Association of rs7041 and rs4588 SNPs of *Vitamin D Binding Protein (VDBP)* Gene and Common Cancers in Thai Patients

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ชื่อวิทยานิพนธ์ ความสัมพันธ์ของ สนิป rs7041 และ rs4588 บนยีน *Vitamin D Binding Protein (VDBP)* กับการเกิดโรคมะเร็งในประชากรไทย

ผู้เขียน นางสาววันวิสาข์ มณีฉาย

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บทคัดย่อ

การศึกษานี้มีวัตถุประสงค์เพื่อศึกษาความสัมพันธ์ระหว่างความหลากหลายของสนิปตำแหน่ง rs7041 (Asp416Glu) และ rs4588 (Thr420Lys) บนยีน *Vitamin D binding protein (VDBP)* กับโรคมะเร็งเต้านม, มะเร็งปอด และมะเร็งลำไส้ใหญ่และไส้ตรง โดยใช้ระเบียบวิจัยแบบ case-control ซึ่งจับคู่อายุและเพศ โดยกลุ่มผู้ป่วยประกอบด้วยมะเร็งลำไส้ใหญ่และไส้ตรง 282 ราย, มะเร็งเต้านม 101 ราย และมะเร็งปอด 113 ราย ทั้ง 3 มะเร็ง ใช้ผู้ป่วยจากโรงพยาบาลสงขลานครินทร์ ในช่วงปี 2011-2013 และในกลุ่มควบคุม เป็นอาสาสมัครที่อาศัยอยู่ในเขตจังหวัดสงขลาและพื้นที่ใกล้เคียง ซึ่งไม่มีประวัติเป็นโรคมะเร็งที่ศึกษา ทั้งในตัวผู้ป่วยเองและในสายตระกูล

ความถี่ minor allele ของ rs7041 (G) และ rs4588 (A) เป็น 0.32 และ 0.24 ตามลำดับ การศึกษาแบบ dominant พบความสัมพันธ์อย่างมีนัยสำคัญระหว่าง minor allele genotypes ของสนิป rs7041 (TG/GG) กับการเกิดโรคมะเร็งปอด (OR 1.78, 95% CI 1.05-3.03, *p*-value 0.032) ซึ่งไม่พบความสัมพันธ์กับโรค ใน genotypes อื่น แต่อย่างไรก็ตาม เมื่อทำการ subgroup และพิจารณาเพศและอายุร่วมด้วย พบความสัมพันธ์อย่างมีนัยสำคัญระหว่าง minor allele genotypes ของสนิป rs7041 (TG/GG) กับโรคมะเร็งลำไส้ใหญ่และไส้ตรง ในผู้ป่วยที่มีอายุมากกว่า 60 ปี (OR 1.67, 95% CI 1.06-2.61, *p*-value 0.023) และพบ minor allele genotypes ของสนิป rs4588 (CA/AA) มีความสัมพันธ์กับการเกิดโรคมะเร็ง ลำไส้ใหญ่และไส้ตรง ในผู้ป่วยเพศชาย อายุ 60 ปีหรือน้อยกว่า (OR 2.34, 95% CI 1.25 - 4.37, *p*-value 0.007) และเมื่อศึกษาด้วยการสร้าง genotype combination ระหว่างสนิปทั้งสอง (rs7041-rs4588) พบ TT-CA มี ความสัมพันธ์อย่างมีนัยสำคัญในเชิงป้องกันมะเร็งปอด (OR 0.44, 95% CI 0.22-0.85, *p*-value 0.014) และผลการศึกษา

ของระดับวิตามินดี ในกลุ่มควบคุม 155 ราย พบระดับวิตามินดีต่ำ ในกลุ่มควบคุมที่มี minor allele ของ rs4588 (CA/AA) อย่างมีนัยสำคัญ

บทสรุปของการศึกษาความหลากหลายบนยีน *VDBP* พบว่ามีความสัมพันธ์กับผู้ป่วยโรคมะเร็งปอด และมะเร็งลำไส้ใหญ่และไส้ตรง ในประชากรไทย ซึ่งระดับวิตามินดีที่ต่ำใน minor variants ของ rs4588 อาจจะอธิบายความสัมพันธ์นี้

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ABSTRACT

This study aimed to determine the association between single nucleotide polymorphisms (SNPs) in *Vitamin D Binding Protein (VDBP)* gene, rs7041 (Asp416Glu) and rs4588 (Thr420Lys), and 3 common cancers in Thai patients (breast, lung and colorectal) by using a case-control study design. Cases were 282 colorectal, 101 breast and 113 lung cancer patients recruited from Songklanagarind Hospital during 2011-2013. The controls were age-matched volunteers living in the same geographic region who had a negative history of index cancers.

The minor allele frequencies of rs7041 (G) and rs4588 (A) were 0.32 and 0.24, respectively. Under dominant model, the study found significant association between minor-allele genotypes of the SNP rs7041 (TG/GG) and lung cancer (odds ratio [OR] 1.78, 95% CI 1.05-3.03, *p*-value 0.032). When subgroup analysis was performed according to sex and age at diagnosis, the study found that the minor-allele genotypes of rs7041 (TG/GG) was 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, *p*-value 0.023) and the minor-allele genotypes of rs4588 (CA/AA) was significantly associated with colorectal cancer in males aged 60 years or less (OR 2.34, 95%CI 1.25-4.37, *p*-value 0.007). When SNP combinations (rs7041-rs4588) were constructed, the TT-CA combination had a significant protective association with lung cancer (OR 0.44, 95% CI 0.22-0.85, *p*-value 0.014). On evaluation of serum 25(OH)D level in 155 individuals without cancer, the level was significantly lower in those harboring minor-allele genotypes of rs4588 (CA/AA).

In conclusion, genetic polymorphisms in *VDBP* had association with lung and colorectal cancers in Thai population. Lower serum 25(OH)D in minor variants of rs4588 may explain this association.

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LIST OF ABBREVIATION AND SYMBOLS

˚C	=	Degree celsius
µg	=	Microgram
µl	=	Microliter
µm	=	Micrometer
µM	=	Micromolar
$1,25(OH)_2D_3$	=	Vitamin D_3
$25(OH)D_2$	=	25 hydroxyvitamin D_2
$25(OH)D_3$	=	25 hydroxyvitaminD_3
BLAST	=	Basic Local Alignment Search Tool
bp	=	Base pairs
C5a	=	Complement factor 5a
Chr	=	Chromosome
CI	=	Confidence Interval
CYP2R1	=	Cytochrome P450, family 2, subfamily R, polypeptide
CYP24A1	=	Cytochrome P450, family 24, subfamily A, polypeptide1
CYP27B1	=	Cytochrome P450, family 27, subfamily B, polypeptide1
CYP450	=	Cytochrome P450
DBP-*maf*	=	Vitamin D binding protein- macrophage activating factor
dH2O	=	Deionised sterile water
DNA	=	Deoxyribose nucleic acid

dNTPs	=	Deoxynucleotide Triphosphate
EDTA	=	Ethylenediaminetetraacetic acid
FAM	=	6-carboxy-fluorescein
Gc	=	Group-specific component
GWAS	=	Genome wide association studies
HWE	=	Hardy Weinberg
IS	=	Internal standard
LC-MS/MS	=	Liquid chromatography-mass spectrometry/mass spectrometry
LD	=	Linkage disequilibrium
M	=	Molar
MAF	=	Minor allele frequency
mg	=	Milligram
MGB	=	Minor groove binder
min	=	Minute
ml	=	Milliliter
mm	=	Millimeter
mM	=	Millimolar
NCBI	=	National Centre for Biotechnology Information
NEB	=	New England BioLabs
ng	=	Nanogram
nmol	=	Nanomole
PCR	=	Polymerase Chain Reaction

RT-PCR	=	Real Time Polymerase Chain Reaction
RXR	=	Retinoid X receptor
SNP	=	Single Nucleotide Polymorphism
TAMRA	=	6-carboxytetramethylrhoda mine
UV	=	Ultraviolet
VDBP	=	Vitamin D Binding Protein
VDR	=	Vitamin D receptor
VIC	=	4,7,2′-trichloro-7′-phenyl-6-carboxyfluorescein

CHAPTER 1
INTRODUCTION

Background and Rationale

Cancer is a significant health problem worldwide. In Thailand, common cancers that have increasing incidence belong to the breast, lung, colorectum, prostate, and hepatobiliary tract (NCI 2011). Among various cancers, lung cancer and breast cancer remain the leading causes of cancer-related death in Thai male and Thai female, respectively, when mortality from colorectal cancer was a problem in both sexes (NCI 2011).

Vitamin D has been reported to lower risk of several cancers in humans. 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 ML et al., 2007, Mocellin S et al., 2011). In addition to its key role in maintaining skeletal homeostasis, emerging evidences suggested that vitamin D play role in reducing the risk of certain cancers, autoimmune diseases and hypertension (Borradale D et al., 2009). The principal active metabolite of vitamin D, 1,25-dihydroxycalciferol [$1,25(OH)_2D_3$] is synthesized from 25-hydroxycholecalciferol [$25(OH)D_3$] in the kidney. Delivery of $25(OH)D_3$ to the kidney is facilitated by vitamin D binding protein (VDBP). As VDBP carries vitamin D metabolites to various sites of action in the human body, the protein regulates bioavailability of vitamin D precursor and thus vitamin D level (Malik S et al., 2013).

VDBP is encoded by the *GC* gene (synonym *VDBP* gene), which belongs to the albumin family, together with human serum albumin and α-fetoprotein. Located on chromosome 4q11-q13, the *GC* gene contains 13 exons, encompassing a 42.5 kilobase nucleotide length that encodes 52–59 kDa VDBP protein. The 458-

amino-acid sequence is arranged in three domains with at least six non-synonymous single nucleotide polymorphisms (SNPs) (Speeckaert M et al., 2006, Sinotte M et al., 2009) and polymorphic VDBP proteins differ in their affinity to $1,25(OH)_2D_3$ metabolite (Pani MA et al., 2002).Two common coding SNPs, rs7041 (Asp416Glu) and rs4588 (Thr420Lys) have correlation with circulating vitamin D levels (Malik S et al., 2013) and risk association of several cancers in various populations.

Review of Literatures

1. Cancer

Cancer is a major health problem. Data from the Ministry of Health in the last five years have shown that cancer is the leading cause of death which likely to increase every years (Figure 1). There are more than 100 types of cancer, including breast cancer, lung cancer, colon and rectum cancer, prostate cancer, and lymphoma etc. (Atlanta G , 2013). The most common cancer in males is colorectal cancer, followed by lung and liver cancer, respectively (Figure 2). And the most common cancer in females is breast cancer followed by cervical and colorectal cancer, respectively (Figure 3).

Cancer have characterized as an abnormal growth of cells, which then can invade adjoining parts of the body and spread to other organs. Cancer cells trying to escape different algorithms based on the theory of the hallmarks of cancer are growing endlessly. The hallmarks of cancer consist of six factors acquired during the several step of developments of human tumors. They include maintaining proliferative activation, evading growth suppression, cell death tolerance, angiogenesis induction, and activating invasion and metastasis (Hanahan D and Weinberg R.A.,2011). These hallmarks are genomic instability, which create the genetic diversity that expedites their acquisition, and inflammation, which enhance multiple hallmark functions. In this study we focusing on three cancers were breast cancer, lung cancer and colon cancer because were common cancer in Songklanagarind Hospital which a major referral center in southern Thailand.

