Association of *BMP6* Methylation and Expression with Clinicopathological Features in Colorectal Cancer

Patcharaporn Sangplod

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Author	Miss Patcharaporn Sangplod
Major Program	Biomedical Sciences

Major Advisor :

.....

(Assoc.Prof.Dr.Surasak Sangkhathat)

Examining Committee :

.....Chairperson

(Dr.Kawin Leelawat)

.....

(Dr.Kanchana Srinitiwarawong)

Co-advisor :

.....

(Dr.Samornmas Kanngurn)

.....

(Assoc.Prof.Dr.Surasak Sangkhathat)

.....

(Dr.Samornmas Kanngurn)

The Graduate School, Prince of Songkla University, has approved this thesis as partial fulfillment of the requirements for the Master of Science Degree in Biomedical Sciences.

.....

(Assoc.Prof.Dr. Teerapol Srichana)

Dean of Graduate School

This is to certify that the work here submitted is the result of the candidate's own investigations. Due acknowledgement has been made of any assistance received.

S. Sangkhathat .....Signature

(Assoc.Prof.Dr.Surasak Sangkhathat)

Major Advisor

พัชราภรณ์ สังข์ปลอด .....Signature

(Miss Patcharaporn Sangplod)

Candidate

I hereby certify that this work has not been accepted in substance for any degree, and is not being currently submitted in candidature for any degree.

.....พัชราภรณ์ สังข์ปลอด.....Signature

(Miss Patcharaporn Sangplod)

Candidate

ชื่อวิทยานิพนธ์ การศึกษาความสัมพันธ์ของการเกิดเมทิลเลชั่นในยีน *BMP6* และการแสดงออกของโปรตีนในมะเร็งลำไส้ใหญ่และลำไส้ตรง

ผู้เขียน นางสาวพัชราภรณ์ สังข์ปลอด

สาขาวิชา ชีวเวชศาสตร์

ปีการศึกษา 2555

บทคัดย่อ

วัตถุประสงค์: ยีน Bone morphogenetic protein 6 หรือ *BMP6* เป็นหนึ่งในสมาชิกของตระกูล transforming growth factor-beta หรือ TGF-beta ซึ่งเป็นที่ทราบกันดีกว่ามีส่วนเกี่ยวข้องกับกระบวนการเจริญเติบโตของเซลล์, การเปลี่ยนแปลงของเซลล์และกระบวนการตายอย่างมีแบบแผนของเซลล์ ทั้งนี้ได้มีงานวิจัยเกี่ยวกับยีน *BMP6* เกี่ยวกับการเกิดเมทิเลชั่นตรงบริเวณตำแหน่งโปรโมเตอร์ในการส่งผลให้เกิดเนื้องอกชนิดไม่ร้ายแรงในเนื้อเยื่อฮีมาโทโพอิทิก นอกจากนี้ยังชักจูงให้เกิดเนื้องอกชนิดร้ายแรงและการพัฒนาไปสู่โรคมะเร็งในที่สุด ในการศึกษานี้จึงมีวัตถุประสงค์เพื่อศึกษาการเกิดเมทิเลชั่นตรงตำแหน่งโปรโมเตอร์และการแสดงออกของยีน *BMP6* ในโรคมะเร็งลำไส้ใหญ่และลำไส้ตรง

วิธีการศึกษา: ศึกษาการเกิดเมทิเลชั่นของยีน *BMP6* โดยวิธี methylation-specific polymerase chain reaction (MSP) และใช้วิธี Immunohistochemistry (IHC) ในการศึกษาการแสดงออกของยีน *BMP6* โดยในการทดลองนี้ใช้ตัวอย่างจากผู้ป่วยมะเร็งลำไส้ใหญ่และลำไส้ตรงเป็นจำนวน 85 ตัวอย่าง

ผลการศึกษา: จากการศึกษาการเกิดเมทิเลชั่นของยีน *BMP6* พบว่ายีนเกิดการเมทิเลชั่นสูงใน 34 ตัวอย่าง คิดเป็นร้อยละ 40 และสภาวะของการเกิดเมทิเลชั่นสูงบนตำแหน่งโปรโมเตอร์ของยีน *BMP6* มีความสัมพันธ์กับการลดลงของการแสดงออกของโปรตีนอย่างมีระดับนัยสำคัญ

สรุป: จากผลการศึกษาแสดงให้เห็นถึงความเป็นไปได้ที่ยีน *BMP6* อาจจะเป็นยีนต้านมะเร็งในมะเร็งลำไส้ใหญ่และลำไส้ตรง

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ABSTRACT

Purpose: Bone morphogenetic protein 6 (*BMP6*) is a member of the transforming growth factor-beta (TGF-beta) superfamily known to regulate cell proliferation, differentiation and apoptosis. Promoter methylation of *BMP6* has been reported in hematopoietic neoplasm and influences carcinogenesis and tumor progression. In the present study, we evaluated the methylation status and expression of *BMP6* in colorectal cancer.

Methods: A methylation-specific polymerase chain reaction (MSP) was used to evaluate the methylation status of *BMP6*. Immunohistochemistry (IHC) was used to determine the *BMP6* protein expression. A total of 85 colorectal cancers (n=85) were included in this analysis.

Results: The methylation study of *BMP6* revealed hypermethylation status in 34 cases (40%). Promoter hypermethylation of *BMP6* was significantly associated with decreased protein expression.

Conclusion: Our findings suggest that *BMP6* is potentially a methylation-silenced tumor suppressor gene for colorectal cancer.

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LIST OF ABBREVIATION AND SYMBOLS

ACF	=	Aberrant crypt focus
ACVR	=	Activin A receptor
ACT	=	Actin-like protein
AJCC	=	American Joint Committee on Cancer
ALK	=	Anaplastic lymphoma receptor tyrosine kinase
APC	=	Adeomatous polyposis coli
Asn	=	Asparagine
bp	=	base pair
BRAF	=	v-Raf murine sarcoma viral oncogene homolog B1
$^{\circ}$C	=	Degree celsius
µg	=	Microgram
µL	=	Microliter
µm	=	Micrometer
µM	=	Micromolar
BMPR	=	Bone morphogenetic protein recrptor
BMP6	=	Bone morphogenetic protein 6
BMSC	=	bone marrow mesenchymal
CIMP	=	CpG island methylator phenotype
CIN	=	Chromosomal instability

CpG	=	Cytosine phosphoguanine
CRC	=	Colorectal cancer
DAB	=	3, 3'-diaminobenzidine
DNA	=	Deoxyribonucleic acid
EDTA	=	Ethylenediaminetetraacetic acid
EMT	=	Epithelial mesenchymal transition
FFPE	=	Formalin-fixed paraffin embedded
hr	=	Hour
IHC	=	Immunohistochemistry
IS	=	Intensity score
KRAS	=	Kirsten rat sarcoma virus oncogene
min	=	Minute
mRNA	=	Messenger ribonucleic acid
MAP	=	Mitogen-activated protein
MLH	=	Human mutL homolog
MMR	=	Mismatch repair
MSI	=	Microsatellite instability
MSH	=	Human mutS homolog
MSP	=	Methylation-specific polymerase chain reaction
ng	=	Nanogram
OD	=	Optical density

p	=	*P*-value
PBS	=	Phosphate buffer saline
PS	=	proportion score
rpm	=	Revolutions per minute
RNA	=	Ribonucleic acid
s	=	Second
TβR	=	Transforming growth factor-beta receptor
TGF-β	=	Transforming growth factor-beta
TAE	=	Tris-acetate buffer
VGR	=	Vegetal Related

CHAPTER 1

INTRODUCTION

Background and Rationale

Cancer is a generic term for a large group of diseases that can affect any part of the body. Other terms used are malignant tumor and neoplasm. One defining feature of cancer is the rapid creation of abnormal cells that grow beyond their usual boundaries, and which can then invade adjoining parts of the body and spread to other organs. This process is referred to metastasis. Metastases are the major cause of death from cancer. Cancer is a leading cause of death worldwide and accounted for 7.6 million deaths (around 13% of all deaths) in 2008 [1].

Colorectal cancer (CRC) is the third most commonly diagnosed cancer in males and the second in females, with over 1.2 million new cancer cases and 608,700 deaths estimated to have occurred in 2008 [2]. CRC death rates have been increasing in Thailand, where CRC is the third most common malignancy in males after hepatobiliary and lung cancers, and the fifth in females after cancers of the cervix, breast, hepatobiliary and lung. The number of colorectal cancer cases in both sexes is increasing and will probably exceed that of lung cancer in the next decade [3]. CRC is one of the cancers that can be prevented by secondary prevention. The precursor of advanced colorectal cancer is either an adenomatous polyp or a flat neoplastic lesion. The majority of cancers arising in the colon and rectum is adenocarcinoma that account for more than 90% of all large bowel tumors. The disease can be cured by the detection at earlier stage and even prevented by the removal of adenomas [3].

Bone morphogenetic proteins (BMPs) belong to the transforming growth factor-β (TGF-β)superfamily. They are multifunctional cytokine that controls cellular responses including the induction of cell growth inhibition, differentiation and apoptosis [4]. *BMP6* is an autocrine stimulator of chondrocyte differentiation [5] and has been implicated in the development of embryonic kidney and urinary systems [6]. Moreover, *BMP6* expression can be

localized to muscle cells in the developing human fetal intestine [7]. In vitro, *BMP6* has been shown to inhibit cell division, to promote terminal epithelial differentiation, and to induce endochondral bone formation, osteoblastic differentiation, and neuronal maturation [8]. Furthermore, in prostatic adenocarcinoma, induction of *BMP6* expression is associated with tumorigenesis [9] and the formation of osteosclerotic deposits in metastatic progression [10]. In addition, *BMP6* has been report that it may play an important role in heterotopic ossification in colon adenocarcinoma. It is prominent in the cytoplasm of tumor cells, and it stains weakly in osteoblast-like cells adjacent to newly formed bone [11]. On the other hand, several reports suggested that *BMP6* promoter methylation status is correlated with cancer.

Recently, promoter methylation of the *BMP6* gene has been demonstrated in aggressive types of cancer, for example, hypermethylation of *BMP6* is common in human prostate cancer [12]. Moreover, the gene may play roles in breast cancer development and metastasis [13]. However, data concerning association of *BMP6* and CRC development was not clear. Therefore, our study will evaluate an expression of *BMP6* in CRC. Furthermore, the study will analyse for any association of the DNA methylation of *BMP6* and its protein expression in this cancer. We hope that the data may help increase understanding role of this gene in CRC pathogenesis and may suggest an opportunity to use it as a disease marker.

Review of Literatures

1. Description of colorectal cancer

CRC, commonly known as colon cancer or large bowel cancer, is a cancer that starts in either the colon or the rectum (parts of the large intestine), or in the vermiform appendix. The majority of CRC cases are sporadic, with hereditary syndromes contributing approximately 5% to 15% of the incidence. There are many known risk factors for sporadic CRC, including non-modifiable and modifiable variables. Preventive measures target at tobacco use, dietary habits, and weight control [14]. The inflammatory bowel disease population is the second major category of patients at increased risk of CRC [15].The two main syndromes accounting for 5% of the

inherited cases are hereditary nonpolyposis colon cancer and familial adenomatous polyposis [16]. The remaining 15% to 20% of inherited CRC cases do not have a clearly defined mechanism and likely result from a combination of gene polymorphisms, alterations in multiple susceptibility loci, and environmental influences [17]. These cases are grouped into familial X CRC syndrome.

