RESEARCH ARTICLE

Single Nucleotide Polymorphisms in the *Gc* Gene for Vitamin D Binding Protein in Common Cancers in Thailand

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Abstract

Background: This case-control study aimed to determine if there were any associations between the two single nucleotide polymorphisms (SNPs) in Gc, rs7041 (Asp416Glu) and rs4588 (Thr420Lys) and 3 common cancers (breast, lung and colorectal) in Thai patients. Materials and Methods: Two hundred and eighty two colorectal, 101 breast and 113 lung cancer patients were recruited from one institute during 2011-2013. The controls were age-matched volunteers who had a negative history of index cancers. In addition, vitamin D levels were compared among different genotypes in the 2 SNPs. Results: The minor allele frequencies of rs7041 (G) and rs4588 (A) were 0.32 and 0.24, respectively. Under the dominant model, the study found significant associations between minor-allele genotypes of the SNP rs7041 (TG/GG) and lung cancer (odds ratio [OR] 1.78, 95% CI 1.05-3.03). When subgroup analysis was performed according to sex and age at diagnosis, the study found that the minor-allele genotypes of rs7041 (TG/GG) were significantly associated with colorectal cancer in patients whose age at diagnosis was more than 60 years (OR 1.67, 95%CI 1.06-2.61) and the minor-allele genotypes of rs4588 (CA/AA) were significantly associated with colorectal cancer in males aged 60 years or less (OR 2.34, 95%CI 1.25-4.37). When SNP combinations (rs7041-rs4588) were examined, the TT-CA combination had a significant protective association with lung cancer (OR 0.44, 95% CI 0.22-0.85). On evaluation of serum 25(OH)D levels in 205 individuals without cancer (males 144, females 61), the proportion of subjects with low serum vitamin D (< 20 ng/ml) in those harboring CA or AA genotypes of rs4588 (41.7%) was significantly higher than the CC genotype (15.5%, p-value < 0.01). Conclusions: Genetic polymorphisms in *Gc* were associated with lung and colorectal cancers in Thai patients. Lower serum 25(OH)D in minor variants of rs4588 may explain this association.

Keywords: Vitamin D - Vitamin D binding protein - colorectal cancer - lung cancer - Gc polymorphisms

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Introduction

Cancer is a significant health problem worldwide. In Thailand, in recent years, there has been an increasing incidence of breast, lung, colorectal, prostate, and hepatobiliary tract cancers (NCI, 2011). Lung and breast cancers remain the leading causes of cancer-related death in Thai males and females, respectively, and mortality from colorectal cancer is the second leading cause of death in both sexes (NCI, 2011).

Vitamin D has been reported to lower the risk of several cancers in humans (Mocellin, 2011). The cellular functions of vitamin D are mediated through the vitamin D receptor, an intracellular transcription-regulating factor that regulates the synthesis of proteins involved in bone mineral homeostasis and cell-cycle regulation (McCullough et al., 2007; Mocellin, 2011). In addition to its key role in maintaining skeletal homeostasis, emerging evidence suggests that vitamin D plays a role in reducing the risk of certain cancers, autoimmune diseases and hypertension (Borradale and Kimlin, 2009). The principle active metabolite of vitamin D, 1,25-dihydroxycalciferol [1,25$(OH)_2$D], is synthesized from 25-hydroxycholecalciferol [25(OH)D] in the kidney. Delivery of 25(OH)D to the kidney is facilitated by the vitamin D binding protein (DBP). As DBP carries vitamin D metabolites to various sites of action in the human body, the protein regulates bioavailability of the vitamin D precursor and thus the vitamin D level (Malik et al., 2013). A meta-analysis have reported that a variant genotype in Vitamin D receptor gene was associated with lower risk of colorectal cancer (Yu et al., 2014).