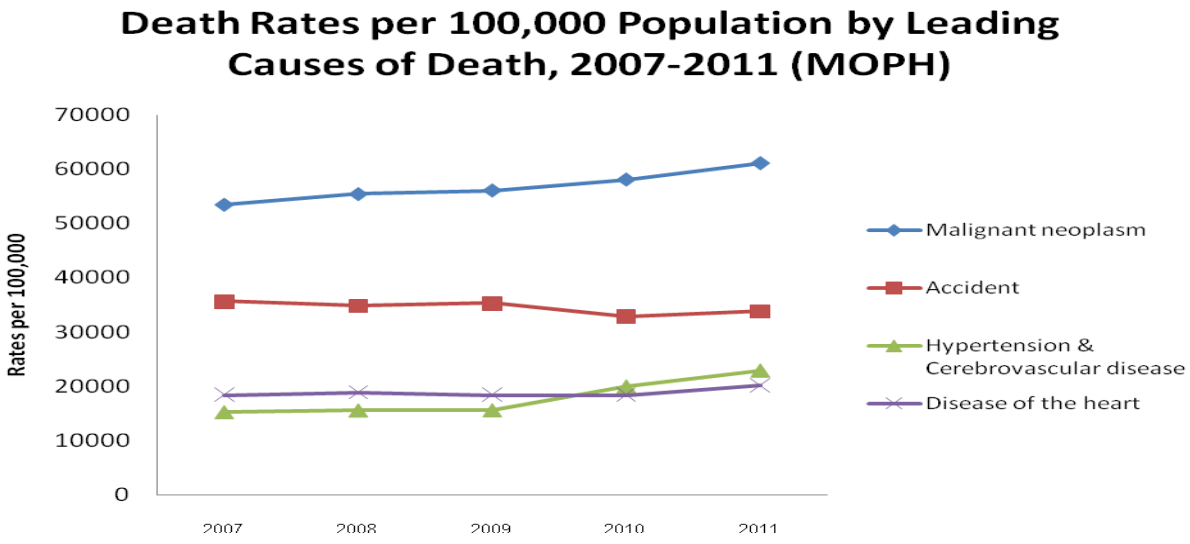

Figure 1: Death rates per 100,000 population by leading causes of death, 2007-2011 (Health Information Unit, Bureau of Health Policy and Strategy)

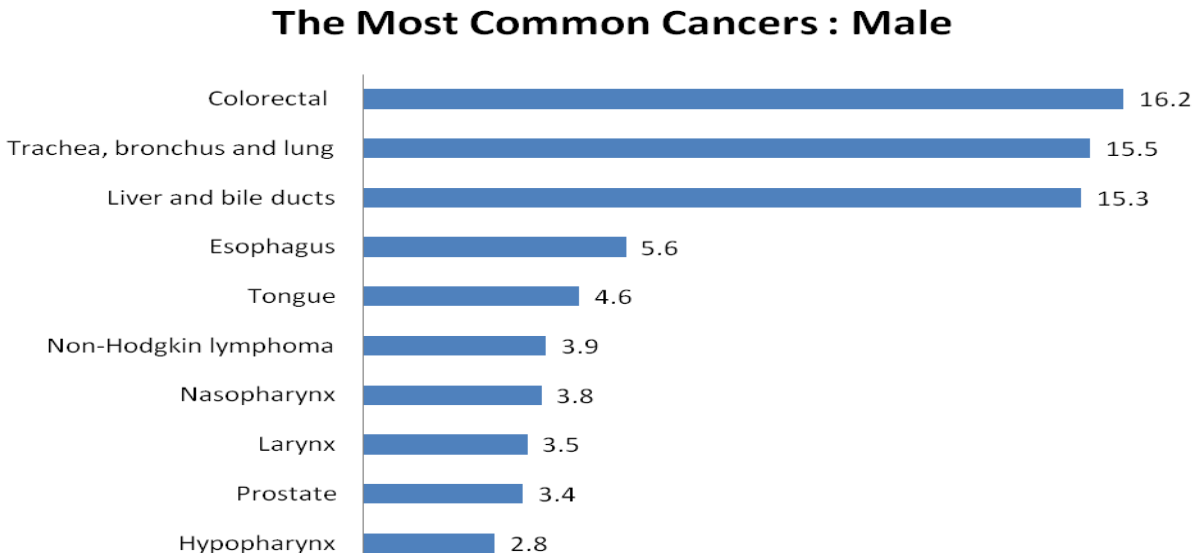

Figure 2: Common cancer in Thai males (National Cancer Institute, Ministry of Public Health; July 2011)

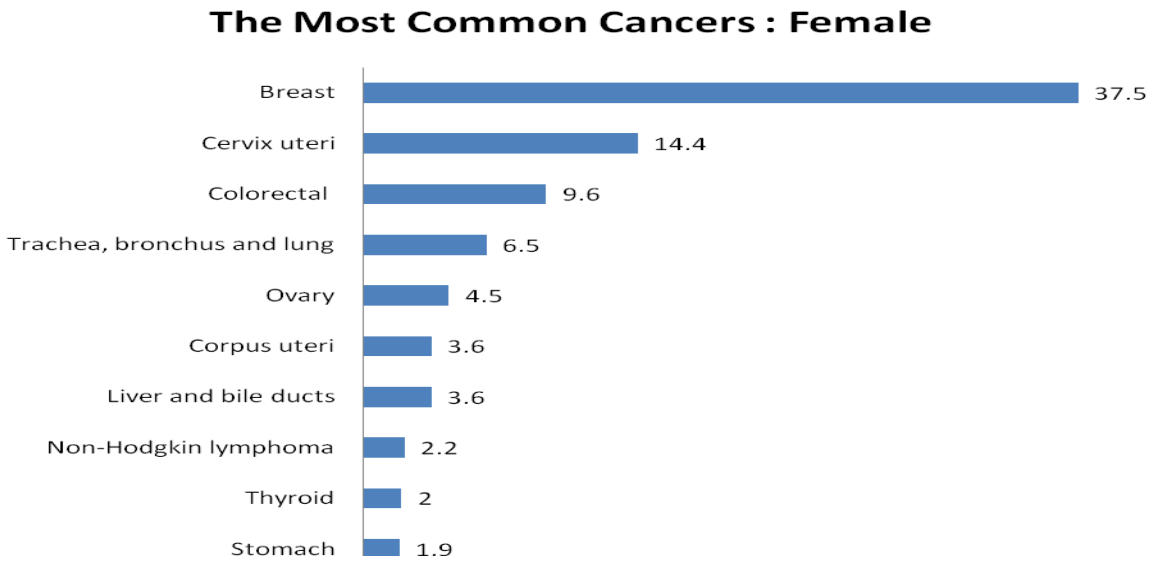

Figure 3: Common cancer in Thai females (National Cancer Institute, Ministry of Public Health; July 2011)

1.1 Breast Cancer

Breast cancer is the most diagnosed cancer, and the second most cause of cancer-related deaths in females (Atlanta G, 2008). Risk factors include hereditary, long menstrual history, high breast tissue density, hormone, reproductive factors, physical inactivity, high BMI, alcohol consumption and weight gain during adulthood (for postmenopausal breast cancer).

The two main breast cancer types include:

- Ductal carcinoma, which starts the breast tube to the nipple. This is a most breast cancers type.
- Lobular carcinoma, which starts in lobules, milk production gland.

1.2 Lung cancer

Lung cancer is malignant neoplasm characterized by the outgrowth of cells in one or both lung tissue. These cells do not maintain the functions of lung cells and cannot develop into normal lung tissue. When the tumor cells grow, they will form a solid tumor and interfere the function of the lung, which provides oxygen to the body via the blood .There are two main types of lung cancer.

Non-small cell lung cancer (NSLC)

NSLC is the most common type of lung cancer comprising about 85% of the patients (Julian R et al., 2008). This type of cancer can be subtyped into squamous cell carcinoma, adenocarcinoma and large cell carcinoma.

. **Small cell lung cancer (SCLC)**

Small cell lung cancer or oat cell cancer is found in about 10%-15% of lung cancers patients (Julian R et al., 2008). This lung cancer type likely has a rapid growth property with quickly spread.

The most important risk factor for lung cancer is smoking. Moreover, the secondhand smoke can affect the nonsmokers on the increased risk of developing lung cancer. Other environmental substances or exposures that can also increase the risk of cancer including tuberculosis, industrial substances, radon, asbestos, air pollution, and genetics (Boffetta P, 2013, Hubaux R et al., 2012).

1.3 Colorectal Cancer

In Thailand**,** colorectal cancer is the most common cancer in males and third in females (National Cancer Institute, Ministry of Public Health; July 2011). Colorectal cancer is one of the common causes of cancer-related deaths. The cancer pathologically starts in the colon or the rectum. Early diagnosis can often lead to a complete cure. Almost all colon cancers start in glands lining in the colon and rectum. The factors increasing a risk of colorectal cancer include:

- Older than 60 years
- Meat over consumption
- Have a polyp
- Inflammatory bowel disease
- Hereditary factors

2. Vitamin D

Vitamin D is an essential precursor for many steroid hormone such as calcitriol. Therefore, should be referred to as a prohormone. The main source of vitamin D is from dietary existing in two forms: vitamin D_3 (cholecalciferol), which presents in animal sources (e.g., meat, eggs, fish), and vitamin D_2 (ergocalciferol), which present in plant sources, which most of the vitamin D are produced in the skin by the sunlight energy (UV) (Borradale D and Kimlin M.G., 2009). Pre-vitamin D_3 is formed by 7-dehydrocholesterol in the skin, catalyzed by UV light. The unstable *cis*-isomer of vitamin D_3 is rapidly changed to the a more stable form, vitamin D_3, which is then bound to and transported by the vitamin D binding protein (VDBP) into the circulation. Vitamin D_3-bound DBP is transported to the liver to be hydroxylated by the mitochondrial cytochrome P450 enzyme, 25 hydroxylase (CYP27A1) leading to generation of 25-hydroxyvitamin D_3 ($25(OH)D_3$) form which is the major circulating type of vitamin D_3 (Hansen C.M et al., 2001). The production of $25(OH)D_3$ is likely uncontrolled and has a correlation with substrate availability rather than with physiological needs. Therefore, evaluating the $25(OH)D_3$ levels in circulation system provides a useful indicator of the actual vitamin D status in the body, which is widely applied in clinical use.

Vitamin D_3 has been associated with a lower risk of several cancers in humans (Hansen CM et al., 2001). The function of vitamin D_3 is mediated through the vitamin D receptor (VDR), a transcription factor that signals the synthesis of proteins involved cell-cycle and bone mineral homeostasis control (McCullough ML et al., 2007, Mocellin S, 2011). Vitamin D_3 deficiency is a known cause of osteopenia in children and adults. In addition, emerging evidence has suggested that vitamin D_3 may play a role in decresing the risk of some cancers, hypertension and autoimmune diseases (Borradale D and Kimlin M, 2009). Vitamin D binding protein (VDBP) is thought to regulate the bioavailability of 25-hydroxycholecalciferol [$25(OH)D_3$] acting as the main transporter from liver to kidney for the synthesis of the principal active metabolite, 1,25-dihydroxycalciferol [$1,25(OH)_2D_3$]. Alternatively, $25(OH)D_3$ delivered to the kidney may undergo catabolic hydroxylation there to the considerably

less active 24,25-dihydroxycalciferol [24,25$(OH)_2D_3$] metabolite (Figure 4) (Deeb KK et al., 2007), which is then subjected to further degradation and renal excretion (Malik S et al.,2013).