2. Molecular pathogenesis pathways in colorectal cancer

CRC is a heterogeneous disease. There are three main pathways included in the development of CRC. It may be broadly categorized into genomic instability, including the chromosomal instability (CIN), the microsatellite instability (MSI), and the CpG island methylator phenotype pathway [18].

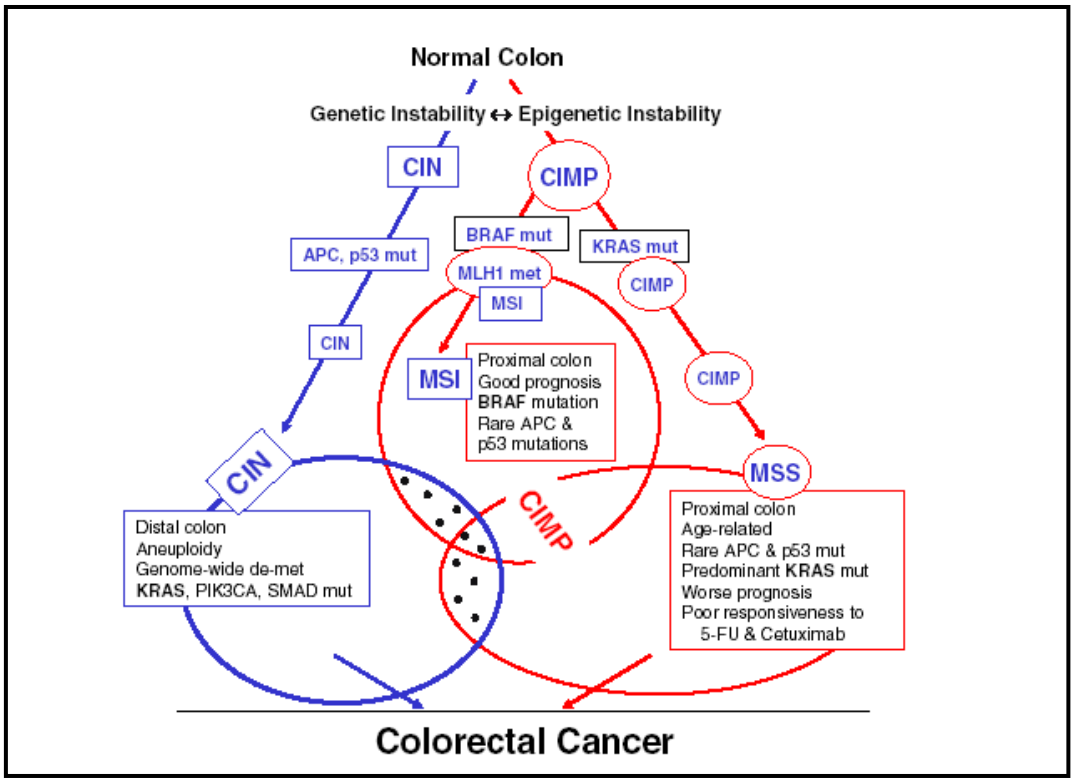


Figure 1. Multiple pathways to progression of colorectal tumors. There are three distinct parallel pathways (CIN, CIMP+/MSI, and CIMP+/MSS) for the progression of CRC from normal colon mucosa.

1.1 The chromosomal instability (CIN) pathway

The CIN pathway, also known as the suppressor pathway, is the most common type of genomic instability. Approximately 70%-85% of CRCs develop by the CIN pathway [19]. In the CIN pathway molecular aberrations occur in significant part through the accumulation of numerical or structural chromosomal abnormalities [20]. The earliest identifiable lesion in this pathway is the dysplastic aberrant crypt focus (ACF), a microscopic mucosal lesion that leads the development of a polyp [21, 22]. The CIN pathway is associated with mutation in APC and/or loss of chromosome 5q that includes the APC gene, mutation of the KRAS oncogene, loss of chromosome 18q and deletion of chromosome 17p. The latter locates an important tumour suppressor gene TP53 [23].

1.2 The microsatellite instability (MSI) pathway

Microsatellites are nucleotide repeat sequences scattered throughout the genome. MSI refers to a discrepancy and instability in the number of nucleotide repeats found within these microsatellite regions in tumor or germline DNA. DNA polymerase has to checks the errors of copying these short repeat sequences and mismatch repair (MMR) dysfunction results in MSI. The MMR system is composed of MutL homolog (MLH), MutS protein homolog (MSH) and postmeiotic segregation protein (PMS), which associate with specific to form functional heterodimers [24]. MLH1 and MSH2 are important in the mismatch repair mechanism and form five functional heterodimeric proteins (MSH2-MSH3; MSH2-MSH6; MLH1-PMS1; MLH1-PMS2; MLH1-MLH3). Mutations in MLH1, MSH2, MSH6 and PMS2 have been involved in hereditary nonpolyposis colorectal cancer (HNPCC). MSI or MSI-high (MSI-H) is defined as MSI at $\geq$2 (40%) of the five specified sites, MSI-low (MSI-l) as MSI at one site and microsatellite stable (MSS) when no instability is demonstrated at these markers. MSI induces to genetic errors and several microsatellites are present in genes implicated in colorectal carcinogenesis [25]. The majority of MSI-H CRCs occur sporadically in the context of DNA

methylation of the MLH1 promoter and the consequent transcriptional silencing of MLH1 expression [26].

1.3 The CpG island methylator phenotype (CIMP) pathway

The CIMP pathway is the second most common pathway underlying sporadic CRCs. The incidence of CIMP pathway involvement is approximately 15% of sporadic cases [27]. The CIMP pathway provides the epigenetic instability for sporadic cancers to methylate the promoter regions of epigenetically inactivate the expression of tumor suppressor genes, such as MLH1. CIMP positive CRCs are defined by using a panel of cytosine-phosphate-guanine (CpG) island methylation markers. They are classified DNA methylation on the basis of certain thresholds. The CIMP panel of genes or markers is corresponding to the panel of microsatellites used to define microsatellite status [28]. Cancers with a percent of methylated reference of $\geq$10 at three or more of these gene promoter sites are classified as CIMP positive [29]. A recent study has suggested that there is an association between CIMP positive and kirsten rat sarcoma (KRAS) and v-raf murine sarcoma viral oncogene homolog B1 (BRAF) mutation [30].

3. Description of Bone morphogenetic protein 6 (*BMP6*)

Bone morphogenetic proteins (BMPs) are multifunctional growth factors belonging to the transforming growth factor-β (TGF-β) superfamily, (Figure1). It has been demonstrated that BMPs had been involved in the regulation of cell proliferation, survival, differentiation and apoptosis. However, their hallmark ability is that they play a pivotal role in inducing bone, cartilage, ligament, and tendon formation at both heterotopic and orthotropic sites [31]. Genetic and functional studies indicated that common components of the BMPs signaling pathway play critical roles in regulating vascular development in the embryo and in promoting vascular homeostasis and disease in the adult. Several BMPs family members have different functions in different tissue [32]. Besides, significant contributions of BMPs, their receptors and interacting molecules have been linked to carcinogenesis and tumor progression. On the other hand, BMPs can also play a role as a tumor suppressor, especially *BMP6* [33].

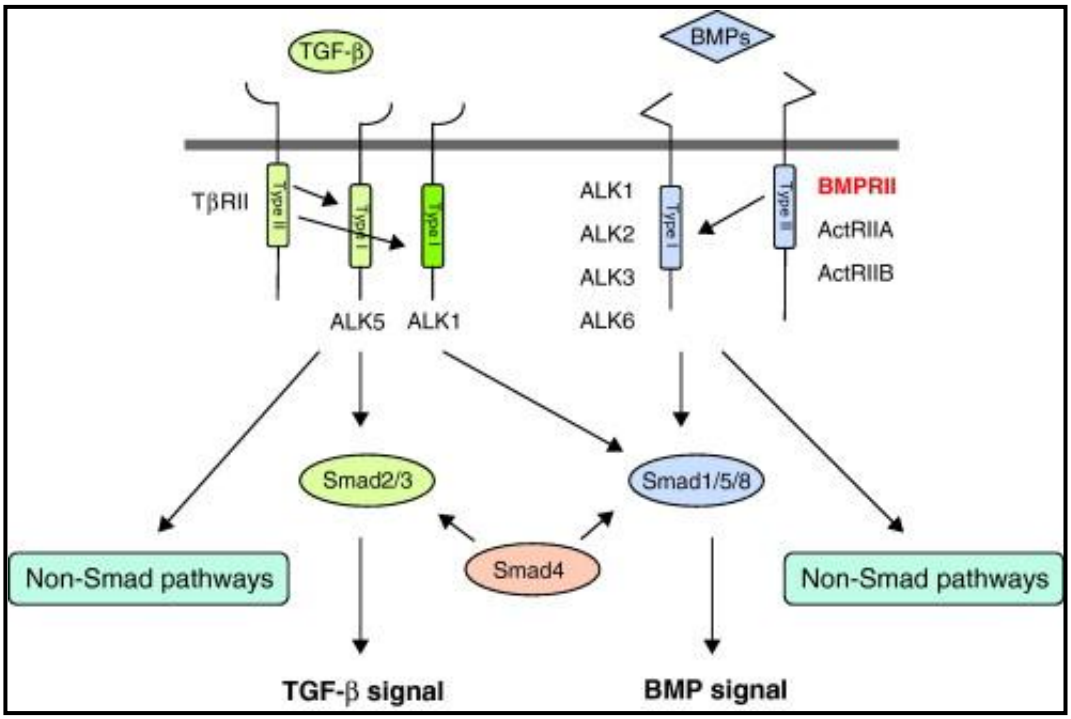


Figure 2. Signaling pathways of the TGF-β/BMP family. TGF-β binds to type II receptor (TβRII), and then TβRII phosphorylates type I receptor (ALK5). Activated ALK5 phosphorylates Smad2/3, which form complex with Smad4. ALK1 expresses only in the arterial endothelium and can transduce the TGF-β signal through Smad1/5/8. The BMP signal pathway is similar to the TGF-β signal pathway. There are three type II receptors (BMPRII, ActRIIA and ActRIIB) and four type I receptors (ALK1, 2, 3 and 6) for BMPs. Both type I and type II receptors are required to activate Smad1/5/8. TGF-β and BMPs also activate non-Smad pathways, including MAP kinase pathways such as p38 and Erk [34].

Bone morphogenetic protein 6 (*BMP6*), also known as Vegetal related1 (VGR1), is on chromosome 6 (6p24-p23), Figure2. It is produced by bone marrow mesenchymal (BMSC) and hematopoietic stem cells, which can differentiate into bone, cartilage, adipose, muscle, hematopoietic, synovial and other tissues [35].