DBP is encoded by the *Gc* gene (synonym *DBP* gene), which belongs to the albumin family, together with human serum albumin and alpha-fetoprotein. Located on chromosome 4q11-q13, the *Gc* gene contains 13 exons, encompassing a 42.5 kilobase nucleotide length that encodes the 52-59 kDa DBP protein. The 458-amino-acid sequence is arranged in three domains with at least six non-synonymous single nucleotide polymorphisms

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(SNPs) (Speeckaert et al., 2006; Sinotte et al., 2009) and these polymorphic DBP proteins differ in their affinity for the 1,25(OH)2D metabolite (Pani et al., 2002). Two common coding SNPs, rs7041 (Asp416Glu) and rs4588 (Thr420Lys), have been found correlated with circulating vitamin D levels (Malik et al., 2013) and associated with several cancers in various populations (Borradale and Kimlin, 2009).

When the two SNPs rs7041-rs4588 are combined, there are 3 combination variants in the *Gc* Gc1s (G-C), Gc1f (T-C) and Gc2 (T-A). Each variant encodes DBP with different glycosylation patterns (galactose and sialic acid in both Gc1s and Gc1f; galactose alone in Gc2) (Abbas et al., 2008). These variants provide different binding affinities with vitamin D metabolites, all resulting in alteration of the plasma 25-hydroxyvitamin D level (Holick, 2007; Engelman et al., 2008; Ahn et al., 2010; Wang et al., 2010; Santos et al., 2013) and cancer risk (Mocellin, 2011). Selected variants in the *Gc* gene have been evaluated in studies on breast (McCullough et al., 2007) gastrointestinal (Zhou et al., 2012) and prostate cancers (Kidd et al., 2005). However, there has to date been no study evaluating their association with certain cancers in the Thai population. Our main objective was to determine if there was any association between the two SNPs in *Gc* and three common cancers, breast, lung and colon, in southern Thailand. In addition, the study also evaluated correlation between the genetic variants and serum vitamin D level in Thai people.

Materials and Methods

Subjects and sample collection

Two hundred and eighty-two colorectal, 101 breast and 113 lung cancer cases were recruited from patients in Songklanagarind Hospital, the major referral center in southern Thailand, during 2011-2013. The controls were a group of age-matched volunteers living in Songkhla and nearby provinces who had a negative history of index cancers, and no familial history of these cancers. The breast cancer controls were limited to females. Blood specimen collections and interviews were performed under informed consent. Ethical approval for this study was obtained from the Ethics Committees of the Faculty of Medicine, Prince of Songkla University.

DNA isolation and genotyping

Genomic DNA was isolated from peripheral blood leukocyte specimens using a QIAamp DNA Mini Kit (Qiagen, Hilden, Germany), following the manufacturer's protocol. Two non-synonymous SNPs in exon 11 of Gc, rs7041 and rs4588, were genotyped. The rs7041 and rs4588 SNPs change the amino acid sequences at codons 416 and 420 which result in a T to G transversion [an aspartic acid (Asp: GAT) to a glutamic acid (Glu: GAG)] and a C to A transversion [a threonine (Thr: ACG) to a lysine (Lys: AAG)], respectively. Genotyping was performed with Taqman genotyping assays on an ABI Prism® 7500 Fast Real-time PCR, ABI GeneAmp® PCR system 7500, using Applied Biosystems reaction system (ABI; Foster City, CA). The assay mixes (including unlabeled PCR primers, FAM™ and VIC® dye-labeled TaqMan MGB probes) of Assays-by-Design were designed and supported by ABI. The reaction contained 50 ng of genomic DNA, 10 µl of 2X TaqMan™ Genotyping Master Mix, 0.5 µl of 40x Assay Mix adjusted with Milli-Q H_2O in a total volume of 20 µl. The PCR conditions consisted of an initial step at 95ºC for 10 min, followed by 40 cycles at 95ºC for 15 sec and 60ºC for 60 sec in a 96-well plate that included negative (no DNA template) and positive controls to ensure genotyping accuracy. The genotyping results were analyzed by 7500 software version 2.0.5 and random samples were selected for confirmation by direct sequencing. (The primers and probes used in this study can be provided on request).