Although the mechanisms of 1,25$(OH)_2D_3$ in the anti-cancer effects still have to be identified, it is now accepted that several signaling pathways may be associated. Some of which can directly regulate target molecules. The anticancer property of vitamin D_3 begins from the ability of vitamin D_3 to affect the expression of genes involved in the regulation of cell proliferation, angiogenesis and induced apoptosis, which are the three key mechanisms of cancer tumorigenesis and progression**.**

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2.1 Anti-proriferation

Vitamin D_3 (1,25$(OH)_2D_3$) inhibits the proliferation of many malignant cells by inducing cell cycle arrest and the accumulation of cells . Claudia G, et al. reported an experiment that synchronized cells were treated with 1,25$(OH)_2D_3$, and EtOH were added, and cells were prepared for fluorescence-activated cell sorting (FACS) analysis, and measured at indicated time points (0, 18, 24, 36, 48 and 72 hr). In squamous cell carcinoma cell lines (SCC), addition of 1,25 $(OH)_2D_3$ resulted in an accumulation of cells in the G_0/G_1 phase, resulting in growth reduction in SCC25, JPPA and HaCaT, after 48 hr. of incubation and was visible until 72 hr., detected by trypan-blue staining. SCC9 cell count was decimated after treatment with 1,25$(OH)_2D_3$ after 72 hr. The viability of cells was permanently 90% (Gedlicka C et al.,2006). Gengfei W. et al. showed concentration dependent inhibition of human breast cancer cells by 1,25$(OH)_2D_3$ and EB1089 (a synthetic analog of vitamin D_3). Breast cancer cell lines were plated in 24 well tissue culture plates at a density of $1.56x10^4$ cells/well, followed by addition of different concentrations of 1,25$(OH)_2D_3$ or EB1089, as indicated. Cells were cultured for 96 hr. and assayed by [^{3}H] thymidine incorporation. The result suggested that breast cancer cell lines showed differential

concentration responses to the compounds. On study of concentration dependence, exponential MCF-7 E and MCF-7 L cells were treated for 96 hr. with increasing concentrations of EB1089 as indicated. On. Western blot analysis of the specific proteins was Cyc A, Cyc E, Cdk2, p27 and p21, alterations in the expression profiles of cell cycle proteins described above indicated that mechanisms by which Cdk2 kinase and Cyc A activity decreased in EB1089 treated MCF-7 E cells was complicated, an increase in p21 and No increase of p27 protein levels (Wu G rt al., 1997).

2.2 Anti-angiogenesis

Vitamin D is a powerful inhibitor of cancer-associated angiogenesis in preclinical trial (Iseki K et al., 1999). Calcitriol can inhibit VEGF-induced endothelia cell tube formation *in vitro* and reduce tumor vascularization *in vivo* in VEGF overexpressed breast cancer bearing mice (Mantell DJ et al., 2000). Kazushige ISEKI et al. also demonstrated the effects of 1-hydroxyvitamin D_3[1α(OH)D_3] and 1,25$(OH)_2D_3$ on the incidence, labeling index and angiogenesis of colon cancer, which is induced by azoxymethane in Wistar rats. Administration of 1α(OH)D_3 and 1,25$(OH)_2D_3$ at higher doses significantly decreased the labeling index, microvessel counts and vascular endothelial growth factor in colonic tumors. Their findings suggested that both 1α(OH)D_3 and 1,25$(OH)_2D_3$ can inhibit the development of colonic tumors (Iseki K et al., 1999).

2.3 Induction of apoptosis

Vitamin D can cause apoptosis in cancer cells. According to Sarah E.et al., calcitriol also induces apoptosis of LNCaP cells (Sarah EB 2000). Bcl_2 family members are critical regulators of apoptosis, which could be measured by Western blotting to study the expression of Bcl_2, Bax, Bcl-X_L, Bcl-X_S, and Mcl-1 as a function of treated with ethanol or 100 nM calcitriol. A down-regulation of Bcl_2 and Bcl-X_L proteins which protect cells from undergoing apoptosis by calcitriol was

accompanied. Other important proteins in apoptotic control such as Bax, Mcl-1, and Bcl-X_S, are unaffected by calcitriol treatment (Sarah EB et al., 2000). Moreover, the cultured MCF-7 cells treated for 4 days with vehicle and 100 nM CB1093 (a synthetic analog of vitamin D_3) were dead from apoptosis because of the interaction between CB1093 and IGF-I. (Xie SP et al., 1999). In addition, Bcl_2 overexpression has been shown to block the pro-apoptosis activity of vitamin D_3 in both prostate cancer cells and breast cancer cells, supporting the important role of Bcl_2 protein family in vitamin D mediated apoptosis (Sarah EB et al., 2000, Xie SP et al., 1999).

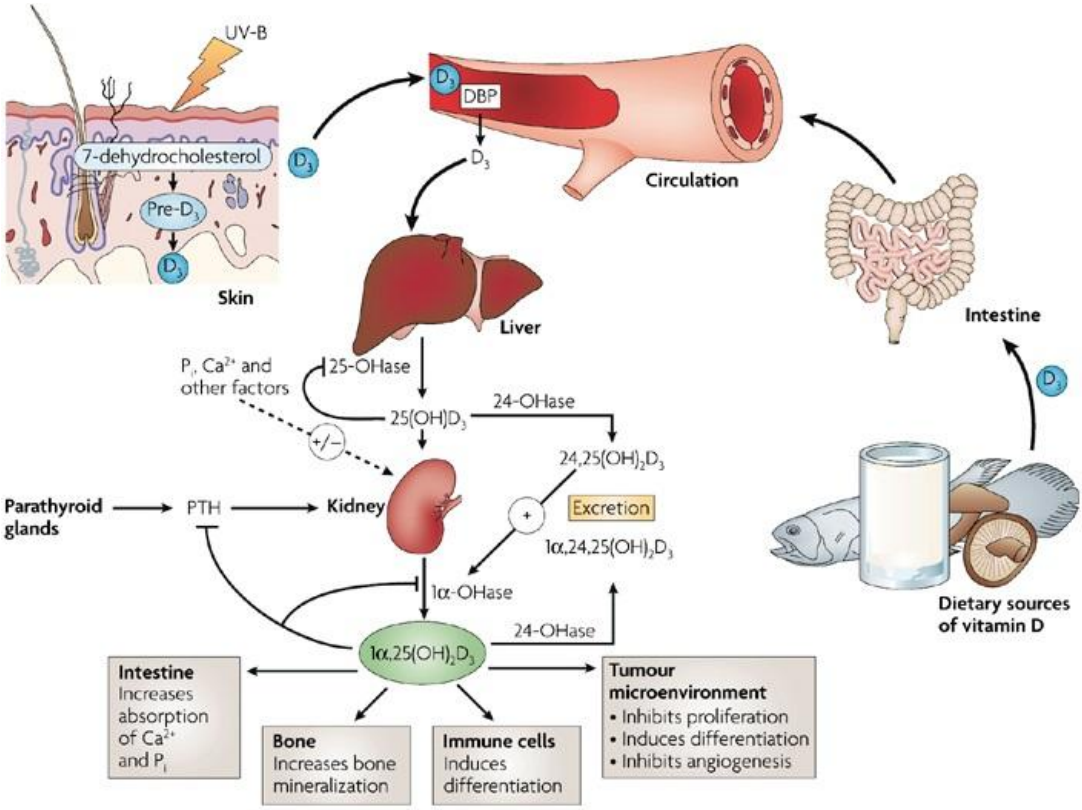

Figure 4: Photochemical synthesis of vitamin D_3 (Deeb KK et al., 2007)

3. Vitamin D binding protein

Vitamin D binding protein (VDBP) is a protein, which has recently achieved increasing attention. DBP is recognized as a member of a multigene family including albumin, α-fetoprotein and α-albumin/afamin. DBP is generated in liver as a single peptide chain and functions in transportation of vitamin D_3. According to Sinotte M et al. in 2009, the *GC* gene, which coding human *GC*, is polymorphic. There are two common single nucleotide polymorphisms (SNP) Thr420Lys (rs4588) and Asp416Glu (rs7041) in this gene. Thr420Lys and Asp416Glu have been demonstrated to change plasma levels of 25(OH)D_3 in candidate gene studies (Sinotte M et al., 2009). In previous reports, selected genetic variants in the *GC* gene, including Thr420Lys and Asp416Glu, have been investigated in breast cancer (Anderson L.N et al., 2011), prostate cancer (Kidd L.C et al., 2005), colorectal cancer (CRC) (Poynter J.N. et al., 2010) and basal cell carcinomas (Flohil S.C et al., 2010). To date, there is, however, no systematic evaluation on how common Thr420Lys and Asp416Glu SNPs are associated in development of gastrointestinal cancers. In 2012, Liqing Zhou and colleagues showed that *GC* Asp416Glu or Thr420Lys polymorphisms are associated with risk of gastrointestinal cancer development. In the study of Chinese Han case-control cohort (964 incident patients with gastrointestinal cancers and 1187 control subjects), the researchers illustrated that Thr420Lys *GC* polymorphism has significant effect on the risk of developing colorectal cancer. Additionally, subjects who was carrying *GC* Asp416 (T) - Lys420 (A) haplotype, which contains the *GC* at-risk 420Lys allele, also shown significant increased risk gastrointestinal tumorigenesis (Zhou L et al., 2012) shown in Table 1.

The non-synonymous rs7041 (T/G transition asparticè to glutamic acid) and rs4588 (C/A transition threonineè lysine) polymorphisms are located in exon 11 of the *VDBP* gene (Speeckaert M et al., 2006). Differences in coding of amino acids of the *VDBP* gene variants (rs7041-G and rs4588-A) were found to either directly affect the concentration of VDBP or its affinity for vitamin D_3 metabolites thus influencing 25(OH)D_3 availability (Sinotte M et al., 2009). Furthermore, both rs7041 and rs4588 exhibit differences in binding properties among themselves. The

rs7041 variant is significantly associated with higher levels of serum 25(OH)D_3 and increased affinity to vitamin D_3 metabolites compared to rs4588 variant (Abbas S et al., 2008).

3.1 Vitamin D Binding Structure

VDBP is the protein encoded by the *GC* gene (synonym *VDBP* gene). It is located on chromosome 4q11-q13. Product of the *GC* gene, the VDBP protein binds to and transports vitamin D to target organs (Sinotte M et al., 2009). There are two common coding single nucleotide polymorphisms (SNP) in exon 11 of the *VDBP* gene, at codons 416 (T>G) and 420 (C>A) (Eloranta JJ et al., 2011).

There are three common alleles (Gc1s, Gc1f and Gc2) shown in Table 1 and more than 120 variants of the *GC* system in the human population. The Genetic variants of the *vitamin D-binding protein* gene (*VDBP*). The transcript consists of 13 exons with flanking untranslated regions at both ends and a key enhancing motif (DNase Hypersensitivity Site IV) in intron 1 shown in Figure 5 (Malik S et al., 2013).