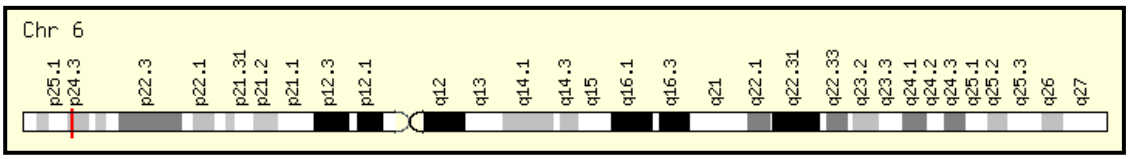


Figure 3. *BMP6* Gene in genomic location. *BMP6* is on chromosome 6 (6p24-p23) and locate at 7,726,332-7,881,961 bp [36].

The promoter region of *BMP6* gene lacks a canonical TATA box, which does not contain a GC rich region and steroid hormonal responsive elements [37]. Like other BMP family members, *BMP6* accessibility is modulated by specific antagonists. Receptor activation is controlled by co-receptors, by localization to distinct membrane microdomains, by endocytosis and by receptor associated proteins [38-40]. Approximately 40 TGF-β superfamily ligands differentially bind and signal through only 12 common receptors indicating that each receptor has multiple ligand-binding partners. *BMP6*, along with BMP5, BMP7 and BMP8, has a high affinity for type II receptors and lower affinity for type I receptors. Conversely BMP2 and BMP4 exhibit higher affinity for their type I receptors. A hydrophobic core is highly conserved between both lower and high affinity type II interfaces including ActRII and ActRIIb, (Figure 3) [41-45].

In the *BMP6* crystal structure, the H3 pre-helix loop region (residues 65–73) presents the largest difference between the *BMP6* and BMP7 structures [47], (Figure 4). Although highly homologous, *BMP6* and BMP7 emerge to have distinct type I receptor specificities with *BMP6* displaying a 20-fold higher affinity to BMPRIA than BMP7, but 20-fold lower than BMP2. This is absolutely an unexpected finding having in mind which *BMP6* shares numerous receptor binding and signaling characteristics with BMP7 [47-49]. N-glycosylation recognition motif of *BMP6* at Asn 73 in the wrist epitope is crucial for the recognition by the ACVRI [50].

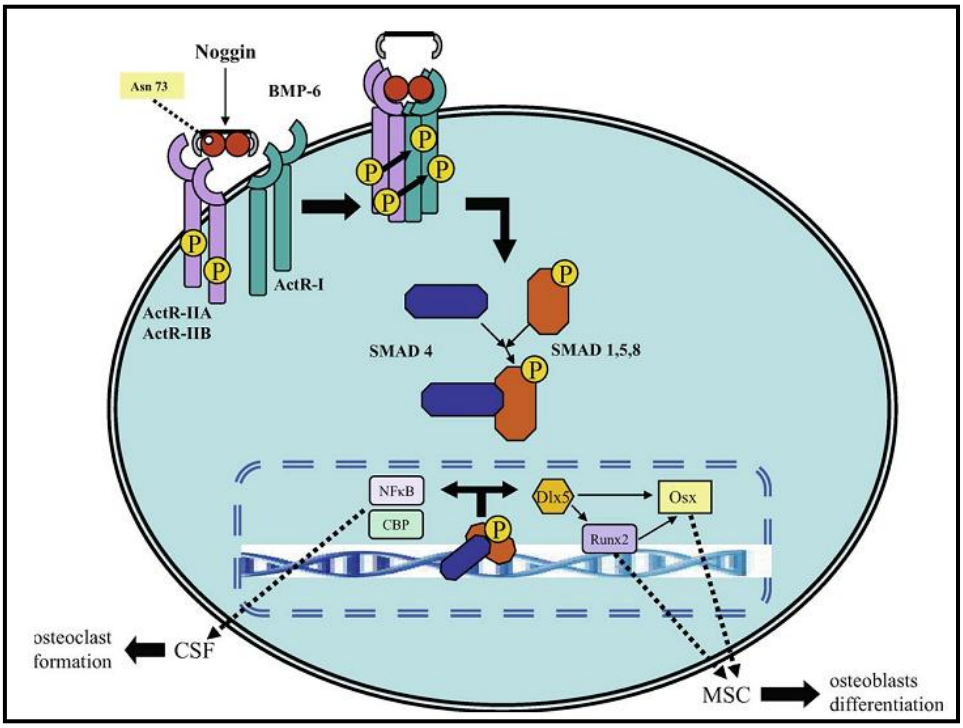


Figure 4. *BMP6* pathway. *BMP6* binds with high affinity to ACVRI, which is presented in many cell types. BMP antagonist noggin binds to *BMP6* and forms a complex, which is not stable upon binding to the receptor due to the specific recognition motif in the *BMP6* wrist epitope. Smads are activated which regulate expression of transcriptional factors and transcriptional coactivators involved in osteoblast (Dlx5, Runx2 and Osx) and osteoclasts formation and differentiation (NFkB and CBP) [46].

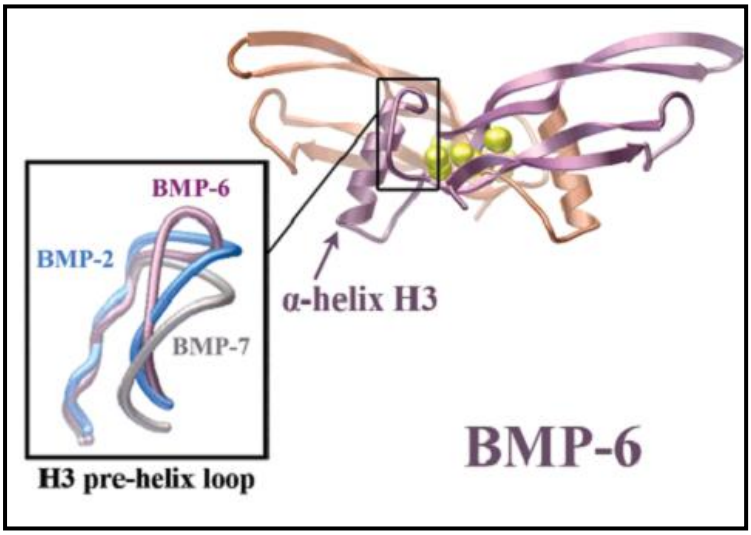


Figure 5. The structure of *BMP6*. The different H3 pre-helix loop conformations of BMP2 bound to BMPRI (blue), unbound BMP7 (gray), and unbound *BMP6* (purple). Sulfur atoms are depicted as yellow spheres.

There are many researches for the role of *BMP6* in cancers. For example, *BMP6* has also been identified as an inhibitor of breast cancer epithelial mesenchymal transition (EMT) through rescuing E-cadherin expression [51]. *BMP6* inhibits human bone marrow B lymphopoiesis upregulation of Id1 and Id3 [52]. Exogenous expression of *BMP6* to DU-145 prostate cancer cell cultures inhibited their growth by up-regulation of several cyclin dependent kinase inhibitors such as p21/CIP, p18, and p19 [53]. Taken together, it is possible that *BMP6* also play tumor suppressor role in colorectal adenocarcinoma.

4. Description of DNA methylation

Epigenetics are heritable changes including gene expression and chromatin organization which are independent of the DNA sequence itself. Epigenetic inheritance is an important mechanism, which allows stable propagation of gene activity from one generation of

cells to the next generation. Epigenetic changes, especially DNA methylation, are a common epigenetic marker in many eukaryotes in the sequence context CpG [54], (Figure 5).

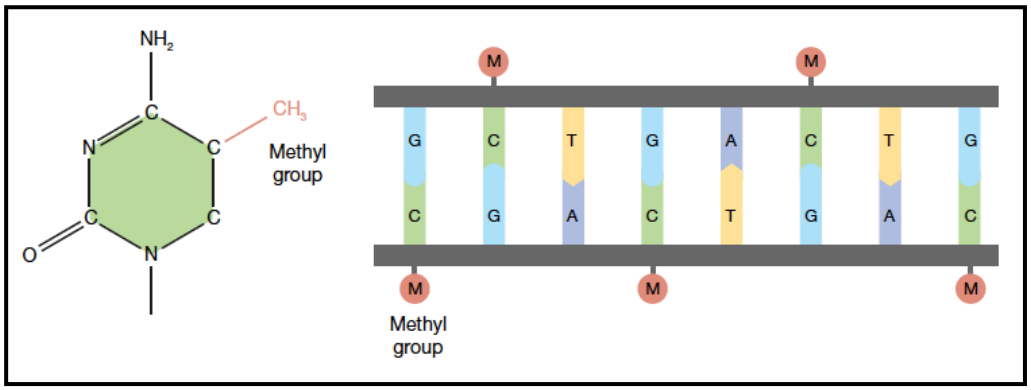


Figure 6. DNA methylation. A methyl group addition to the cytosine carbon 5 in cytosine-phosphate-guanine (CpG) and other nucleotide sequences inhibits the binding of transcription factors to promoters [55].

DNA methylation located at gene promoters is usually kept repressed. It is controlled by DNA methyltransferases with highly conserved catalytic motifs. In general, only a subset of potential target sequences in the genome is methylated, hence the distribution of methylation status can be used as epigenetic information demarcating the regions of transcriptional silence or transcriptional expression potential. Evaluation of DNA methylation patterns and broader DNA methylation profiles has important implications for understanding aberrant expression patterns and a certain disease [56]. Moreover, the epigenetic modification of DNA methylation is recognized as a crucial event in altering gene expression associated with carcinogenesis. It is more frequent in cancer than genetic changes [57]. All tissue types in carcinogenesis have methylation of promoter CpG islands (CGI) [58], (Figure 6). Therefore, DNA methylation leads to transcriptional silencing of genes involved in tumor suppression, cell cycle control, DNA repair, apoptosis, and invasion [59]. Expression of tumor suppressors, such as p16, p14, MGMT, and hMLH1 are frequently inactivated by this epigenetic event [60].

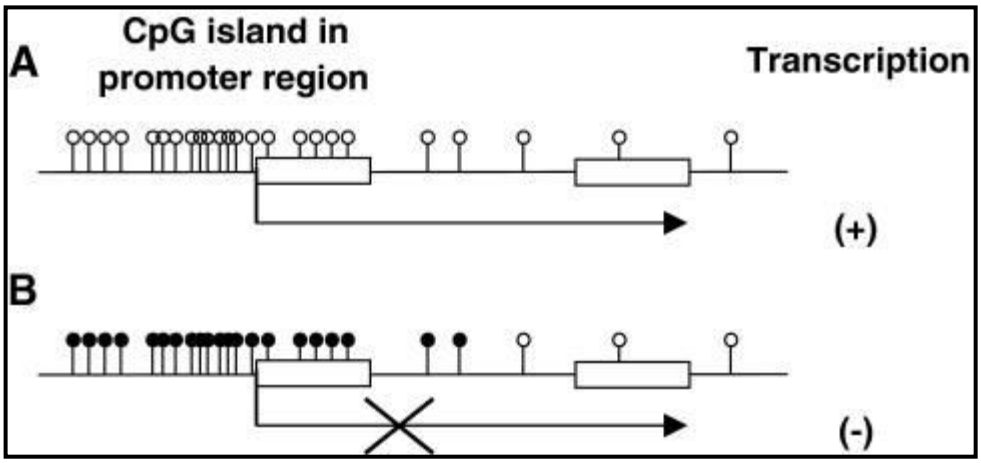


Figure 7. Methylation of a promoter CpG island (CGI) and transcription of its downstream gene. Open and closed circles unmethylated and methylated CpG sites, respectively. **(A)** In a normal cell, most CpG sites within a promoter CGI are unmethylated. **(B)** Methylation of most CpG sites (dense methylation) of the promoter CGI completely blocks transcription. If such methylation occurs in a tumor suppressor gene, it leads to inactivation of the tumor suppressor gene [61].