Table 1. Numbers of Cases And Controls Together with Mean Age (± standard deviation) and Sex Distribution

Cancer		Controls	Cases	*p*-value
Breast	Number	101	101	
	Mean age	49.6±11.81	49.8±10.51	0.91
	Sex: Male	0	0	N/A
	Female	101	101	
Lung	Number	113	113	
	Mean age	62.5±11.5	62.8±11.7	0.90
	Sex: Male	76	76	1.00
	Female	37	37	
Colorectal	Number	282	282	
	Mean age	57.7±16.7	62.0±14.5	<0.01
	Sex: Male	174	174	1.00
	Female	108	108	

Table 2. Genotype Distributions and Minor Allele Frequencies of the Single Nucleotide Polymorphisms (SNP) rs4588 and rs7041 on Gc, and *p*-values of Allelotype Association Studies

SNP	Controls	%	MAF (%)	Cases	%	MAF (%)	*p*-value
rs4588:							
Breast	N=101		27.72	N=101		19.31	0.11
CC	54	53.47		65	64.36		
CA	38	37.62		33	32.67		
AA	9	8.91		3	2.97		
Lung	N=113		26.11	N=113		21.24	0.45
CC	60	53.10		69	61.06		
CA	47	41.59		40	35.40		
AA	6	5.31		4	3.54		
Colorectal	N=282		21.81	N=282		20.74	0.53
CC	172	60.99		173	61.35		
CA	97	34.40		101	35.82		
AA	13	4.61		8	2.84		
rs7041							
Breast	N=101		31.68	N=101		36.63	0.52
TT	51	50.50		43	42.57		
TG	36	35.64		42	41.58		
GG	14	13.86		16	15.84		
Lung	N=113		27.88	N=113		35.84	0.10
TT	58	51.33		42	37.17		
TG	47	41.59		61	53.98		
GG	8	7.08		10	8.85		
Colorectal	N=282		32.45	N=282		37.23	0.26
TT	134	47.52		116	41.13		
TG	113	40.07		122	43.26		
GG	35	12.41		44	15.60		

MAF: minor allele frequency

Measuring serum 25(OH)D levels

To evaluate the influence of genotype variants on vitamin D levels, we measured serum level of 25(OH)D in 205 samples from the control group randomly selected by genotypes. Samples, calibrators, and quality controls were prepared according to the clinical laboratory standards of Songklanagarind Hospital (ISO15189). Briefly, 25 µl of the precipitation reagent and 200 µl of the internal standard were added to a 100 µl serum sample. After a 10-minute incubation, the sample was centrifuged at 15000 g for 5 min, and finally 200 µl of serum was transferred into a vial and 20 µl was injected into the LC-MS/MS system.

Statistical analysis

The required sample size was calculated using the comparison of two-proportion method. Descriptive statistics were used to compare demographic data between cases and controls. Statistical analysis of any associations between genotype frequencies of each SNP and the occurrence of disease and the agreement of genotype frequencies with Hardy-Weinberg equilibrium for each SNP were performed using Chi-square test. Odds ratios (OR) and 95% confidence intervals (95%CI) were calculated using unadjusted univariate logistic regression analysis. Unpaired Student's-t-test was used to compare serum vitamin D levels between genotypes. A *p*-value of less than 0.05 was considered statistically significant. All statistical calculations were performed with Stata version 13.1 (Texas, USA).

Results

Study population

The number of cases and controls in each cancer type, together with age and sex data, are shown in Table 1. No significant deviations from Hardy-Weinberg expectations were observed for polymorphisms rs4588 and rs7041 in either cases or controls. Linkage disequilibrium between the two SNPs was almost complete (D'=1). The ages of cases and controls were comparable in breast and lung cancers, however, the average age of the controls was significantly younger than that of the case group in the colorectal cancer patients.

Genotyping results

The Taqman SNP genotyping method used in this study gave a call rate greater than 99% and an accuracy rate of 100% when compared with genotypes derived by direct sequencing (Figure 1). Minor allele frequencies in the rs7041 and rs4588 SNPs were 0.32 and 0.24, respectively. The genotype distribution of the two SNPs in each cancer is displayed in Table 2.

Individual SNP analysis

When each individual cancer was analyzed, no significant disease association was found between the 2 SNPs and either colorectal or breast cancer. However, the rs7041:TG/GG group was found to have a disease association with lung cancer at an OR of 1.78 (95%CI 1.05-3.03). When individual genotypes were calculated, only TG had a significant association with lung cancer, at an OR of 1.79 (95%CI 1.03-3.10) (Table 3).