Table 1 : Common *GC* alleles in the vitamin D binding protein and their glycosylation pattern (Abbas S et al., 2008)

***GC* allele (haplotype)**	**rs7041 codon (amino acid)**	**rs4588codon (amino acid)**	**Glycosylation pattern**
Gc1s	G(Glutamin acid)	C (Threonine)	galactose and sialic acid
Gc1f	T (Aspartic acid)	C (Threonine)	galactose and sialic acid
Gc2	T (Aspartic acid)	A(Lysine)	galactose only

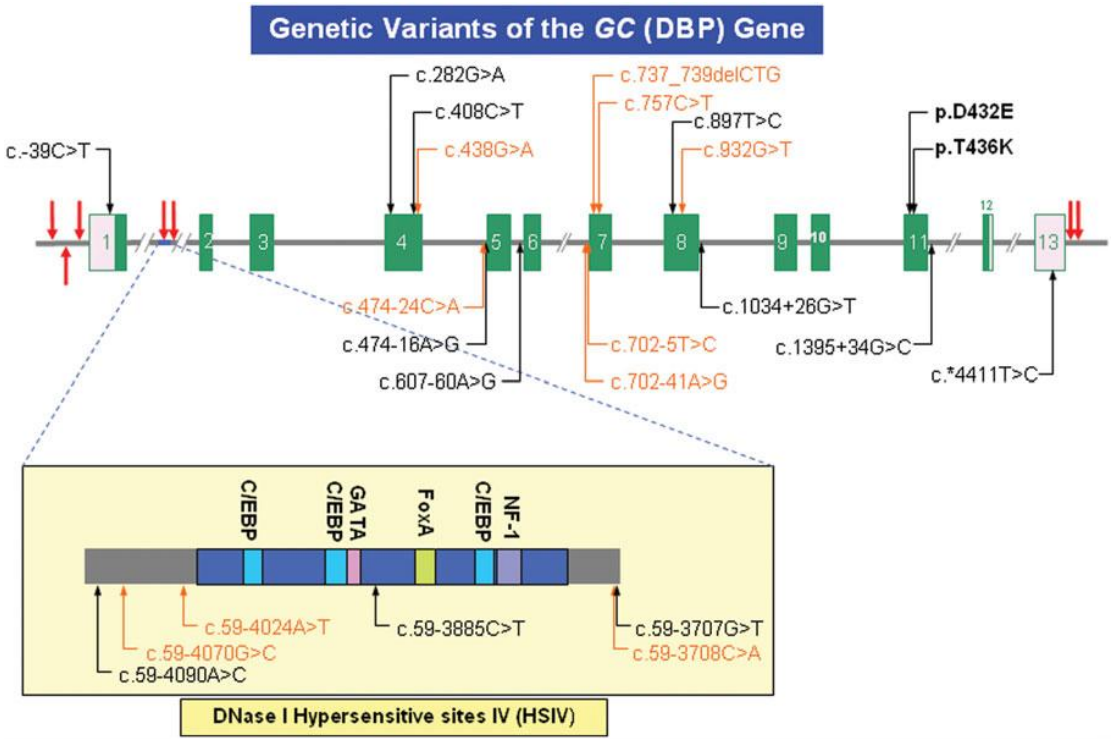

Figure 5: Genetic variants of the *vitamin D-binding protein* gene (*VDBP*). The variant shown in this schematic are the 13 exons (Malik S et al., 2013).

Table 2: Association between common genetic variants of the vitamin D binding protein gene (GC) and cancers

Cancer	Referance	Sample size (N)		Country	Ethnicity	GC polymorphisms studied	Findings
		Cases	Controls				
Breast Cancer	Abbas et al.(2008)	1402	2608	Germany	Caucasiana	Gc1F, Gc1S, Gc2	Genotype Gc2-2 was associated with decreased risk of postmenopausal breast cancer, [OR=0.72 (CI=0.54–0.96, p=0.04)], when compared to the most frequently observed genotype, Gc1S-1S. Gc2 carriers had an OR of 0.88(CI=0.77–1.01, p=0.02) compared to non-carriers.
Breast Cancer	Anderson et al.(2011)	1560	1633	Canada	Caucasian	rs7041, rs4588	TT genotype of rs7041 was associated with breast cancer, OR=1.23 (CI=1.01–1.51, p<0.05). Resulting genotype Gc2-2 had OR=1.22 (CI=0.93–1.59), when compared to genotype Gc1-1.
Gastrointestinal	Zhou et al.(2012)	964	1187	China	Han Chinese	D432E, T436K	Homozygous KK genotype of T436K had elevated risk for colorectal cancer in comparison to 436 T/T genotype, OR=3.41 (CI=1.85–6.57, p<0.001). When all gastrointestinal cancer types were combined, KK genotype had 1.15 fold increased risk over T/T (CI=1.02–1.30, p=0.020) after adjustment for age, sex and smoking status. Carriers of D432- 436K haplotype (associated with Gc2 allele) also had an increased risk for developing gastrointestinal cancer,OR=1.22 (CI=104–1.39,p=0.015)
Prostate Cancer	Dimopoulos et al.(1984)	115	155	Greece	Caucasian	Gc1F, Gc1S, Gc2	Increased disease risk for carriers of Gc2 allele; RR=1.81 (p<0.01)

3.2 Functions of Vitamin D Binding Protein

The human *VDBP* gene or *GC* gene encodes the plasma protein responsible for the binding and transport of vitamin D_3 and its metabolites to target cells (Speeckaert et al., 2006, Wang et al., 2010). The DBP is secreted from hepatocytes as a polymorphic glycoprotein that constitutes one of the most abundant serum proteins (Hiroki et al., 2006). Despite its main function in the mobilization of vitamin D_3 metabolites, VDBP has been found to modulate certain immune and inflammatory responses including hemotaxis, actin scavenging and macrophage activation (DiMartino and Kew , 1999).

3.2.1 Vitamin D binding

The main function of vitamin D binding protein is to solubilize and transport vitamin D and its metabolites. The principal component of vitamin D_3, binds VDBP about 88% and $1,25(OH)_2D_3$ bind VDBP about 85% (Speeckaert M et al., 2008, White P and Cooke N, 2000).

3.2.2 Actin scavenging action

The polymerization of G-actin into F-actin leading to the obstruction of blood vessel and organ dysfunction. VDBP and gelsolin, members of the extracellular actin scavenger system, can co-operate to protect the body from these events by binding to G-actin, resulting in the inhibition of filament formation (White P and Cooke N, 2000, Lee WM and Galbraith RM , 1992).

3.2.3 Fatty acid transport

VDBP can mainly bind to saturated and unsaturated fatty acids. Less than 5% of the total amount of fatty acids (Ena JM et al., 1989). The affinity of VDBP to bind $25(OH)D_3$ and $1,25(OH)_2D_3$ is decreased by unsaturated fatty acids, but is unaffected by saturated fatty acids (Bouillon R et al., 1992).

3.2.4 VDBP-macrophage activating factor (GcMAF)

The GC protein contains one trisaccharide composed of N-acetylgalactosamine with dibranched galactose and sialic acid at codon 420 threonine residue. This saccharide can be hydrolyzed by the inducible membranous B cells and T cells to produce a macrophage activating factor (MAF), which can activate macrophage to present the antigen to B and T lymphocytes (Yamamoto N., 2008). Thus, GC protein is the precursor of MAF activity in cancer patient is reduced or absent, because the deglycosylation of their serum GC protein by α-N-acetylgalactosaminidase (Nagalase), which is secreted from cancer cells.

3.2.5 Chemotaxis

The VDBP have a comparable co-chemotactic activity which is released from tissue damaged sites and also exerts a chemotactic function on vascular smooth muscle cells and acts as a growth factor. $25(OH)D_3$ and $1,25(OH)_2D_3$ inhibit this activity by competing binding site on VDBP (Raymond MA , 2005).

On combining the two SNPs rs7041-rs4588, there are 3 combination variants in the VDBP including Gc1s (G-C), Gc1f (T-C) and Gc2 (T-A). Each variant encodes VDBP with different glycosylation patterns (galactose and sialic acid in both Gc1s and Gc1f; galactose only in Gc2) (Abbas S et al., 2008). These variants provide different binding affinity to vitamin D metabolites, which results in alteration of plasma 25-hydroxyvitamin D level (Ahn J et al., 2010, Wang TJ et al.,2010, Holick MF et al., 2007, Santos BR et al., 2013, Engelman CD et al., 2008) and cancer risk (Mocellin S et al., 2011). Selected variants in *Gc* gene have been evaluated in studies on breast (McCullough ML et al., 2007) gastrointestinal (Zhou L et al., 2012) and prostate cancers (Kidd LC et al., 2005).

However, there is no report on their association with certain cancers within the Thai population.

Objectives

1. To study the association of rs7041 and rs4588 SNPs on *Vitamin D Binding Protein* gene with the occurrence of common cancers in Thailand.
2. To study the association between rs7041 and rs4588 SNPs and Vitamin D level in Thai**.**

CHAPTER 2
RESEARCH METHODOLOGY

1. Materials and Reagents

1.1 Reagents

Absolute Ethanol
Acetic acid
Agarose gel
Ammonium chloride
Bromophenol blue
Disodium Hydrogen Phosphate
Distilled water
EDTA
Ethidium Bromide
Isopropanol
PBS buffer
Potassium chloride
Potassium Dihydrogen Phosphate
Sodium Chloride
Sucrose
TE buffer
Tris acetate

1.2 Commercially provided kits

DNA ladder 100 bp (New England Biolabs)
dNTP mix (Qiagen)

FlaxiGene DNA kit (Qiagen)

HotStarTaq DNA polymerase (Qiagen)

QIAquick PCR Purification Kit (Qiagen)

Taqman® Genotyping Master Mix (Life Technologies)

1.3 Scientific instruments

7500 Fast Real time PCR (Applied Biosystems®)

Auto micropipettes

Freezer -80 ^{o}C

Freezer -20 ^{o}C

Gel Document (Major Science)

Gel electrophoresis system (Mupid-Exu)

Liquid Chromatography

Mass Spectrophotometry

Microcentrifuge (Hitachai)

Mini PCR plate spinner (Labnet)

Mini spin down (Biosan)

Nanodrop 2000 Spectrophotometer (Thermo Fisher Scientific)

Pipet tips

Refrigerated Centrifuge (3780 Kubota, Dynamica)

Refrigerator +4 ^{o}C

Sequencer

Temperature Shaker (Biosan)

Thermo Cycler PCR (S1000 BioRad)

Vortex (Genie)

2. Methods

2.1 Participants

Sample size calculation

For sample size, the two proportion score was used to estimate the number of samples.

$$n = \frac{\left\{ z_{\frac{\alpha}{2}} \sqrt{2P(1-P)} + z_{\beta} \sqrt{P_1(1-P_1) + P_2(1-P_2)} \right\}^2}{(P_1 - P_2)^2}$$

When

$z_{\frac{\alpha}{2}}$ = 95% confidence (1.96)

z_{β} = Type II-error not more then 20% (0.84)

P_1 = Proportion of study outcome in the first group (minor allele frequency case)

P_2 = Proportion of study outcome in the second group (minor allele frequency control)

$$P = \frac{(P_1 + P_2)}{2}$$

However, the actual sample sizes used in this study were depended on an availability of collected samples, as described in the following section.

Ethics Statement

This study was approved by the Research Ethics Committee Faculty of medicine Songklanagarind University, and written. Informed consent was obtained from each subject and each participant was then interviewed to collect the information on demographic characteristics.