5. DNA methylation of *BMP6* in cancers

Various researchers evaluate association between DNA methylation of *BMP6* and cancarcinogenesis. Zhang M and colleagues have investigated epigenetic regulation of *BMP6* gene expression in breast cancer cell lines. They found that *BMP6* gene expression can be activated dose-dependently by estrogen in estrogen receptor positive (ER^+) breast cancer cell line MCF-7, but not in ER negative (ER^-) cell line MDA-MB-231. Moreover, the endogenous level of *BMP6* mRNA in ER^- cell line MDA-MB-231 was relatively lower than that in ER^+ MCF-7 and T47D cell lines. After the treatment with 5-aza-2′-deoxycytidine, the *BMP6* mRNA expression in MDA-MB-231 was up-regulated. They used enzyme restriction PCR (MSRE-PCR) and bisulfite sequencing (BSG) methods. The methylation of human *BMP6* gene promoter was detected in MDA-MB-231while in MCF-7 and T47D, *BMP6* gene promoter remained demethylated status. In 33 breast tumor specimens, promoter methylation of *BMP6* was detected

by methylation-specific PCR, hypermethylation of BMP-6 was observed in ER negative cases (16 of 16 cases (100%)), while obviously lower methylation frequency were observed in ER positive cases (3 of 17 cases (18%)), indicating that *BMP6* promoter methylation status is correlated with ER status in breast cancer [13].

Taniguchi A and colleagues reported that promoter methylation of the *BMP6* gene associated with adult T-cell leukemia. They investigated *BMP6* promoter methylation in patients with various types of leukemia. The *BMP6* methylation was found preferentially in adult T-cell leukemia (ATL) (49 of 60, 82%) compared with other types of leukemia studied including acute myeloid leukemia (3 of 67, 5%), acute lymphoblastic leukemia (6 of 38, 16%) and chronic lymphocytic leukemia (1 of 21, 5%). Among subtypes of ATL, the *BMP6* gene was more frequently methylated in aggressive ATL forms of acute (96%) and lymphoma (94%) types than less malignant chronic ATL (44%) and smoldering ATL (20%). They analyzed the methylation status of peripheral blood mononuclear cells from healthy donors and nonmalignant lymph nodes with reactive lymphadenopathy, none of which showed detectable *BMP6* methylation. The *BMP6* methylation was correlated with decreased mRNA transcript and protein expression. Expression of *BMP6* was restored by the demethylating agent 5-aza-20-deoxycy-tidine, suggesting that methylation was associated with the transcriptional silencing. Serial analysis demonstrated an increasing methylation of CpG sites in the*BMP6* promoter and the resultant suppression of *BMP6* expression as ATL progressed. They suggested that *BMP6* promoter methylation is likely to be a common epigenetic event at later stages of ATL and that the methylation profiles may be useful for the staging of ATL as well as for evaluation of the individual risk of developing the disease [4].

Daibata M and co-workers have investigated promoter hypermethylation of the *BMP6* gene in malignant lymphoma. Their experimental designs are investigation of *BMP6* promoter methylation and its gene expression in various histologic types of 90 primary lymphomas and 30 lymphoma cell lines and evaluation the effect of *BMP6* promoter hypermethylation on clinical outcome. They found that *BMP6* was epigenetically inactivated in

subsets of lymphomas. The silencing occurred with high frequency in diffuse large B-cell lymphoma (DLBCL) and Burkitt's lymphoma in association with aberrant *BMP6* promoter methylation. The methylation was observed in 60% (21of 35) of DLBCL cases and 100% (7 of 7) of DLBCL cell lines, and in 83% (5 of 6) of Burkitt's lymphoma cases and 86% (12 of 14) of Burkitt's lymphoma cell lines. Whereas, other histologic types of primary lymphomas studied had little or no detectable methylation (1of 49; 2%). The presence of *BMP6* promoter hypermethylation in DLBCL statistically correlated with a decrease in disease-free survival and overall survival. Multivariate analysis showed that the methylation profile was an independent prognostic factor in predicting disease-free survival and overall survival. They suggested that *BMP6* promoter was hypermethylated more often in aggressive types of lymphomas, and the hypermethylation is likely to be related to the histologic type of lymphomas. *BMP6* promoter methylation may be a potential new biomarker of risk prediction in DLBCL [62].

Kimura K and et al reported that the expression and methylation status of *BMP6* associate with malignant pleural mesotheliomas (MPMs). The expression status of *BMP6* mRNAs was examined in seven MPM cell lines by RT-PCR assay. The expression of *BMP6* was partly suppressed in 2 cell lines. Furthermore, partial methylation of *BMP6* was found in 2 cell lines whose expression was partly suppressed. Methylation status was found in 57 surgically resected MPM cases, in addition, aberrant methylation of *BMP6* in 4 (24%) cases from Japan and 12 (30%) cases from USA, showing significant difference in frequency of *BMP6* methylation between MPMs of the two countries. They indicated that *BMP6* genes were suppressed by DNA methylation and were significantly frequent in Japanese MPMs, presenting its pathogenic role and the ethnic difference in MPMs [63].

Recently, promoter methylation of the *BMP6* gene has been shown to be associated with various cancers. However, data about association of *BMP6* in CRC was not clear. Therefore, our study will examine the association of the DNA methylation and its protein expression of *BMP6* in CRC. We hope that *BMP6* will be used in the diagnosis in patients with CRC.

Objectives

1. To evaluate methylation status of *BMP6* in colorectal cancer specimen

2. To evaluate expression of *BMP6* in colorectal cancer specimen

3. To examine for any association between *BMP6* expression and *BMP6* methylation of colorectal cancer patients

4. To evaluate association between *BMP6* expression and clinicopathological features in colorectal cancer.

CHAPTER 2

RESEARCH METHODOLOGY

Materials

1. Fresh tissue and formalin-fixed paraffin embedded (FFPE) tissue samples

A total of 85 cases from patients who underwent a surgery in Songklanagarind Hospital from 2008 to 2010 were included in this study. Fresh tissue and formalin-fixed paraffin embedded (FFPE) tissue samples were used to study *BMP6* methylation and expression, respectively. All specimens were collected under informed consent. The research has been approved by the Institutional Review Board of the Faculty of Medicine, Prince of Songkla University.

2. Primer for methylation specific PCR (MSP)

The primer set for both methylation and unmethylation forms are reported by Kimura K, et al in 2008. In addition, MethPrimer3 program was used for designing primers. The primers were shown in Table 1.

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

Primer Name	Primer Sequence (5' to 3')	Size (bp)
BMP6-MF	GGTTTGTTGGGTAGTCGGG	19
BMP6-MR	GCCCCTCCCCAAATCG	16
BMP6-UF	TTGGGTAGTTGGGTGATTGTT	21
BMP6-UR	ACACCCCTCCCCAAATCA	18

3. Reagents

3.1 Reagents for methylation specific PCR (MSP)

HotStarTaq DNA polymerase (Qiagen)

dNTP mix (Qiagen)

Milli-Q water

Agarose powder (OmniPur)

TAE buffer (0.040M Tris-acetate and 0.001M EDTA)

Ethidium bromide (Invitrogen)

DNA ladder 100 bp (New England Biolabs)

6X Gel loading dye, blue (New England Biolabs)

3.2 Reagents for immunohistochemistry (IHC)

Xylene (J.T. Baker)

Absolute ethanol (Merck)

Distilled water

Tris EDTA buffer, pH 9.0

30% H_2O_2 (Merck)

PBS buffer (137mM NaCl, 2.7mM KCl, 10mM Na_2HPO_4, 2mM KH_2PO_4)

Normal horse serum (Gibco)

Primary antibody: Mouse monoclonal anti-BMP6 antibody (Abcam)

Secondary antibody: EnVision ™+ kit (Dako)

DAB Substrate (Sigma)

Hematoxylin (Merck)

Permount

4. Commercially provided kits

4.1 DNA extraction and purification

QIAamp DNA mini kit (Qiagen)

QIAquick gel extraction kit (Qiagen)

4.2 Bisulfite modification of DNA

EZ DNA Methylation™ kit (Zymoresearch)

5. Scientific instruments

5.1 Scientific instruments for Bisulfite modification of DNA

Automatic pipettes

Pipet tips

Gloves

Microcentrifuge tubes

Racks

Nanodrop 2000 Spectrophotometer (Thermo Fisher Scientific)

Sample boxes

Ice box

Refrigerated centrifuge (Kubota)

Heat block (Major Science)

5.2 Scientific instruments for methylation specific PCR (MSP)

Automatic pipettes

Pipet tips

Gloves

Microcentrifuge tubes

PCR tubes

Racks

Vortex genie 2 (Scientific Industries)

PCR Thermocycler (Bio-Rad)

Spin down centrifuge (Biosan)

Gel electrophoresis system (Mupid-Exu)

Gel document (Major Science)

5.3 Scientific instruments for immunohistochemistry (IHC)

Slides

Cover glass

Staining jar

Heat block

Slide Staining System

Staining dish

Humidified chamber

Automatic pipettes

Pipet tips

Immunopen

Beaker 500 mL

Microwave oven

Microscope

Methods

1. DNA extraction from fresh tissues

QIAamp DNA mini kit (Qiagen) was used for extraction of DNA from fresh tissue according to an enclosed manufacturer's protocol. First, the tissue sample was cut up into small pieces of about 25 mg. It was. When it was placed in a 1.5 mL microcentrifuge tube and 180 µl of buffer ATL was added. Next, 20 µL of proteinase K was added and mixed by vortex, followed by 3 hours incubation at 56 °C. The mixture was added with 200 µL of buffer AL and

mixed by vortex for 15 s. After it was then incubated at 70 °C for 10 min, it was added with 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 incubated for 10 min. After it was centrifuged at 10,000 rpm for 1 min at room temperature, the flow though was discarded. Next, buffer AW1 was added to the filtrate column, which was centrifuged at 10,000 rpm for 1 min at room temperature, followed by a re-centrifugation once at 13,000 rpm for 3 min at room temperature to eliminate the remaining buffer AW2. The column was placed in a new 1.5 microcentrifuge tube and added with 100 µL of buffer AE and incubated for 10 min at room temperature. DNA was eluted by centrifugation at 10,000 rpm for 3 min at room temperature.

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. The electrophoresis used 135 volts for 15 min in 0.5X TAE buffer bath. The electrophoresed gel was stained with ethidium bromide and visualized by exposing the gel to UV light. DNA samples were stored at -20 °C.