Considering age as a strong factor associated with cancer occurrence, subgroup analysis was performed according to sex and age at diagnosis using a cut-off at 60 years, and it was found that the dominant risk genotypes

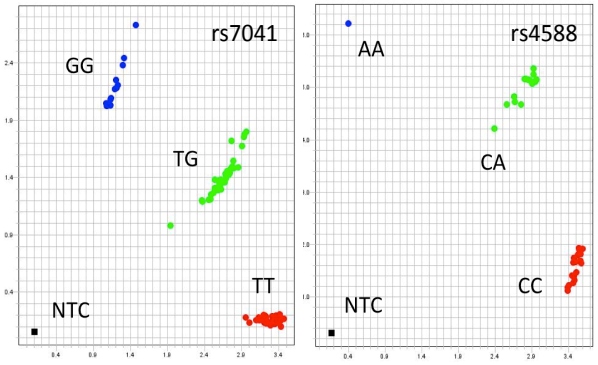

Figure 1. Examples of Allelic Discrimination Plots in rs7041 and rs4588 Analyzed by Taqman SNP Genotyping Method

Table 3. *Gc* Genotype Frequencies among Cases and Controls and their Risk Association with the Three Cancers Studied

Cancer type	Thr420Lys (rs4588 C/A)				Asp416Glu (rs7041 T/G)			
	Genotype	Controls/Cases	OR (95%CI)	*p*-value	Genotype	Controls/Cases	OR (95%CI)	*p*-value
Breast		101/101				101/101		
	CC	54/65	Reference		TT	51/43	Reference	
	CA	38/33	0.72(0.40-1.30)	0.28	TG	36/42	1.38(0.76-2.53)	0.29
	AA	9/3	0.28(0.07-1.07)	0.05	GG	14/16	1.35(0.59-3.09)	0.47
	p-trend			0.07	*p*-trend			0.30
Lung		113/113				113/113		
	CC	60/69	Reference		TT	58/42	Reference	
	CA	47/40	0.74(0.43-1.28)	0.28	TG	47/61	1.79(1.03-3.10)	*0.037
	AA	6/4	0.58(0.16-2.15)	0.41	GG	8/10	1.73(0.63-4.74)	0.29
	p-trend			0.21	*p*-trend			*0.04
Colorectal		282/282				282/282		
	CC	172/173	Reference		TT	134/116	Reference	
	CA	97/101	1.04(0.73-1.47)	0.85	TG	113/122	1.25(0.87-1.78)	0.23
	AA	13/8	0.61(0.25-1.51)	0.28	GG	35/44	1.45(0.87-2.42)	0.15
	p-trend			0.80	*p*-trend			0.10

OR; Odds ratios *p<0.05

Table 4. Genotyping Results for rs7041 and rs4588 in the *Gc* and Assignment to Combined *Gc* Genotypes (Abbas et al., 2008)

Combined genotype	Genotype		Breast			Lung			Colorectal		
	rs7041	rs4588	Controls/ Cases	OR(95%CI)	*p*-value	Controls/ Cases	OR(95%CI)	*p*-value	Controls/ Cases	OR(95%CI)	*p*-value
Gc1s-1s	GG	CC	8/11	1.42(0.55-3.69)	0.47	8/10	1.27(0.48-3.36)	0.62	29/42	1.50(0.92-2.53)	0.09
Gc1s-1f	TG	CC	21/32	1.77(0.93-3.34)	0.07	31/38	1.30(0.76-2.37)	0.31	81/78	0.95(0.66-1.37)	0.78
Gc1f-1f	TT	CC	25/22	0.85(0.44-1.62)	0.62	21/21	1.00(0.51-1.95)	1.00	62/53	0.82(0.54-1.24)	0.35
Gc2-1s (or Gc1f-x)	TG	CA	14/10	0.68(0.29-1.62)	0.38	16/24	1.63(0.81-3.27)	0.16	32/43	1.40(0.86-2.29)	0.17
Gc2-1f	TT	CA	18/19	1.06(0.52-2.18)	0.86	31/16	0.44(0.22-0.85)	*0.014	60/56	0.92(0.61-1.38)	0.68
Gc2-2	TT	AA	8/2	0.23(0.05-1.13)	0.052	6/4	0.65(0.18-2.38)	0.52	12/8	0.66(0.26-1.63)	0.36
Gc1s-x	GG	CA	6/4	0.65(0.18-2.39)	0.52	0/0	N/A	N/A	5/2	0.39(0.08-2.06)	0.25
Gcx-x	GG	AA	0/1	N/A	0.32	0/0	N/A	N/A	1/0	N/A	N/A
Gc2-x	TG	AA	1/0	N/A	0.32	0/0	N/A	N/A	0/0	N/A	N/A