Case – Control Sample

Healthy control individuals and cancer patients were invited to participate in this case-control study. Cancer cases consist of 282 patients with colorectal cancers, 101 patients with breast cancers and 113 patients with lung cancer. All were recruited from patients in Songklanagarind Hospital, a major referral center in southern Thailand, during the year 2011-2012. Controls were cancer-free volunteers in three districts of Songkhla province and Blood Bank unit, Department of Pathology, Faculty of Medicine, Prince of Songkla University. Controls selected to be age-matched to the case. Numbers for the control group were 101 for breast cancer, 113 for lung cancer and 282 for colorectal cancer. All individuals had no history of cancer confirmed by interviews. Collecting blood specimens and interviews were performed under informed consent.

2.2 Blood Collection

Whole blood samples

We collected approximately 3 ml of blood which is carefully transfer from the syringe to the EDTA tube and gently inverted 2-3 times to thoroughly mix the anticoagulant with the blood. We used white blood cells from whole blood by centrifuge the sample at 4^{o}C and 2000 g for 10 minutes prepare for DNA extraction.

Serum samples

We collected approximately 3 ml of blood to preserve for measure $25(OH)D_3$ and let the tubes stand at room temperature about 1 hr. The sample were centrifuged at 2000 rpm for 10 minutes. The clear serum was retrieved (Figure 2) and freezed at -80 ^{o}C.

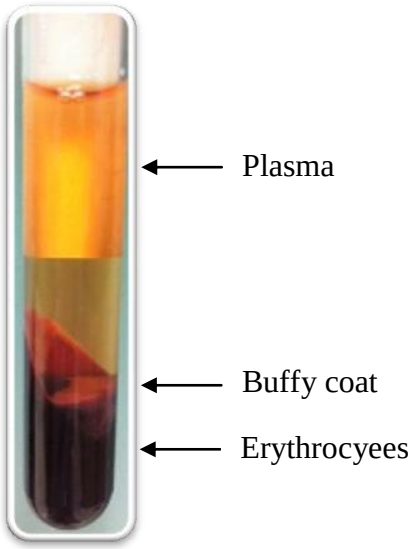

Figure 1 : Schematic of centrifuged whole blood showing different cell fractions

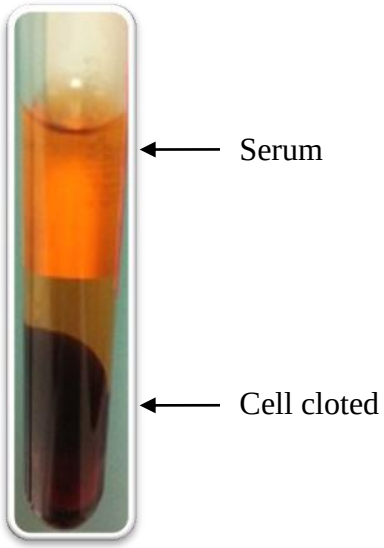

Figure 2 : Schematic of centrifuged whole blood showing serum and clot blood

2.3 DNA extraction

Genomic DNA was extracted from buffy coat of whole blood and the pellet was washed with 1 ml of PBS centrifuged at 10000 rpm for 1 min. The supernatant was discarded again and the pellet cell were used for DNA extraction by the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany), according to an enclosed manufacturer's protocol. Briefly, the pellet cell was transferred into 1.5 ml tube and adds 180 µl of ATL buffer. Next, 20 µl of proteinase K was added and mixed by vortex, followed by 10 min incubation at 65 °C. 200 µL of AL buffer was added to the mixture, which was mixed by vortex for 15 s. After that, it was then incubated at 70 °C for 10 min, following by adding 200 µl of absolute ethanol and mixed by vortex for 15 s.

The mixture was carefully loaded to the QIAamp Mini spin column (in a 2 ml collection tube) and centrifuged at 10,000 rpm for 1 min RT, the flow though was discarded and added AW1 buffer to the filtrate column, which was centrifuged at 10,000 rpm for 1 min at RT, followed by a re-centrifugation once at 13,000 rpm for 3 min at RT to eliminate the remaining buffer. The column was placed in a new 1.5 microcentrifuge tube and added with 100 µl of AE buffer and incubated for 10 min at room temperature. DNA was eluted by centrifugation at 13,000 rpm for 3 min at room temperature. DNA samples were stored at -20 °C.

DNA yield, length and purity were determined by Nanodrop 2000 Spectrophotometer (Thermo Fisher Scientific) analysis at 260 nm and 280 nm, and 1% agarose gel electrophoresis at 135 volts for 15 min in 0.5x TAE buffer. The electrophoresed gel was stained with ethidium bromide and visualized by exposing the gel to UV light.

2.4 Single Nucleotide Polymorphism (SNP) selection and design

SNP selection

Data on 2 SNPs were accessed from Ensembly (http://www.ensembl.org), Information was estimated using data from the Han Chinese population. Therefore SNP variations in Thai populations would likely have a similar representation with Chinese HapMap data.

Haploview

Haploview 4.2 software was utilized to produce linkage disequilibrium (LD) plots for gene variants using information obtained from the international HapMap project- phase I, II and III (http://www.hapmap.org).

SNP primer design

All primers in Table 1 were designed using the oligocalculator software (http://www.basic.northwestern.edu/biotools/oligocalc.html)

Primers were also tested for their binding location by performing a nucleotide-nucleotide BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) search to ensure that each primer had strong sequence homology only to the desired chromosomal region. Sequencing primer are synthesized by BioDesign Co., Ltd. Primers and Probe for genotyping analysis were purchased from Applied Biosystems (ABI; Foster City, CA).

Table 1. Nucleic acid sequences of oligonucleotide primers used in this study

Primer	**Rs 7041**	**Rs 4588**
Primer VDBP sense	5’CTGGCAGAGCGACTAAAAG3’	5’GTGGAGGGTTACATTTTCCTAC3’
Primer VDBP antisense	5’ TCAGACTGGCAGAGCGA3’	
Primer VDBP Probe	6-FAM-5’GGTGTGGC (C) TCAGGCAATT 3’-TAMRA	6-FAM-5’ TAACCAGCTTTGCCAGTTCC (T) TGG3’-TAMRA
	VIC-5’GGTGTGGC (A) TCAGGCAATT 3’-TAMRA	VIC-5’ TAACCAGCTTTGCCAGTTCC (G) TGG3’-TAMRA

2.5 Prepare sample for sequence

Polymerase Chain Reaction (PCR) amplification

The concentration of the template DNA used was 50 ng/µl. and Polymerase chain reactions were performed using thin-walled 200 µl tubes. The concentrations of reagents, using HotStarTaq DNA Polymerase from QIAGEN (Qiagen, Hilden, Germany), are shown in Table 2. The master mix contains all reagents except DNA template. In each reaction, 100 ng of DNA was added to each well followed by 18 µl of PCR cocktail.

All PCR procedures for this research were performed using automated S1000 thermal cycler obtained from Biorad (Bio-Rad Laboratories USA). Temperature sensitive steps involved in the PCR to amplify specific regions of genomic DNA are shown in Table 3.

Table 2: The PCR master mix cocktail reaction.

Reagent	Stock Concentration	Volume (µl)	Final Concentration
PCR buffer	10x	2	1x
MgCl2	25 mM	2	2.5 mM
dNTPs	10 mM	0.4	2 mM
Forward Primer	10 µM	0.4	2 µM
Reverse Primer	10 µM	0.4	2 µM
Taq Polymerase	250 units	0.05	5 unit/µl
ddH2O	Up to 18 µl		
Template DNA	50 ng/µl	2	
Total		20	

Table 3: Thermocycler parameter requirements for PCR amplification.

Temperature (°C)	Program	Time	Number of Cycles
95	Denaturing	15 min	1
95	Denaturing	30 sec	40
60	Annealing	30 sec	
72	Extension	45 sec	
72	Final extension	10 min	1

The amplified PCR product were detected by 2% agarose gel using electrophoresis. Gels were run using 135 volts for approximately 30 min and stained with ethidium bromide for visualizations under ultra-violet light.

PCR product purification

The PCR products were purified using QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) First, 5 volumes of PBI Buffer were added to 1 volume of the PCR sample and mixed by vortex. Next, the mixture was loaded to the QIAquick spin column. After that, it was centrifuged at 10,000 rpm for 1 min at RT. After discarding flow-through, the column was added with 750 µL of PE buffer to wash and centrifugated at 10,000 rpm for 1 min at RT. The column was re-centrifuged at maximum speed for 2 min. QIAquick columns were placed into 1.5 ml tube, 30 µl of EB buffer was added to the column for DNA elusion and the column was centrifuge at 13,000 rpm for 2 min. Purified PCR product was stored in microcentrifuge tube at 4 ^{o}C for sequencing.

Sequencing

Samples were selected for sequencing by using the Sanger method shown in Figure 3.

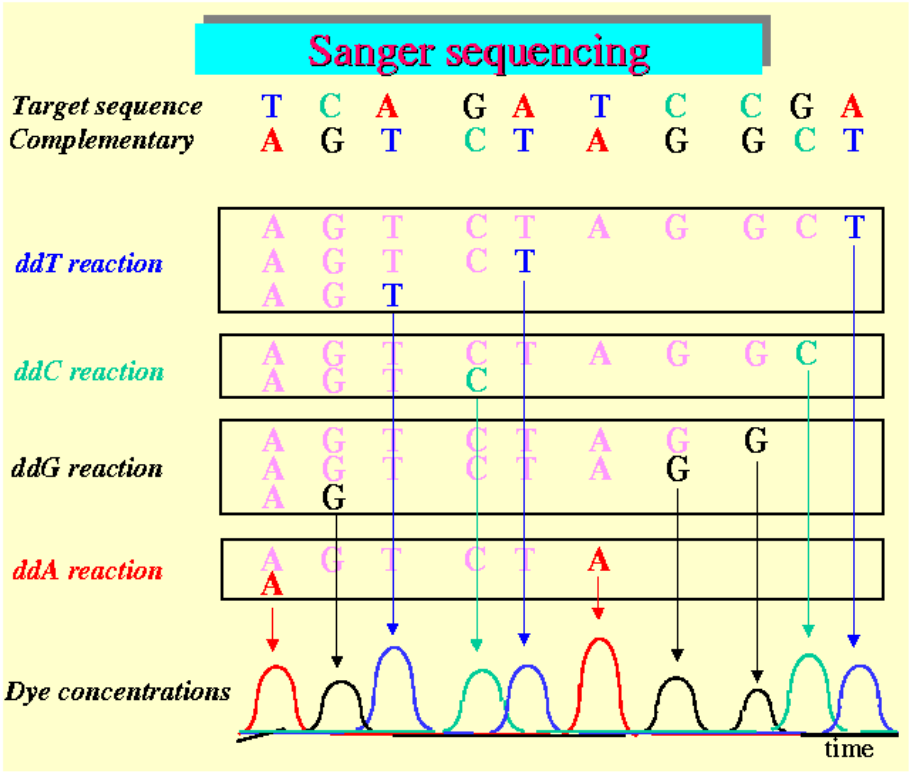

Figure 3: Sanger's sequencing technique (http://stat.fsu.edu)

2.6 SNP genotyping assays

Genotyping was performed with Taqman genotyping assays. The assay mixes (including unlabeled PCR primers, FAM™and VIC® dye-labeled TaqMan MGB probes) were designed and supported by Applied Biosystems (ABI) shown principle in Figure 4. Allelic discrimination is achieved using the TaqMan® SNP genotyping reaction mix as shown in Table 4. Amplification step was shown in Table 5. Genotyping was performed in a 96-well plate including negative (no DNA) and positive controls to ensure genotyping accuracy using ABI Prism®7500 Fast Real-time PCR, ABI GeneAmp® PCR system 7500.