2. Bisulfite modification of DNA

DNA modification was prepared by using sodium bisulfite to convert unmethylated cytosines to uracils. The methylated cytosine in modified DNA was subsequently detected by using methylation specific PCR (MSP) technique. First, M-dilution buffer was added to the DNA sample and adjusted the total volume to 50 µL with sterile distilled water. The sample was mixed by flicking or pipetting up and down and incubated at 42 °C for 30 min. After the sample was incubated at 30 min, it was added 100 µL of the prepared CT conversion reagent to each sample and lightly vortex. The sample was incubated in the dark at 50 °C for 12 – 16 hr.

After 12 – 16 hr, the sample was incubated on ice for 10 min afterwards the sample was added 400 µL of M-binding buffer and mixed by pipetting up and down. The sample was loaded into a Zymo-spin I column and placed column into a 2 mL collection tube. The sample was centrifuged at 14,000 rpm for 1 min henceforth it was discard the flow-through. After 200 µL of M-wash buffer was added to the column, it was spin at 14,000 rpm for 1 min. Next, the column was added with 200 µL of M-desulphonation buffer and let stand at room temperature for 20 minutes. After the incubation, the column was spin at 14,000 rpm for 1 min. 200 µL of wash buffer was added to the column and spin at 14,000 rpm for 1 min at 2 times. Finally, 10 µL of M-elution buffer was added in the column and spin at 14,000 rpm for 1 min. DNA yield, length and purity were determined by Nanodrop 2000 Spectrophotometer (Thermo Fisher Scientific) analysis at 260 nm and 280 nm. DNA treated bisulfite samples were stored at -80 °C.

3. Methylation specific PCR (MSP)

Methylation Specific PCR (MSP) is a bisulfite conversion based PCR technique for the study of DNA CpG methylation. For MSP experiment, two pairs of primers are needed with one pair specific for methylated DNA (M) and the other for unmethylated DNA (U). To complete discrimination for methylated and unmethylated DNA, in each primer sequence, one or more CpG sites are included. First, DNA is modified with sodium bisulfite and purified. Then, two PCR reactions are performed using M primer pair and U primer pair. Successful amplification from M pair and U pair indicated methylation and unmethylation, respectively.

In this study, The primers were 5'-TTGGGTAGTTG GGTGATTGTT-3' (sense) and 5'-ACACCCCTCCCCAAATCA-3' (antisense) for unmethylated form and 5'-GGTTTGTTGGGTAGTCGGG-3' (sense) and 5'-GCCCC TCCCCAAATCG-3' (antisense) for the methylated form which give a PCR product of 248 bp, encompassing the BMP6 promoter region -836 to -589 bp relative to the transcriptional start site, (figure 7). Two pairs of primer, methylated and unmethylated, were used to amplify by HotStar Taq DNA polymerase (Qiagen). MSP reaction used the composition in table 2. The PCR was performed under the following

conditions: 95 °C 15 min, followed by 39 cycles at 94 °C for 30 s, 64.4 °C for 30 s and 72 °C for 45 s. Human methylated & non-methylated DNA sets (Zymoresearch) were used as negative and positive controls, respectively. Finally, PCR products were detected by 2% agarose gel electrophoresis at 135 volts for 30 min and stained with ethidium bromide. Thereafter, the remaining PCR products were purified by a QIAquick gel extraction kit (Qiagen), following the manufacturer protocol.

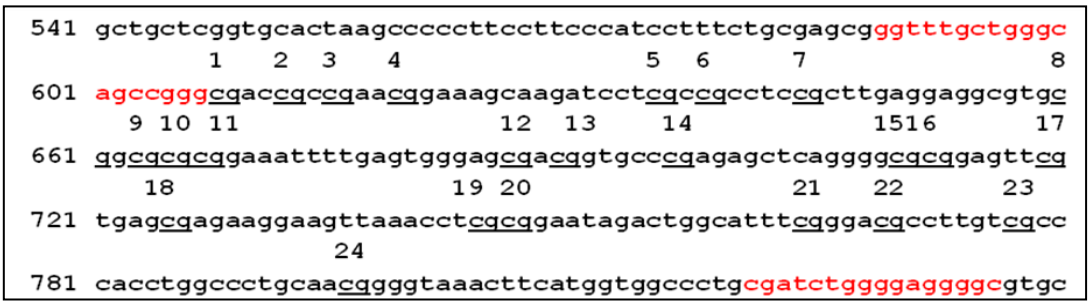


Figure 8. A map of the CpG islands in relation to the promoter of the BMP6 gene, primer locations and CpG sites. The positions of sense and antisence primers are indicated by red. The primers give a 248 bp PCR product with 24 CpG binucleotides (underlined and numbered 1 to 24).

Table 2. The composition of MSP reaction used in this study

Materials	Conc.	Final conc.	1x/20 µL
1. 10x buffer	10x	1x	2.0
2. 25 mM $MgCl_2$	25 mM	1.5 mM	1.2
3. 10 mM dNTP	10 mM	0.125 mM	0.25
4. Primer BMP6- F	10 µM	0.2 µM	0.4
5. Primer BMP6- R	10 µM	0.2 µM	0.4
6. HotStar Taq	5 unit/uL	0.0125 unit/ µL	0.05
7. Milli Q H_2O	-	-	14.7
8. DNA		50 ng	1

4. Immunohistochemistry (IHC)

First of all, the tissue was fixed in 10% neutral buffered formalin for at least 24 hr and embeds in paraffin wax according to embedding machine manufactures instructions. Second, the tissue was prepared 4 - 12 μm sections on the microtome and place on slides and heated in drying oven for 15 min at 60 °C. Third, the slides were deparaffinized by xylene at 2 times for 5 min. After that the slides were rehydrated by 100% ethanol at 2 times for 3 min and 70% ethanol at one for 3 min, and rinsed by distilled water. Then the slides were antigen retrieved by 1X Tris-EDTA buffer (pH 9.0) in staining dish. They were heated to boiling by microwave oven for 10 min and cold down at room temperature about 15-20 min and rinsed by distilled water. On the next the slides were blocked of endogenous peroxidase by 3% H_2O_2 for 5 min and rinsed by distilled water and washed by PBS buffer for 5 min. After that the slides were blocked of non-specific protein by 10% normal horse serum for 30 min. Then the slides were incubated by mouse monoclonal anti-BMP6 antibody (antibody: PBS; 1: 100) at 4 °C for overnight. Thereafter, the slides were washed by PBS buffer at 2 times for 5 min. Next, the slides were incubated by EnVision ™+ kit (Dako) for 30 min and washed by PBS buffer at 2 times for 5 min. After that the slides were dropped by DAB substrate for 5-10 min and dipped in hematoxylin for 15 s and swayed them in water. Next, the slides were rehydrated by 95% ethanol for 3 min and 100% ethanol at 2 times for 3 min and xylene at 3 times for 5 min, respectively. Finally, the slides were dropped permount mounting medium about 1-3 drops and closed the slides with cover glass slide.

5. Application study

5.1 BMP6 gene methylation analysis

DNA from CRC fresh tissue samples were subjected to amplification by MSP. The bands were shown on 2% agarose gel that presented methylation and unmethylation of BMP6 in samples. Results were investigated to pattern of methylation of BMP6 in CRC patients.

5.2 BMP6 gene expression analysis

After FFPE tissue samples of CRC were stained, BMP6 expression was detected under light microscopy. The expression was quantitated by Allred score. The Allred score is evaluated two categories (stain intensity and stain pattern). The numerical value for overall intensity (intensity score (IS)) is based on a 4 points system: 0, 1, 2, and 3 (for none, light, medium, or dark staining). The numerical value for percent stained (proportion score (PS)) is determined by a geometric rather than linear division; no stain = 0, $\geq$1/100 cells stained = 1; $\geq$1/10 cells stained = 2; $\geq$1/3 cells stained = 3, $\geq$2/3 cells stained = 4; all cells stained = 5. Addition of the two values gives the total Allred score; therefore, the Allred score can vary between 0 and 8. These scores are related with expression of *BMP6*. Results were displayed as pattern of expression of *BMP6* in CRC patients.

6. Statistical analysis

Sample size was calculated by this formula.

$$N = (Z_{\alpha/2})^2 \, p(1-p)/ \, d^2$$

N = Sample size

$Z_{\alpha/2}$ = Reliability coefficient

P = Probability of event in previous study

d = Acceptable error

In this study, the incidences of BMP6 methylation in other cancers have been 5-94 %**.** Thus, sample size was calculated as shown below:

5% incidences**; N =** $\frac{1.96^2(0.05)(0.95)}{0.05^2}$ = 72.99 = 73 cases

For clinicopathological parameters, data was presented as mean or percent. In part percent methylation was calculated as follows: M or (M + U)/100. In part the association between the BMP6 methylation status and immunohistochemical score, and clinicopathological parameters and immunohistochemical score was determined by using the Chi-square or Fisher's exact test, as appropriate. Survival analysis was performed using Log-rank test. P value < 0.05 was considered statistically significant. The analyses were performed on Intercool Stata program version 6.0 statistical package. (Stata Corporation, USA)

CHAPTER 3

RESULTS

1. Clinicopathological characteristics of patients

A total of 85 CRC (44 females and 41 males) were included in this analysis. The mean age of the patients was 63 years (range from 32 to 87 years) with 47 cases (55%) aged more than 60 years. The pathologic classification of stage and grade of tumor were defined according to the TNM staging system of the American Joint Committee on Cancer (AJCC). Approximately 65% of the cases were categorized as stage 3-4 on diagnostic work-up. Regarding the sites of the primary tumor, 50 cases (60%) were rectal in origin and 33 cases (40%) were colonic cancer. The demographic data of CRC patients included in this study was summarized in table 3.

2. Purity and concentrations of DNA

2.1 Genomic DNA

The genomic DNA from 85 frozen tissue samples collected in Songklanagarind hospital during the years 2008-2010 periods were included in this evaluation. The concentration and purity of genomic DNA was estimated by measuring the absorbance of DNA solution at 260 nm and 280 nm (OD_{260}/OD_{280}) with Nanodrop 2000 spectrophotometer. The concentration ranged from 40-4000 ng/μL and the ratio of OD_{260}/OD_{280} was in the range of 1.6-2.1.

2.2 DNA treated bisulfite

Spectrophotometric characteristics of bisulfite-treated DNA were similar to RNA. Therefore, detection used the same method as RNA measuring. The concentration and purity of bisulfite-treated DNA was estimated by measuring the absorbance of DNA solution at

260 nm and 280 nm (OD_{260}/OD_{280}) with Nanodrop 2000 spectrophotometer. The concentration ranged from 10-500 ng/µL and ratio of OD_{260}/OD_{280} was in the range of 1.6-2.4.

3. MSP optimization for the amplification of *BMP6*

The compositions of MSP reaction were optimized as shown in Table2. Besides, two pairs of primer were used in the amplification of *BMP6*, which were pre-optimized for annealing temperature (Tm). The optimal annealing temperature varied from the estimated Tm. Tm was calculated from formula of 2 °C X (A+T) + 4 °C X (C+G), which A+T is a number of A and T nucleotide, and C+G is the number of C and G nucleotide in the primer sequence. The actual Tm was 1-2 °C below the calculated Tm for primer. Tm for two pairs of primer (BMP6-MF, BMP6-MR, BMP6-UF and BMP6-UR) was 64.4 °C.