OR; Odds ratios; * p<0.05

Table 5. Comparison of Serum 25(OH)D Level between Genotypes and Risk Association between Genotypes of *Gc* and Low Serum Level (<20 ng/ml) of 25(OH)D

SNPs (genotype)	n (%)	25(OH)D level				
		mean (ng/ml)	*p*-value	<20 ng/ml n (%)	≥20 ng/ml n (%)	*p*-value
rs4588						
CC	120 (58.54)	26.55	0.02	16 (13.33)	104 (86.67)	0.005
CA/AA	85 (41.46)	24.19		25 (29.41)	60 (70.59)	
rs7041						
TT	88 (42.93)	25.48	0.89	18 (20.45)	70 (79.55)	0.89
TG/GG	117 (57.07)	25.64		23 (19.66)	94 (80.34)	

of rs7041 (TG/GG) were significantly associated with colorectal cancers whose age at diagnosis was more than 60 years (OR 1.67, 95%CI 1.06-2.61, *p*-value 0.02). The CA/AA genotypes group of the rs4588 showed a positive association with colorectal cancer in males aged 60 years or less (ORs 2.34, 95%CI 1.25-4.37, *p*-value<0.01).

Genotype-combination analysis

Using our genotype data, 9 genotype-combinations could be constructed and named according to previously described electrophoretic variants (Malik et al., 2013; Abbas et al., 2008) (Table 4). The 3 most common variants found in our cases were Gc1s-1f, Gc1f-1f and Gc2-1f. When SNP combinations (rs7041-rs4588) were constructed and analyzed, the Gc2-1f combination (TT-CA) had a significant protective association with lung cancer (OR 0.44, 95%CI 0.22-0.85).

25(OH)D level

The serum levels of 25(OH)D were evaluated in 205 healthy volunteers (144 males and 61 females). The mean age of the volunteers was 35 years (range 17-87 years). The average level of 25(OH)D in the males (27.67 ng/ml) was significantly higher than in the females (20.68 ng/ml) (*p*-value < 0.01). In rs4588, the average 25(OH)D level in CC was significantly higher than in CA/AA (Table 5). Moreover, it was found that the proportion of cases with serum 25(OH)D less than 20 ng/ml was higher in those with CA/AA genotypes in rs4588. In rs7041, neither the average level of 25(OH)D nor the proportion of those with low serum 25(OH)D level showed any significant difference between genotype groups (Table 5).

Discussion

In this study, we examined 2 SNPs in *Gc* for their association with 3 common cancers in Thai patients. Our main findings were that the risk-genotypes (TG/GG) in rs7041 showed a disease association with lung cancer. The same rs7041 risk-genotypes group was associated with colorectal cancer when detected in patients over 60 years of age. Moreover, in males aged less than 60 years, the A-allele of rs4588 was significantly associated with a higher colorectal cancer risk. The minor allele frequencies of rs7041 (0.32) and rs4588 (0.24) were comparable with those reported in Asians in a large genome database (http://browser.1000genomes.org/) which reported frequencies at rs7041 at 0.29 and rs4588 at 0.28, and is also comparable with a previous report in Thai subjects (rs7041; 0.31 and rs4588; 0.19) (Chupeerach et al., 2012).