The assay design incorporates a minor groove binder moiety (MGB) attached to the non fluorescent quencher at the 3’ end of the probe. A 6-carboxy-fluorescein (FAM) reporter dye is affixed on the 5’ end of the probe. The MGB molecule binds to the minor groove of the DNA helix enhancing the stability of the MGB-probe and DNA template complex. This feature increases the melting temperature without altering the probe length. This method allows for accurate allelic discrimination and the increased stability allows the use of probes as short as 13 bases. The close proximity of the reporter dye to the quencher results in suppression of the reporter fluorescence. During replication, allele detection is achieved via exonuclease cleavage of the 5’ end releasing the FAM dye generating an assay signal. The increase in fluorescence signal occurs only if the target sequence is complementary to the probe and is therefore displaced during PCR.

Table 4: TaqMan SNP genotyping cocktail reaction.

Reagent	Stock Concentration	Volume (µl)	Final Concentration
TaqMan™ Genotyping Master Mix	2x	5	1x
Primer & Probe	40X	0.25	1x
ddH2O	Up to 8.5 µl		
Template DNA	50 ng/µl	1.5	
Total		10	

Table 5: Thermocycler parameter requirements for real-time PCR amplification.

Temperature (°C)	Program	Time	Number of Cycles
60	Pre-PCR Read	1 min	1
95	Holding Stage	10 min	
95	Cycling Stage	15 sec	40
60	Annealing	1 min	
60	Post-PCR Read	1 min	1

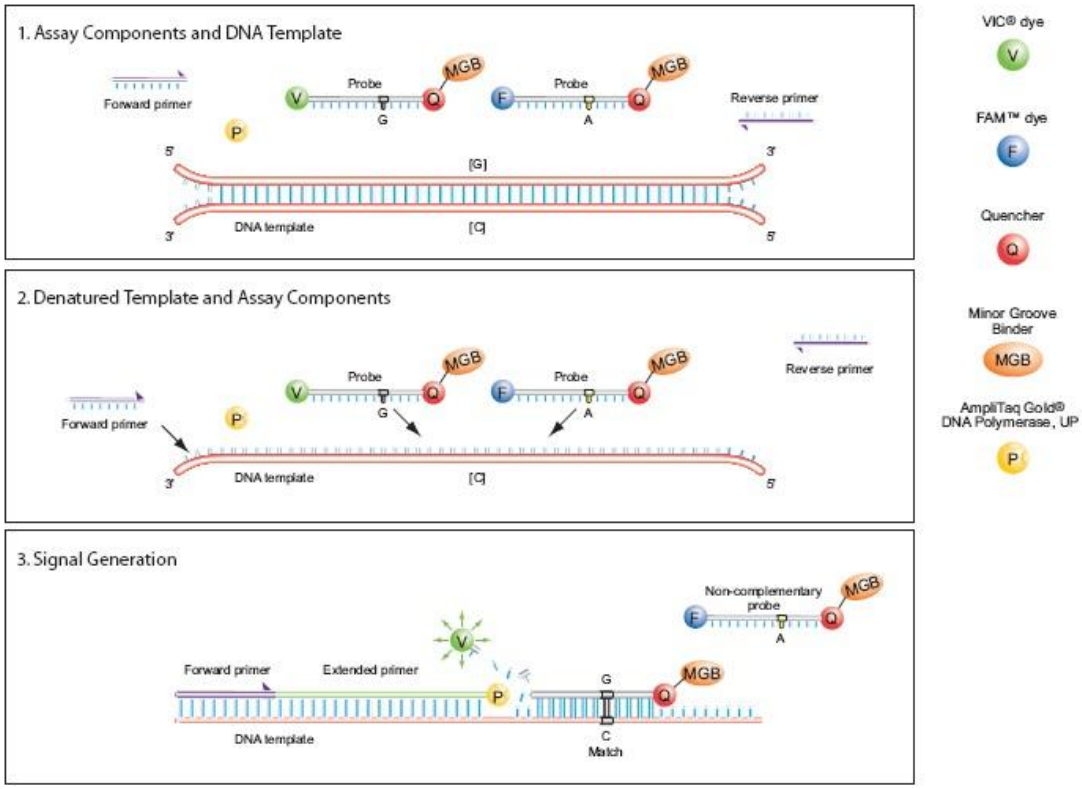

Figure 4: Principle of TaqMan probe (Applied Biosystems)

2.7 Sample preparation for Vitamin D measurement

Aliquots 250–500 µl of blood serum were shipped on dry ice to Helena Thai Laboratories for serum 25(OH)D_3 measurements by liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) System. 100 µL of patient's serum were pipetted into disposable borosilicate tubes. To each sample , 75 µl of 60 nmol/l internal standard (IS) in methanol - propanol (80:20 ratio volume) solution was added. The samples were vortex- mixed for 10 sec. 500 µL of hexane was added to each. The samples were re- vortexed for 10 sec and centrifuged for 15 min at 1600 g. 400 µL of each hexane layer was transferred to a sterile auto sampler vial. The solvent was evaporated to dryness under a stream of nitrogen heating block at 75^{o}C. The residual precipitate was reconstituted in 300 µl of methanol-water (70:30 ratio). Vials were sealed, vortex-mixed and 20 µL was injected into the LC-MS/MS system assayed by LC-MS/MS WATERS ACQUITY®Xevo^{TM} TQ-S UPLC^{TM} model and analyted were MassLynx Version 4.1.

2.8 Statistical analysis

Sample size was calculated by using comparison of two-proportion method. Descriptive statistics were used to compare demographic data between cases and controls. Statistical analysis of association between genotype frequency 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. An Odds ratio (OR) and 95% confidence interval (95% CI) was calculated by using unadjusted univariate logistic regression analysis. Unpaired-t-test was used to compare serum vitamin D level between genotypes. A *p*-value of less than 0.05 was considered statistically significant. All statistical calculation was performed on Intercool Stata version 6.

CHAPTER 3
RESULTS

1. Study population

Numbers of cases and controls in each cancer type, together with age and sex data are shown in Table 1. No significant deviation from Hardy-Weinberg expectations was observed for polymorphisms rs4588 and rs7041in both cases and controls. Linkage disequilibrium between the two SNPs was almost complete (D'=1). Age of cases and controls were comparable in breast and lung cancers. However, the average age in controls was significantly younger than that of the case group in colorectal cancer.

2. Sequencing results

In every real time PCR experiment, we used known DNA sequence as standard controls. The sequences were shown in Figure 1-3

Table 1 : Numbers of cases and controls in this study together with mean age (± standard deviation) and sex distribution

Cancer	Control	Case	*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±11.52	62.8±11.66	0.90
Sex: Male	76	76	1.00
Female	37	37	
Colorectal			
Number	282	282	
Mean age	57.7±16.70	62.0±14.49	0.001
Sex: Male	174	174	1.00
Female	108	108	

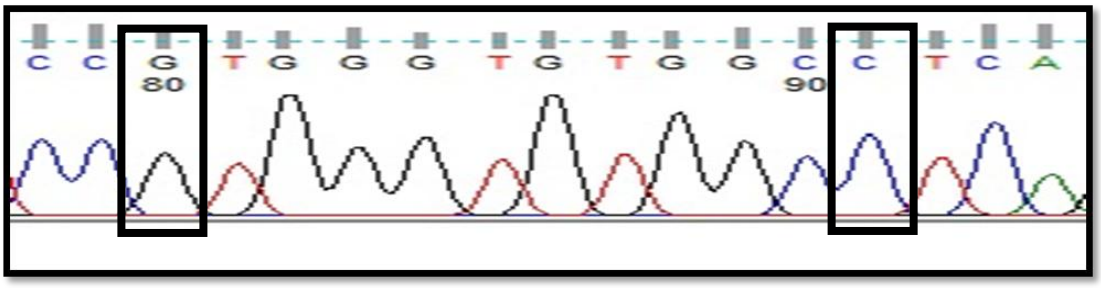

Figure 1: The sequencing result of rs7041 and rs4588 SNPs are GG and CC genotype, respectively on *VDBP* gene

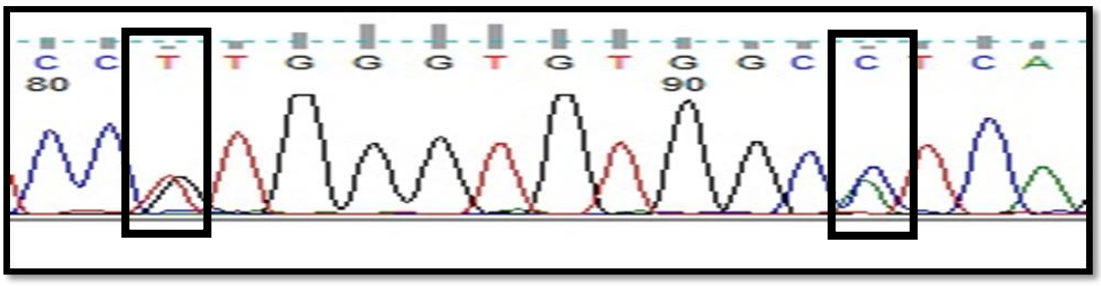

Figure 2: The sequencing result of rs7041 and rs4588 SNPs are TG and CA genotype, respectively on *VDBP* gene

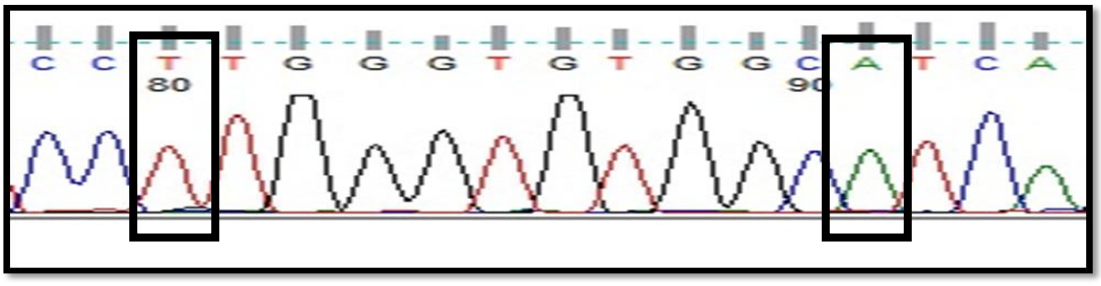

Figure 3: The sequencing result of rs7041 and rs4588 SNPs are TT and AA genotype, respectively on *VDBP* gene

3. Genotyping result

Taqman SNP genotyping used in this study gave call rate greater than 99% and an accuracy rate of 100% when compared with genotypes derived by direct sequencing. Minor allele frequencies in the SNP rs7041 and rs4588 were 0.32 and 0.24, respectively. The figure show allelic discrimination plot of the SNP rs7041 (Figure 4) and the SNP rs4588 (Figure 5) by 7500 Fast Real time PCR (Applied Biosystems®)

Genotype distribution of the two SNPs in each cancer is displayed in Table 2. When allelic distribution was compared between cases and controls, C allele (major allele) in rs4588 was significant associated with breast cancer (*p*-value 0.049).