4. Incidence of *BMP6* methylation

In the present study, the methylation study of *BMP6* revealed hypermethylation status in 34 out of 85 cases (40%). There was no statistically significant association between *BMP6* hypermethylation and clinicopathological parameters of the CRCs studied as shown in table 3.

Table 3. Clinicopatological data and *BMP6* methylation status of 85 colorectal cancer cases studied

Parameter		**Methylation status**		**p-value**
		negative	**positive**	
All		51 (60)	34 (40)	
Sex	Female	29 (66)	15 (34)	0.25
	Male	22 (54)	19 (46)	

Age	≤ 60 > 60	24 (63) 27 (57)	14 (37) 20 (43)	0.59
Site of tumor	Rectum Colon	31 (62) 20 (39)	19 (38) 13 (41)	0.90
Differentiation	Well Moderate Poor	24 (69) 19 (58) 6 (60)	11 (31) 14 (42) 4 (40)	0.63
AJCC stage	Stages 1-2 Stages 3-4	16 (55) 35 (65)	13 (44) 19 (35)	0.39
T	T1-2 T3-4	7 (54) 44 (63)	6 (46) 26 (37)	0.54
N	N0-1 N2	27 (54) 24 (73)	23 (46) 9 (27)	0.09
M	M0 M1	37 (60) 14 (67)	25 (40) 7 (33)	0.57

5. Expression of *BMP6*

On immunohistochemical study, slides of CRC specimens were analyzed in parallel by two investigators in an effort to provide a consensus on staining patterns under light microscopy, Figure 8. The *BMP6* protein was also localized in the cytoplasm which was detected in stroma cells and epithelial cells. In this study, prostate cancer tissues were used to positive and negative control which shown in figure 9. The *BMP6* expression protein of CRC was shown in 1-5 score of PS, 0-3 score of IS and 3-8 score of AR (sum of IS and PS) as shown in figure 10.

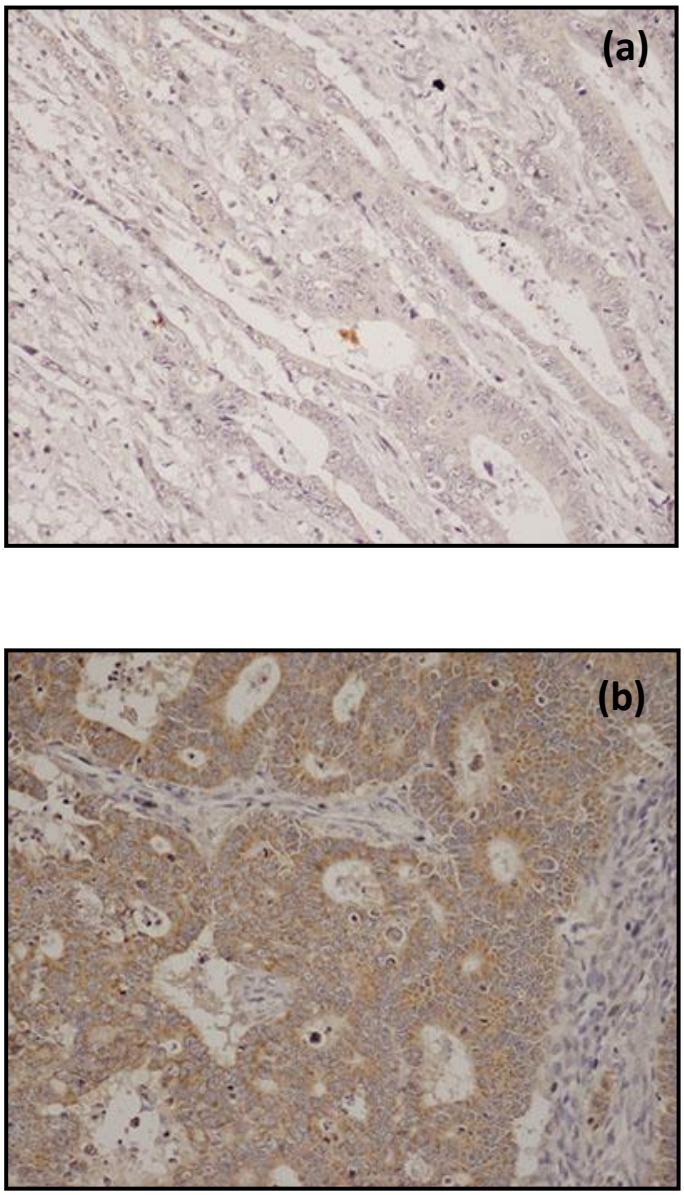
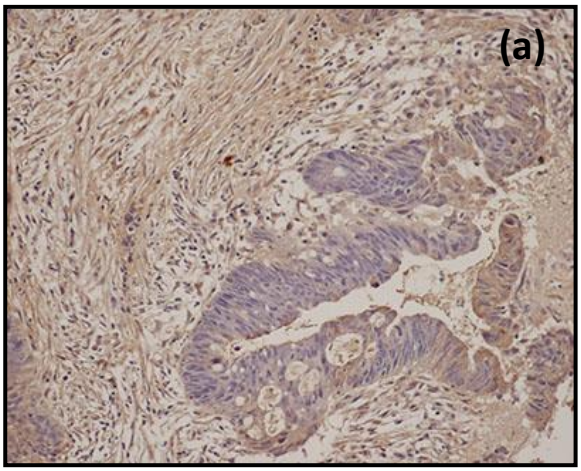
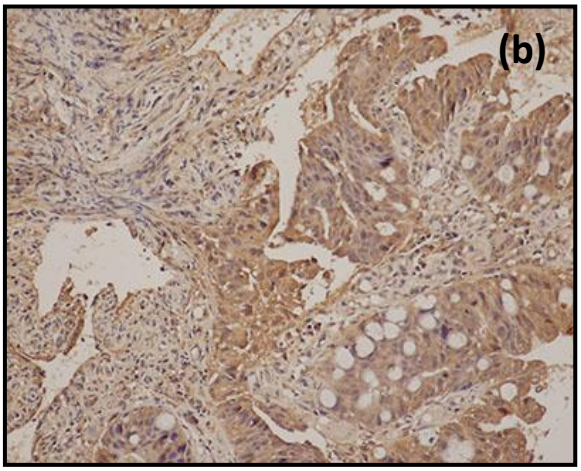


Figure 9. Positive and negative control of BMP6. (a) negative control was the prostate cancer tissues which wasn't incubated by primary antibody (PS = 0 score, IS = 0 score and AR = 0 score) (b) positive control was the prostate cancer tissues which was incubated by primary antibody (PS = 5 score, IS = 3 score and AR = 8 score). Magnification: X20.