Disease associations have been demonstrated for rs4588 with various cancers including breast (McCullough et al., 2007), gastrointestinal (Zhou et al., 2012) and prostate (Kidd et al., 2005). However, other studies have found more inconsistent results (Poynter et al., 2010; Mahmoudi et al., 2014). Inverse relationship between serum vitamin D level and colorectal cancer risk has been demonstrated by a recent study (Weinstein et al., 2014). As the A allele in this SNP was associated with lower serum 25(OH)D in mainland Chinese (Zhang et al., 2013) and Chinese-Singaporean populations (Robien et al., 2013), it could be expected that the minor allele genotypes (CA/AA) would hold a higher risk of cancer development. Our study found that A-allele carriers in rs4588 had a significant risk of low serum 25(OH)D level, at least in

males. Taken together, the results from those previous works and our study could explain the correlation between genetic variants and lower serum 25(OH)D levels and the risk of colorectal cancer in our patients. This study chose to measure 25(OH)D level in young healthy volunteers with an attempt to reduce heterogeneity of subjects caused by cancer related change in nutritional status and senile related co-morbidity. The number of females from whom we measured serum 25(OH)D levels was not enough for a clear interpretation, however, the result suggest that the effect of the A-allele on serum 25(OH)D level may not be consistent with their male counterparts. This disparity between sexes needs further study, as it may explain the sex interaction in our disease-association model. The interaction with age in the rs4588 association in our colorectal cancers might be explained by the fact that, with aging, there are increasing senility factors associated with cancer occurrence, and those factors could overcome the effect of vitamin D level.

A significant association between the T allele in rs7041 and breast cancer has been reported in Caucasian women in Canada (Anderson et al., 2011). To our knowledge, our current study reports a significant association between rs7041 and lung cancer for the first time. According to recent genome wide association studies, the SNP is the strongest marker associated with the serum level of 25(OH)D (Ahn et al., 2010; Wang et al., 2010). A study in premenopausal women in Canada indicated that the T allele (a minor allele in that population) was associated with lower serum concentrations of 25(OH)D (Sinotte et al., 2009). Similar results were reported in a study in healthy Brazilian girls (Santos et al., 2013) and Chinese-Singaporeans (Robien et al., 2013). Moreover, a recent study in post-menopausal women in Thailand reported a higher 25(OH)D level in subjects homozygous for G in rs7041 (26.0 ng/ml compared to 23.5 ng/ml in TT/TG), although the difference did not reach statistical significance (Chupeerach et al., 2012). Our study found no difference in the 25(OH)D levels in rs7041 genotypes. However, it should be noted that most of the participants in the vitamin D level tests were young males instead of women in previous studies. We speculate that rs7041 may not have been directly associated with serum 25(OH)D levels in our subjects, and the lung cancer association we found with rs7041 might have been an effect of its linkage disequilibrium with the other SNPs. A study in black and white American suggested that polymorphisms in *Gc* are also associated with the level of DBP protein itself, which in turn affects bioavailability of free vitamin D (Powe et al., 2013). In terms of cancer risk, recent evidences have suggested that DBP level was a risk-modifier of 25(OH)D in colorectal (Anic et al., 2014) and prostate cancer (Weinstein et al., 2013).

Apart from the three cancers in our study, previous studies demonstrated association between *Gc* polymorphisms and other cancers in various populations although results remained inconclusive. Prostate cancer is among those with reported association in American (Taylor et al., 1996), Chinese (Bai et al., 2009) and Koreans (Ohi et al., 2014). In addition, *Gc* polymorphisms were demonstrated on a meta-analysis for their association with skin cancer susceptibility in European (Zhaoi et al., 2014). Interestingly, a risk genotype of *Gc* in one cancer can be a protective genotype in other cancers (Gandini et al., 2014).

A limitation of our study was the relatively low number of subjects, especially in the breast and lung cancer groups, which decreased the power of the study. In addition, our goal to have age-matched controls in the colorectal cancer group was not achieved because the majority of colorectal cancer patients were in an extreme age group. However, we have compensated for this weak point by subgroup analysis, and found disease association in both SNPs.

In summary, the study evaluated 2 SNPs in DBP for their association with 3 common cancers in Thai patients. The study detected significant disease associations between rs 7041 and lung cancer, and between rs4588 and colorectal cancer in young males. Additional study on 25(OH)D levels suggested that the associations are linked by differences in vitamin D levels.

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