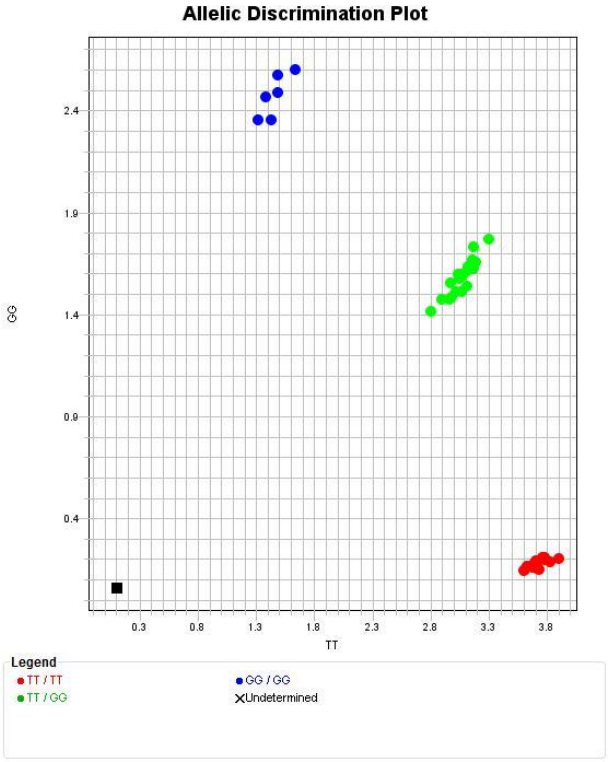

Figure 4: Genotype discrimination plot of the rs7041 SNP on *VDBP* gene. The blue dots represent homozygous for GG and red dots represent homozygous for TT, green dots for heterozygous TG and black dot represents negative control.

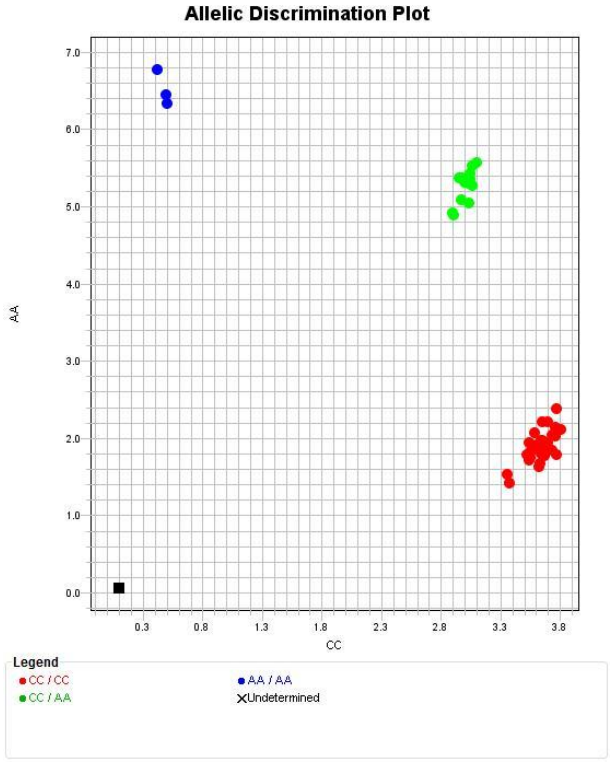

Figure 5: Genotype discrimination plot of the rs4588 SNP on *VDBP* gene. The blue dots represent homozygous for AA and red dots represent homozygous for CC, green dots for heterozygous CA and black dot represents negative control

Table 2: Genotypes distribution and minor allele frequencies of the single nucleotide polymorphisms (SNP) rs4588 and rs7041 on *VDBP* and *p*-value of allelotype association study

SNPs	Control	%	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

4. Individual SNP analysis

When all cancer cases were pooled together, the genotype AA in rs4588 had protective association with the cancers at the OR 0.50 (95% CI 0.26-0.95) and G containing genotypes of rs7041 (TG and GG) were significantly associated with the cancers at the ORs 1.39 (95%CI 1.06-1.81) and 1.48 (95%CI 1.01-2.21), respectively (*p*-trend 0.01) shown in Table 3. When each individual cancer was analyzed, no significant disease association was found between the 2 SNPs and colorectal nor breast cancer. However, the rs7041:TG/GG group was found to have disease association with lung cancer at an OR 1.78 (95%CI 1.05-3.03) (Table 4).

Considering age as a strong factor associating with cancer occurrence, subgroup analysis was performed according to sex and age at diagnosis, the study found that the dominant risk genotypes of rs7041 (TG/GG) was significantly associated with colorectal cancer and lung whose age at diagnosis was more than 60 years (OR 1.67, 95%CI 1.06-2.61, *p*-value 0.02). Paradoxically, the CA/AA genotypes group of the rs4588 showed positive association with colorectal cancer in males aged 60 years or less (OR 2.34, 95%CI 1.25-4.37, *p*-value 0.007) shown in Table 4.

Table 3: *GC* genotype frequencies among cases and controls and their association with risk of three cancers

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)	0.50	**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
Total		**496/496**				**496/496**		
	CC	286/307	Reference		**TT**	243/201	Reference	
	CA	182/174	0.89(0.68-1.16)	0.39	**TG**	196/225	1.39(1.06-1.81)	*0.016
	AA	28/15	0.50(0.26-0.95)	*0.032	**GG**	57/70	1.48(1.01-2.21)	0.050
	***p*-trend**			0.11	***p*-trend**			0.01

Table 4 Genotype association analysis of the two SNPs and three cancers studied

	Breast	***p*-value**	**Lung**	***p*-value**	**Colorectal**	***p*-value**
All cases	Controls/ Cases		Controls/ Cases		Controls/ Cases	
rs4588 (Dominant)		0.07		0.23		0.93
CC	54/65		60/69		172/173	
CA/AA	47/36		53/44		110/109	
rs4588 (Recessive)		0.12		0.52		0.27
CC/CA	92/98		107/109		269/274	
AA	9/3		6/4		13/8	
rs7041 (Dominant)		0.26		*0.032		0.13
TT	51/43		58/42		134/116	
TG/GG	50/58		55/71		148/166	
rs7041 (Recessive)		0.69		0.62		0.28
TT/TG	87/85		105/103		247/238	
GG	14/16		8/10		35/44	
Age < 60 years						
rs4588 (Dominant)		0.19		0.57		0.11
CC	44/51		20/22		79/55	
CA/AA	38/29		20/17		47/50	
rs4588 (Recessive)		0.32		0.57		0.36
CC/CA	76/77		38/38		121/103	
AA	6/3		2/1		5/2	
rs7041 (Dominant)		0.34		0.57		0.53
TT	41/34		20/17		56/51	
TG/GG	41/46		20/22		70/54	
rs7041 (Recessive)		0.95		0.75		0.86
TT/TG	70/68		35/35		109/90	
GG	12/12		5/4		17/15	
Age > 60 years						
rs4588 (Dominant)		0.38		0.44		0.19
CC	9/11		38/43		92/110	
CA/AA	9/6		28/24		61/54	
rs4588 (Recessive)		0.08		0.64		0.68
CC/CA	15/17		63/65		146/158	
AA	3/0		3/2		7/6	
rs7041 (Dominant)		0.6		*0.044		*0.023
TT	9/7		33/22		76/61	
TG/GG	9/10		33/45		77/103	
rs7041 (Recessive)		0.33		0.48		0.23
TT/TG	16/13		63/62		135/137	
GG	2/4		3/5		18/27	

5. Genotype-combination analysis

Based on our genotypes data, 9 genotype-combinations could be constructed and named according to the previously described electrophoretic variants (Abbas S et al., 2008, Malik S et al., 2013) (Table5). Three most common variants found in our cases included Gc1s-1f, Gc1f-1f and Gc2-1f. When SNP combinations (rs7041-rs4588) were constructed and analyzed, Gc2-1f combination (TT-CA) had significant protective association with lung cancer (OR 0.44, 95% CI 0.22-0.85).In addition, Gc1s-1s combination (GG-CC) was significantly associated with colorectal cancer in males aged more than 60 years (OR8.31, 95% CI 1.07-64.47) and Gc1s-1fcombination (TG-CC) was significantly associated with breast cancer in patients aged less than 60 years (OR2.10, 95%CI 1.03-4.30) (Table 6).

Table 5: Genotyping results for rs7041 and rs4588 in the *VDBP* gene and assignment to combined *GC* genotypes. (Abbas S 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

Table 6: Combine genotype association subgroup analysis to sex and age at diagnosis

Cancer	Genotype		Sex	Age (years)	Combination genotype frequency (%)	Cases	Controls	OR(95%CI)	*p*-value
	rs7041	rs4588							
Breast	TG	CC	Female	≤ 60	33.8	27/80	16/82	2.10(1.03-4.30)	0.04
Lung	TT	CA	Both	All	14.1	16/113	31/113	0.44(0.22-0.85)	0.01
Colon	GG	CC	Male	> 60	15.9	17/107	1/45	8.31(1.07-64.47)	0.02

6. **25(OH)D_3 level**

The serum level of 25(OH)D_3 was evaluated in 155 healthy controls including 136 males and 19 females (Table7, Figure 6). Mean age of the volunteers was 24.4 years (range 17-75 years). Average 25(OH)D_3 in males (28.0 ng/ml) was significantly higher than females (22.6 ng/ml) (*p*-value < 0.01). In rs4588, the average 25(OH)D_3 level in CC was significantly higher than that of CA/AA (Table 8). Moreover, it was found that the proportion of cases with serum 25(OH)D_3 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_3 nor the proportion of those with low serum 25(OH)D_3 level showed no significant difference between genotype groups (Table 8).

Table 7: Association between risk combination of *GC* gene and serum levels of 25(OH)D in control samples (Sex: 0 = Male, 1=Female)

Code	25(OH)D level (ng/ml)	Sex	Age (year)	Genotype	
				rs4588	rs7041
mc01	28.019	0	26	CC	GG
mc02	21.807	0	52	CA	TT
mc04	20.863	0	24	CC	TG
mc05	23.103	0	21	CA	TG
mc06	30.304	0	22	CC	TG
mc07	26.564	0	24	CC	GG
mc08	24.186	0	21	CC	TG
mc09	29.924	0	21	CA	TT
mc10	27.604	0	21	CC	GG
mc11	30.772	0	21	CC	TT
mc12	32.482	0	21	CC	TT
mc14	25.341	0	24	CC	TG
mc15	34.681	0	20	CC	GG
mc16	34.389	0	21	CC	TT
mc18	31.918	0	21	CC	TG
mc19	29.503	0	21	CA	TT
mc20	37.758	0	22	CC	TG
mc21	28.404	0	21	CC	TT
mc23	30.808	0	21	CC	TT
mc24	35.892	0	23	CA	TG
mc25	42.748	0	23	CC	TT
mc26	28.022	0	24	CA	TT
mc27	33.304	0	22	CA	TT
mc30	21.859	0	53	CA	TT
mc31	48.718	0	52	CA	TT
mc32	45.046	0	22	CA	TG
mc33	39.821	0	21	CC	TT
mc34	27.682	0	21	CC	TG
mc35	45.885	0	23	CC	GG
mc37	17.024	0	28	CA	TG
mc38	29.809	1	22	CA	TT
mc39	32.392	0	22	CC	TG
mc40	28.208	0	21	CC	TT
mc41	36.018	0	21	CC	TG
mc42	36.753	0	21	CC	TT
mc43	48.198	0	21	CC	TG
mc44	27.245	0	22	CA	TT