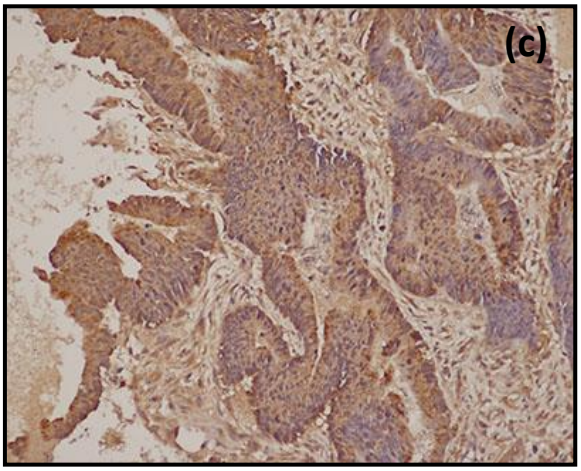
Score: IS = 2, PS = 4 and AR = 6

Methylated status



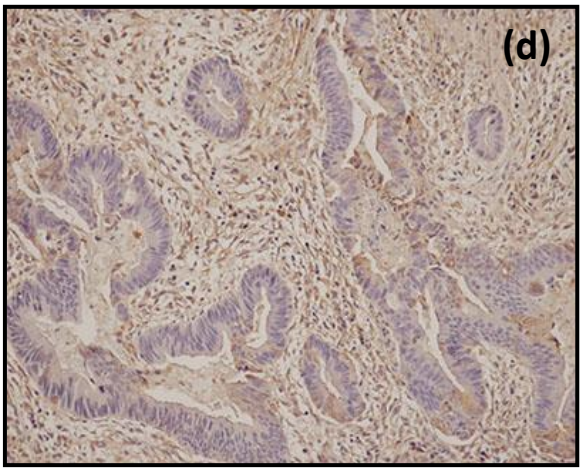
Score: IS = 3, PS = 5 and AR = 8

Unmethylated status



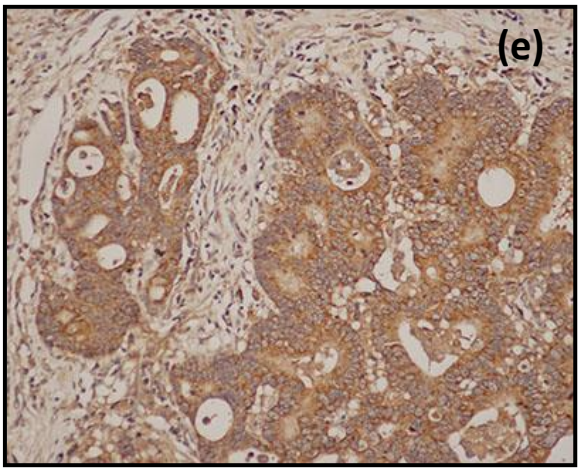
Score: IS = 3, PS = 5 and AR = 8

Unmethylated status



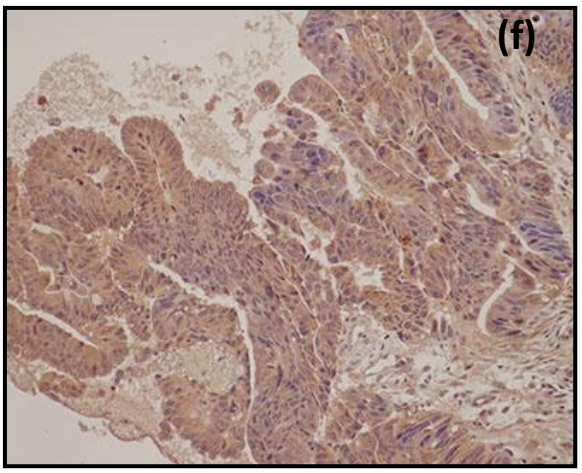
Score: IS = 1, PS = 3 and AR = 4

Methylated status



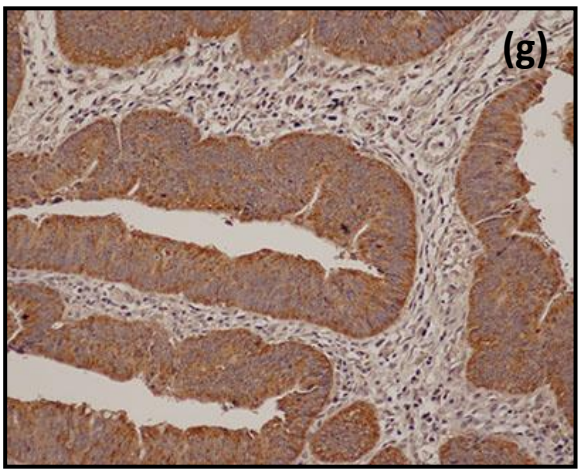
Score: IS = 3, PS = 5 and AR = 8

Unmethylated status



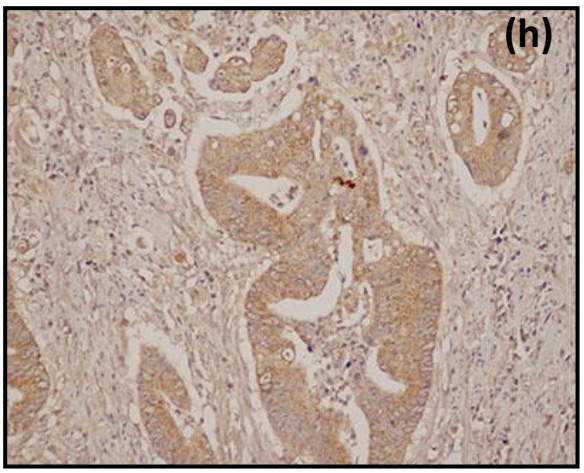
Score: IS = 3, PS = 5 and AR = 8

Unmethylated status



Score: IS = 3, PS = 5 and AR = 8

Methylated status

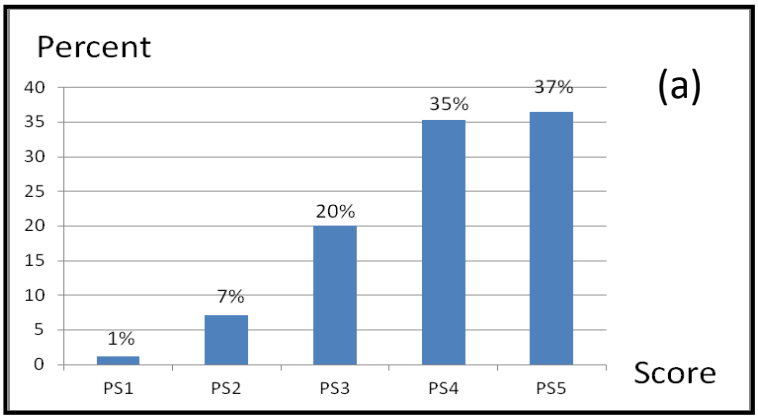


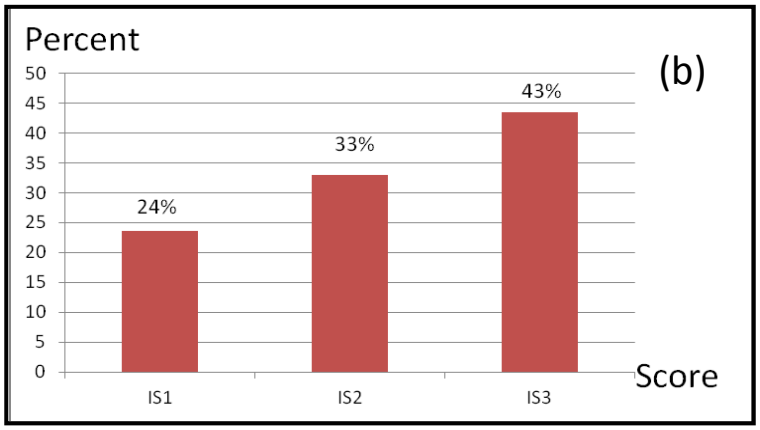
Score: IS = 3, PS = 5 and AR = 8

Unmethylated status

Figure 10. Representative pictures of immunohistochemistry on CRC specimens stained for *BMP6* antibody. (a)-(h) show methylation status and expression of *BMP6* in tumor cells. Magnification: X20.

The PS study, we found a PS of 5 in 31 cases (37%), 4 in 30 cases (36%), 3 in 17 cases (20%), 2 in 6 cases (7%) and 1 in 1 cases (1%). Furthermore, the IS study presented IS score of 3 in 37 cases (43%), 2 in 28 cases (33%) and 1 in 20 cases (24%). Taken together, AR score was 8 in 23 cases (27%), 7 in 18 cases (21%), 6 in 16 cases (19%), 5 in 15 cases (18%), 4 in 6 cases (7%) and 3 in 7 cases (8%) as shown in figure 11.





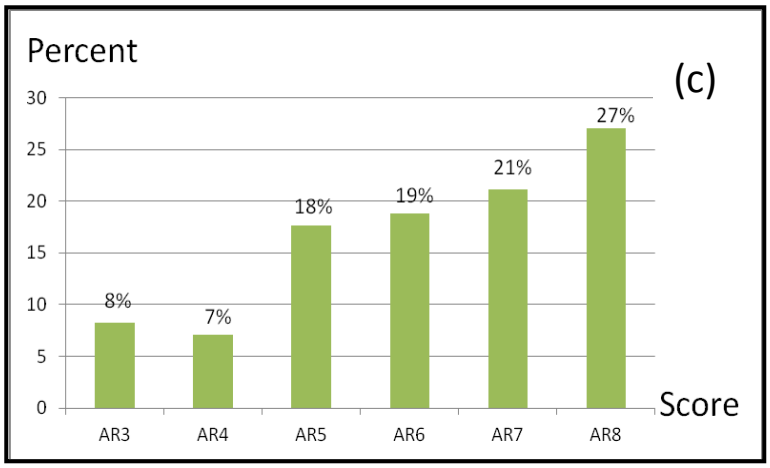


Figure 11. The distribution of percent of positivity score (PS), Intensity score (IS) and Allred score (AR). (a) the distribution of PS. (b) the distribution of IS and (c) AR.

6. The association between the *BMP6* methylation status and immunohistochemical score

CRC samples with PS1-2, 3-4 and 5 had an incidence of hypermethylation at 57%, 45% and 29%, respectively. The IS of 1, 2 and 3 had an incidence of hypermethylation at 55%, 46% and 27%, respectively. IS of more than 3 was significantly correlated with hypermethylation status (p-value 0.03). Overall, hypermethylation had a tendency to have inverse correlation with both factors, however, the correlation did not reach a statistically significant level, Figure 12.

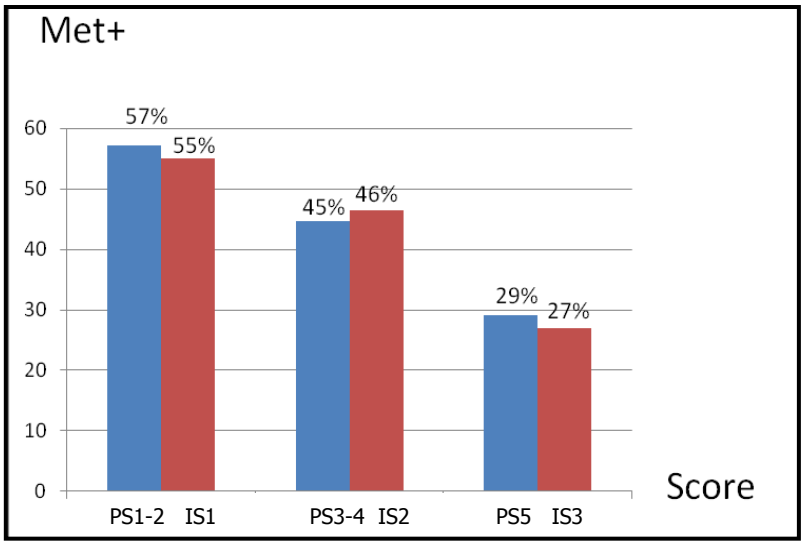


Figure 12. Correlation between positivity score (PS) and intensity score (IS) stratification and methylation status of *BMP6*

When PS and IS were taken together as the AR, and analyzed for its correlation with the methylation status, there seemed to be inverse correlation between the score and the hypermethylation status as shown in figure 10 and 13. Serial analysis revealed that a cut-off value at 6 provided the lowest p-value on the Chi-square test (p-value = 0.05). Tumors with an AR of 6

or less had a 50% incidence of hypermethylation when 30% of tumor with higher score had the status.

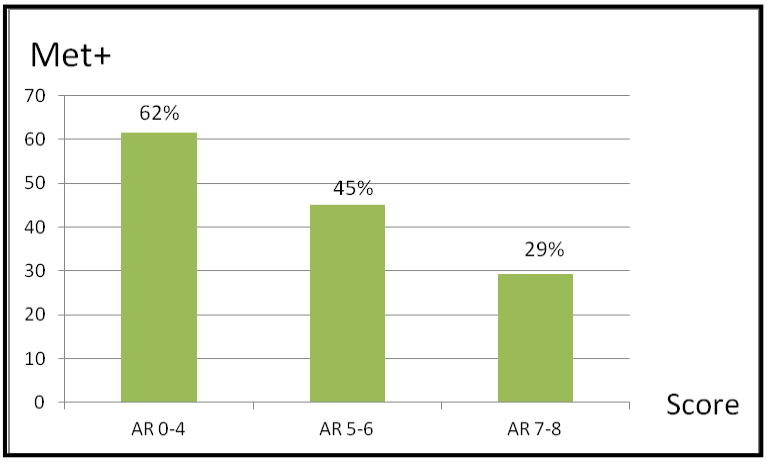


Figure 13. Frequency of *BMP6* hypermethylation according to Allred score (AR)

7. The association between clinicopathological parameters and immunohistochemical score

In this study, PS, IS and AR had no statistically significant correlation with age, sex, tumor location and stage of tumor in CRC patients, Table4-6. However, it should be noticed that lower T tumor tended to have more frequent hypermethylated status of *BMP6* (p-value 0.05).

Table 4. Clinicopathological parameters and incidence of methylated *BMP6* in each positivity score (PS) stratification (PS0-4 and PS5). Percentage is shown in the parentheses.

Parameter		**PS0-4**	**PS5**	**p-value**
All		54 (64)	31 (36)	
Sex	Female	28 (64)	16 (36)	0.98
	Male	26 (63)	15 (37)	
Age	≤ 60	26 (68)	12 (32)	0.40
	> 60	28 (60)	19 (40)	
Site of tumor	Rectum	33 (66)	17 (34)	0.44
	Colon	19 (58)	14 (42)	
Differentiation	Well	22 (63)	13 (37)	0.76
	Moderate	19 (58)	14 (42)	
	Poor	7 (70)	3 (30)	
AJCC stage	AJCC 1-2	18 (62)	11 (38)	0.94
	AJCC 3-4	34 (63)	20 (37)	
T	T1-2	5 (38)	8 (62)	0.05
	T3-4	47 (67)	23 (33)	
N	N0-1	31 (62)	19 (38)	0.88
	N2	21 (64)	12 (36)	
M	M0	39 (63)	23 (37)	0.94
	M1	13 (62)	8 (38)	

Table 5. Clinicopathological parameters and incidence of methylated *BMP6* in each intensity score (IS) stratification (IS1-2 and IS5). Percentage is shown in the parentheses.

