA (GC Globulin) and GG (*CYP2R1*) indicated a probable influence on dysphagia grade severity of the studied cohort (Table 4).

Discussion

Vitamin D is involved in different biological processes like metabolism, modulation of immune response, regulation of cell proliferation and differentiation. Vitamin D has significant function in overall human health, including cancer occurrence (Trowbridge et al., 2013). Vitamin D controls cellular differentiation and proliferation in normal tissue and regulates proliferation, apoptosis and cell adhesion at the tumour cell level, along with decreasing oxidative DNA damage (Krajewski et al., 2016). Vitamin D maintains its biological action via the vitamin-D-receptor (VDR) and an extensive study has been conducted on VDR polymorphism and its association with various genetic diseases (Elzehery et al., 2017). The most commonly studied VDR polymorphisms include Fok1 (rs2228570), Apa1 (rs7975232), Bsm1 (rs1544410), Bgl1 (rs739837), and restriction fragment length polymorphisms rs7975232 (G/T substitution), rs1544410 (A/G substitution), and rs739837 (G/T substitution) (Denzer et al., 2011). In our study, we found VDR haplotype heterozygous TC was strongly associated with the carcinoma group (OR: 1.09, 95% CI: 0.67-1.75). Stepwise logistic regression analysis, indeed, revealed that the CC mutant was most likely associated with cancer, with an OR of 0.876 at 95% CI (0.673-1.144).

There are two commonly reported non-synonymous SNPs, Glu416Asp (rs7041) and Thr420Lys (rs4588) in the coding of the GC gene in humans. Previous research has linked Glu416Asp and Thr420Lys to changes in 25(OH)D plasma concentrations (Engelman et al., 2008; Sinotte et al., 2009). The risk factor analysis using the GC globulin rs4588 phenotype found a robust correlation in terms of mutant AA's harmful influence on the cancer cohort, [OR of 1.125 (95% CI, 0.573-2.206)]. The wild type variant CC was also found to be associated with the group (OR: 0.957, at 95%CI 0.7424-1.234). Genetic and epigenetic factors can influence several crucial steps along the metabolic pathway of vitamin D. Several genes like DHCR7, *CYP2R1*, VDR, CYP24A1, CYP27B1 directly involved in the vitamin D pathway as their aberrant expressions have been demonstrated to be associated with vitamin D concentrations and cancer in several past studies (Afshan et al., 2021; Gnagnarella et al., 2021; Latacz et al., 2020; O'Brien et al., 2017; Voutsadakis, 2020). A number of genome-wide association studies (GWAS) among European populations (Ahn et al., 2010; Anderson et al., 2014; O'Brien et al., 2018) and some other ethnic populations (Robien et al., 2013; Xu et al., 2015) have identified the *CYP2R1**2 SNP of the *CYP2R1* gene as being associated with changes in vitamin D levels.

Among the identified SNPs, an association between a SNP located in the non-coding region of the 5′-UTR (rs10741657) and 25(OH)D levels has been established(Duan et al., 2018; Slater et al., 2017; Wang et al., 2010; Ye et al., 2015), with individuals with the GG genotype demonstrating a decreasing trend in 25(OH) D levels in comparison to the no-risk genotype AA. It is believed that SNP in this 5′-UTR region are likely to regulate gene expression and therefore, the levels of activity and expression of 25-hydroxylase may be affected (Ramos-Lopez et al., 2007). Two SNPs, rs10766197 and rs12794714, have been identified in the coding region of introns, and these polymorphisms may be associated with changes in selective splicing regulation and gene regulation (Ramos-Lopez et al., 2007). We also looked at the influence of the *CYP2R1* rs10741657 polymorphism on the malignant cohort and discovered that the GG mutant had a significant negative impact on the carcinoma group, suggesting that the gene phenotype has a causal role in disease severity (OR: 1.736, at 95% CI; 0.368-8.180). The wild type AA, on the other hand, may have a protective or no effect on the development of cancer in the diseased group (OR: 0.964, at 95% CI, 0.867-1.073). In addition, we also investigated the link between haplotype distributions and dysphagia grade, albeit based on the Odd's ratio (OR). The results revealed a positive correlation between the mutant TC of the VDR gene and dysphagia grade (OR 1.09; p=0.203, Table 4). Furthermore, with OR 1.394 and 1.736, haplotypes AA (GC Globulin) and GG (*CYP2R1*) also indicated a probable influence on the dysphagia grade severity of the studied cohort (Table 4). From the study of polymorphism of VDR, the effects were found in the metabolism, modulation of immune response, regulation of cell proliferation and differentiation. Vitamin D has a substantial function in overall human health, including cancer occurrence. The

polymorphism of GC-globulin influences its binding to vitamin D and its plasma metabolites and transports them to target tissues. he GC-globulin polymorphism influences macrophage phagocytotic activity during inflammation. This polymorphism also affects the macrophage activating factor (GcMAF) by the stepwise action of β-galactosidase of B cells and T cells sialidase. GcMAF could stimulate the phagocytotic activity of macrophages in inflammation. GcMAF has also shown anti-cancer activities in mice and may be a potential immunotherapeutic reagent for metastatic breast cancer (Paduraru et al., 2019).

In conclusion, the present study showed the association of VDR gene polymorphism in the progression and development of esophageal cancer. However, further studies with adequate sample size validation could accurately predict a definite result.

Author Contribution Statement

All authors contributed equally in this study.

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Ethical declarations

This study was approved by the institutional review board of the Gauhati Medical College and Hospital, Guwahati, Assam and all procedures performed in studies were in accordance with the ethical standards of the institutional research committee and with the 1964 Helsinki Declarations. All patients gave written informed consent.

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