Code	25(OH)D level (ng/ml)	Sex	Age (year)	Genotype	
				rs4588	rs7041
mc45	34.085	0	22	CA	TG
mc46	25.008	0	20	CC	TT
mc47	34.955	0	21	CA	TG
mc48	24.255	0	23	CC	TG
mc49	28.884	0	25	CC	TG
mc50	32.676	0	21	CA	TG
mc51	29.913	0	21	CA	TG
mc52	35.513	0	21	CA	TT
mc53	25.973	0	18	CA	TG
mc55	23.279	0	18	AA	TT
mc56	26.478	0	23	CA	TG
mc57	28.192	0	22	CA	TT
mc59	45.989	0	21	CA	TG
mc60	20.810	0	30	CC	TG
mc61	38.297	0	18	CC	GG
mc62	39.626	0	17	CC	TG
mc63	32.607	0	18	CC	TT
mc64	22.904	0	18	CC	TG
mc65	34.532	0	18	CC	TT
mc66	18.787	0	17	CA	TG
mc67	24.974	0	19	CA	TG
mc68	38.678	0	18	CC	TG
mc69	29.217	0	18	CA	TT
mc70	29.897	0	17	CA	TT
mc71	29.563	0	18	CC	TG
mc72	31.930	0	18	CC	TG
mc73	21.221	1	18	CC	GG
mc74	27.481	0	18	CC	TG
mc76	22.080	0	18	CC	TG
mc77	25.984	0	18	CA	TT
mc78	17.851	0	18	CA	TT
mc79	22.832	1	18	CA	TG
mc80	22.741	1	18	CC	TG
mc81	27.387	0	18	CC	TT
mc82	26.966	0	18	CC	GG
mc83	28.383	0	18	CC	TG
mc84	19.186	1	18	CA	TT
mc85	25.474	1	18	CC	TG
mc86	27.042	1	18	CC	TG
mc87	17.213	1	27	CC	TG

Code	25(OH)D level (ng/ml)	Sex	Age (year)	Genotype	
				rs4588	rs7041
mc88	19.950	0	26	CC	TG
mc89	24.332	0	18	CA	TT
mc90	27.165	0	18	CC	TG
mc91	23.738	0	18	CA	TT
mc92	26.111	0	18	CC	GG
mc93	24.347	0	19	CC	TT
mc94	31.803	0	19	CC	TG
mc95	23.844	0	18	CA	TG
mc96	25.950	0	18	CC	GG
mc97	21.052	0	59	CA	TT
sc02	30.070	0	50	CC	TT
sc06	29.355	0	21	CA	TT
sc07	33.750	0	23	CC	TT
sc08	25.721	0	51	CA	TT
sc09	23.751	0	20	CC	TG
sc11	30.666	0	23	CC	GG
sc12	39.871	1	22	CC	TT
sc13	21.080	0	22	CA	TG
sc15	23.950	0	34	CC	GG
sc16	22.274	0	22	CA	TT
sc17	24.340	0	25	CC	TT
sc20	21.318	0	21	CA	TG
sc21	19.215	0	25	CA	TT
sc24	19.311	0	29	CC	TG
sc29	25.018	0	31	CA	TT
sc30	23.989	0	23	CA	TG
sc34	14.422	0	53	CC	TG
sc36	26.277	0	27	CC	TT
sc37	22.742	0	37	CC	TG
sc39	27.369	0	31	CA	TG
sc44	30.851	0	24	CC	TG
sc45	20.336	1	56	CC	TT
sc46	29.149	0	21	CC	TG
sc47	24.126	0	21	CC	TG
sc48	35.549	0	21	CC	TT
sc49	28.969	0	21	CC	TT
sc50	27.457	0	22	CC	TG
sc51	25.523	0	21	CC	TT
sc52	25.669	0	21	CC	TG
sc53	25.392	0	22	CC	TG

Code	25(OH)D level (ng/ml)	Sex	Age (year)	Genotype	
				rs4588	rs7041
sc54	21.427	0	21	AA	TT
sc55	28.314	0	21	CC	TT
sc56	25.477	0	21	CC	GG
sc58	30.008	0	22	CA	TT
sc59	21.155	0	22	CA	TT
sc60	27.887	0	18	CC	TT
sc67	17.628	0	18	CA	TT
sc68	31.047	0	18	CC	TG
sc69	24.099	0	18	CA	TT
sc71	24.660	0	18	AA	TT
sc72	25.127	0	17	CC	TG
sc73	30.661	0	17	CC	GG
sc74	20.007	0	18	CC	TG
sc75	34.469	0	18	CA	TG
sc76	25.017	0	18	CC	TG
sc77	21.270	0	19	CC	TT
sc78	21.490	0	18	CA	TG
sc80	18.896	0	18	CC	TG
sc81	20.742	0	18	CC	TG
sc82	18.342	1	18	CA	TG
sc84	30.747	0	18	CC	TG
sc85	19.826	0	18	CC	TT
sc86	22.341	1	18	CC	TG
sc88	18.319	0	17	CA	TT
sc89	27.593	0	19	CC	GG
sc90	24.405	1	18	CC	TT
sc91	21.187	1	17	CA	TT
sc94	19.894	0	18	CA	TT
SI 008	15.410	1	31	CA	TG
SI 009	37.309	0	32	CC	GG
NA 25	21.587	0	75	AA	TT
NA 42	22.801	1	57	AA	TT
NA 76	18.576	1	53	CA	GG
NA 101	22.287	0	57	AA	GG
NB 16	33.376	1	34	CA	GG
R 016	19.940	1	53	CA	GG
R 096	18.623	1	59	AA	TG
R 118	37.402	1	61	AA	TT

Table 8: Comparison of serum 25(OH)D_3 level between genotypes and association between risk genotypes of *VDBP* and low serum level (<20 ng/ml)

SNPs (genotype)	**N(%)**	**25(OH)D_3 levels**				
		Mean (ng/ml)	***p*-value**	**≤20 ng/ml n (%)**	**>20 ng/ml n (%)**	***p*-value**
rs4588 (dominant)						
CC	90(58.1)	28.3	0.03	6(6.7)	84(93.3)	0.01
CA/AA	65(41.9)	26.0		13(20.0)	52(80.0)	
rs7041 (dominant)						
TT	65(41.9)	27.4	0.84	7(10.7)	58(89.2)	0.63
TG/GG	90(58.1)	27.2		12(13.3)	78(86.7)	

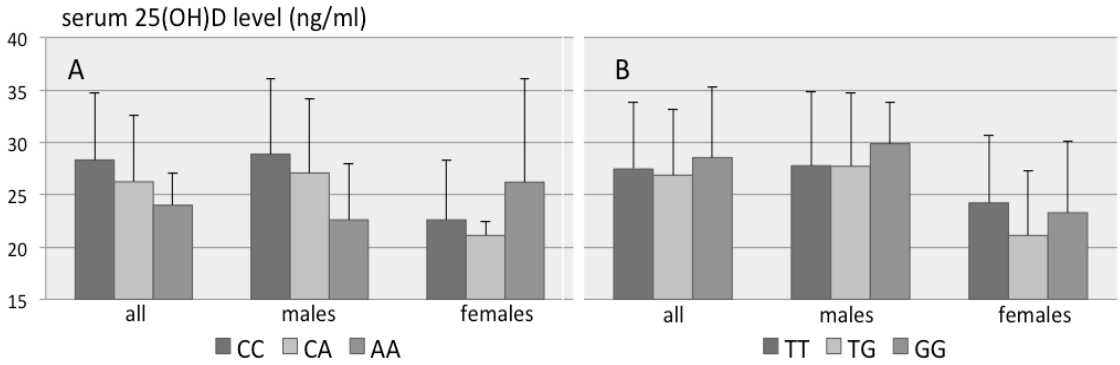

Figure 6 Difference in 25(OH)D_3 among genotypes according to sexes A) rs4588 B) rs7041

CHAPTER 4
DISCUSSION

In this study, we examined 2 SNPs in DBP for their association with 3 common cancers in Thai patients. Our main findings were that the risk-genotypes (TG/GG) in rs7041 showed disease association with lung cancer. The same rs7041 risk-genotypes group was associated with colorectal cancer when detected in patients over 60 years. Moreover, in males aged less than 60 years, A-allele of rs4588 was significantly associated with 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 the frequencies at rs4588: 0.28 and rs7041: 0.29.

A significant association between the T allele in rs7041 and breast cancer has been reported in Caucasian women in Canada (Anderson LN 2012). However, to our knowledge, the current study reports a significant association between rs7041 and lung cancer for the first time. According to recent genome wide association studies, the SNP was known as the strongest marker associated with a serum level of 25(OH)D_3 (Ahn J 2010, Wang TJ 2010). A study in premenopausal women in Canada indicated that the T allele (a minor allele in that population) was associated with lower serum concentration of 25(OH)D_3 (Sinotte M 2009). Similar results were reported in a study in healthy Brazilian girls (Santos BR 2012) and Chinese-Singaporean (Robian K 2013). Taken together data from those previous studies, although our study could not demonstrate a difference in vitamin D levels in different rs7041 genotype groups, the data suggest that the cancer risk was higher in groups with lower vitamin D levels.

Disease associations have been demonstrated for rs4588 in various cancers including breast (McCullough ML 2007), gastrointestinal (Zhou L 2012) and prostate (Kidd LC 2005). However, other studies have found more inconsistent results

(Poynter JR 2010, Mahmoudi T 2014). As the A allele in this SNP was associated with lower serum of 25(OH)D_3 in mainland Chinese (Zhang Z 2013) and Chinese-Singaporean populations (Robian K 2013), it could be expected that the minor allele genotypes (CA/AA) held a higher risk of cancer development. Our study found that A-allele carriers in rs4588 had a significant risk of low serum vitamin D level, at least in male. This metabolic risk explained risk of colorectal cancer in our patients. The number females who participated in serum vitamin D measuring was not enough for a clear interpretation, however, the result suggested that the effect of A-allele on the serum 25(OH)D_3 may not be the same as the male counterpart.

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. Moreover, our goal to collect 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 corrected this weak point by subgroup analysis and found disease association in both SNPs.

CHAPTER 5
CONCLUSIONS

In conclusion, this case-control study detected association between genotype polymorphisms in the vitamin D binding protein and the risk of common cancers in Thai population. The study was consistent with a previous study in Chinese population which found association between the A-containing genotypes in the rs4588 as a risk in Chinese colorectal cancer. Interestingly, when AA genotype had protective association with the pool cancer case, it was in the risk group in colorectal cancer.

In summary, the study evaluated 2 SNPs in DBP for their association with 3 common cancers in Thai patients. The study detected significant disease association between the polymorphisms and the cancer studied.

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List of Publications:

1. Pornsuksiri K, Chewatanakornkul S, Kanngurn S, **Maneechay W**, Chaiyapan W, Sangkhathat S.(2012). Clinical outcomes of gastrointestinal stromal tumor in southern Thailand. *World J Gastrointest Oncol* 4(11): 216-222.

2. Sangkhathat S, Kanngurn S, Chaiyapan W, Gridist P, **Maneechay W**. (2010). Wilms' tumor 1 gene (WT1) is overexpressed and provides an oncogenic function in pediatric nephroblastomas harboring the wild-type WT1. *Oncology Letters* 1(4): 615-9.

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