Parameter		**IS1-2**	**IS3**	**p-value**
All		48 (56)	37 (44)	
Sex	Female	24 (55)	20 (45)	0.71
	Male	24 (58)	17 (41)	
Age	≤ 60	24 (63)	14 (36)	0.26
	> 60	24 (51)	23 (49)	
Site of tumor	Rectum	26 (52)	24 (48)	0.30
	Colon	21 (63)	12 (36)	
Differentiation	Well	21 (60)	14 (40)	0.19
	Moderate	14 (42)	19 (58)	
	Poor	7 (70)	3 (30)	
AJCC stage	AJCC 1-2	17 (59)	12 (41)	0.79
	AJCC 3-4	30 (55)	24 (44)	
T	T1-2	6 (46)	7 (54)	0.41
	T3-4	41 (59)	29 (41)	
N	N0-1	26 (52)	24 (48)	0.30
	N2	21 (63)	12 (36)	
M	M0	36 (58)	26 (42)	0.65
	M1	11 (52)	10 (48)	

Table 6. Clinicopathological parameters and incidence of methylated *BMP6* in each Allred score (AR) stratification (AR0-6 and AR7-8). Percentage is shown in the parentheses.

Parameter		**AR0-6**	**AR7-8**	**p-value**
All		44 (52)	41 (48)	
Sex	Female	21 (48)	23 (52)	0.44
	Male	23 (56)	18 (44)	
Age	≤ 60	20 (53)	18 (47)	0.88
	> 60	24 (51)	23 (49)	
Site of tumor	Rectum	26 (52)	24 (48)	0.75
	Colon	16 (48)	17 (51)	
Differentiation	Well	17 (49)	18 (51)	0.72
	Moderate	15 (45)	18 (55)	
	Poor	6 (60)	4 (40)	
AJCC stage	AJCC 1-2	15 (52)	14 (48)	0.88
	AJCC 3-4	27 (50)	27 (50)	
T	T1-2	5 (38)	8 (61)	0.34
	T3-4	37 (53)	33 (47)	
N	N0-1	22 (44)	28 (56)	0.14
	N2	20 (61)	13 (39)	
M	M0	31 (50)	31 (50)	0.85
	M1	11 (52)	11 (48)	

8. Association between BMP6 hypermethylation and clinical outcomes

Median follow-up period was 39 months. Two-year progress-free survival (2Y-PFS) in CRC stage I-II (100%) was significantly higher than those of stage III-IV (63%) (p-value < 0.01). 2-Y PFS in CRC with *BMP6* hypermethylation (74%) was not different from those without hypermethylation (76%) (p-value 0.63)

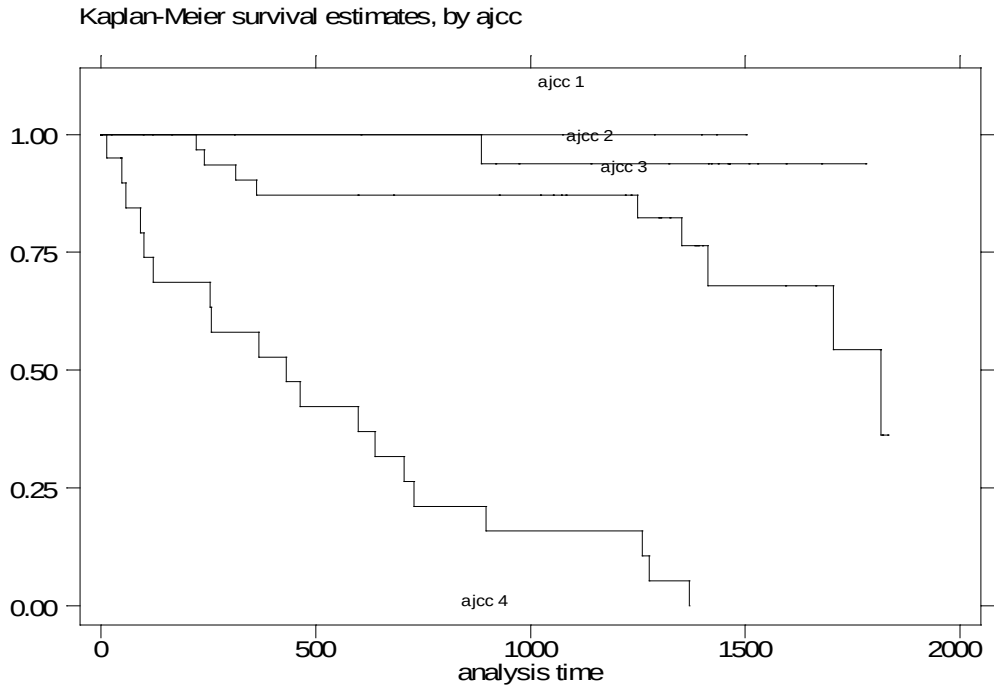


Figure 14. Kaplan Myer Curve showing progress-free survival in CRC patients stage I-IV

Progress-free survival were compared between CRC stage I-III cases who had methylated BMP and non-methylated BMP by using Log-rank test. The analysis showed that there was no statistical significant different between survival of the 2 groups.

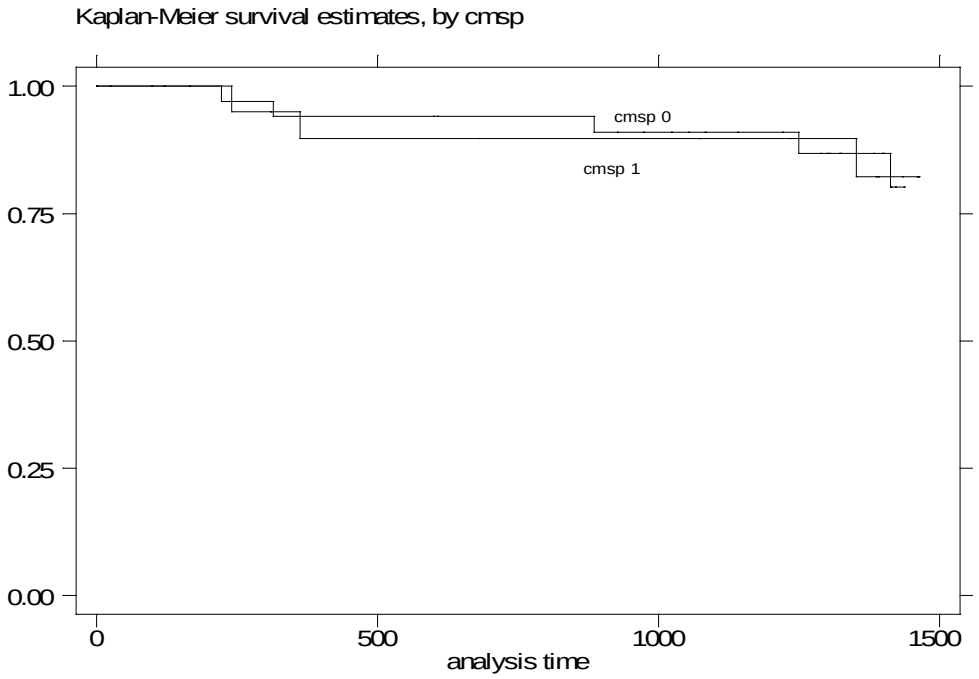


Figure 15. Kaplan Myer Curve showing progress-free survival comparison in CRC patients stage I-IV

CHAPTER 4

DISCUSSION

BMP is a member of the TGF-β superfamily of signaling molecules which are important to inhibit cellular proliferation and is involved in organogenesis, particularly of the lung, heart and kidney [63-64]. *BMP6* is similar to other BMP members which signals through ligation of type I and type II serine-threonine kinase receptors (BMPR) and signal downstream by phosphorylation of Smad1, 5, and 8. These Smads then form complexes with the Smad4 and are translocated into a nucleus where they exert regulation of target genes specific for the BMP pathway. Thus, BMP signaling is similar to the paradigm established by TGF-β signaling. It is logical, therefore, to suppose that any functional impairment by genetic alterations or epigenetic inactivation of genes involved in the BMP/TGF-β pathway may induce the development of cancers. There are many reports that described genetic alterations or epigenetic inactivation of *BMP6* which was shown to be associated with tumorigenesis and/or disease progression in several cancers [65]. *BMP6* inhibited proliferation of prostate cancer cells by up-regulation of several cyclin-dependent kinase inhibitors. Loss of sensitivity to *BMP6* is necessary to achieve the malignant phenotypes [66-68]. Furthermore, epigenetic inactivation of *BMP6* by gene promoter hypermethylation promoted lung tumor development [69].

In this study, we have analyzed the methylation status of the BMP6 promoter region in CRC samples. We found intensive promoter methylation with significant high frequency in CRC which suggests that the *BMP6* promoter methylation seems to be tumor specific. We also showed methylation-dependent loss of BMP6 expression protein levels. In fact, methylation was observed to play a role in mediating gene expression. Evidence of this has been found in other studies that show that methylation near gene promoters varies considerably depending on cell type, with more methylation of promoters correlating with low or no transcription of the gene. Thus, our findings implied a causal relationship between methylation

of the *BMP6* promoter and transcriptional repression. Our data are, to the best of our knowledge, the first demonstration of epigenetic inactivation of a BMP family member in CRC.

On the other hand, although the study found that the *BMP6* methylation status had a tendency to be correlated with lower T staging, correlation between the methylation and other clinical or pathological parameters was not clear. We explained this negative finding by the fact that there are other molecules that play their roles in CRC development. Moreover, the number of cases in our study was still less than expected earlier. Including more cases to the study and more factors to the analysis may provide clearer picture.

Early detection is one of the most important approaches to reduce mortality of colon cancer patients while the disease is still localized and curable. Furthermore, longer survival and better quality of life can be achieved with earlier detection of progressive CRC. A subset of genes harboring genetic or epigenetic alterations during colorectal carcinogenesis has some potential to serve as early detection markers or markers for therapy monitoring. In contrast to detection of genetic mutations, which requires a large number of possible mutations, DNA methylation alterations appear to be simpler and easier and particularly amenable to sensitive detection. DNA methylation can be detected in tumor-derived DNA found in the bloodstream or in samples of body fluids obtained from cancer patients. If optimal sensitivity and specificity is gained through the identification of markers that show the highest differences in methylation between the cancer and the background, testing for methylated DNA in blood or stool samples may have great potential as a new screening marker for CRC as well as a tool for disease monitoring in colon cancer patients [70].

In addition, the use of many different technical approaches may lead to inconsistent results in various reports. MSP is the most commonly used technique for methylation analysis due to its relative simplicity and safety and high sensitivity and specificity. Similar to other PCR techniques, a drawback of MSP is a possible contamination of the analyzed sample and obtaining false-positive results, but it has been proved to be as efficient as MethyLight to define CIMP subgroups. Real-time-PCR (Q-MSP) approaches such as MethyLight and the

TaqMan-MSP assay, which involve fluorescent signals during the process of DNA amplification, have been used to quantify the number of methylated alleles (in a single region) among wild-type DNA. However, the use of different cutoffs for statistical analysis of CIMP has not been sufficiently discussed [71].

Finally, the study should also determine methylation status and expression of *BMP6* in normal colorectal tissue samples and compare the methylation pattern with the expression of *BMP6* in CRC cases. The results will provide insights into the underlying biology of cancer and open the door to translational diagnostic, prognostic, and therapeutic applications for cancer patients.

CHAPTER 5

CONCLUSIONS

This study aimed to determine the methylation status of the BMP6 promoter region and expression of the *BMP6* in CRC. Moreover, the study analyzed for association between methylation status and expression of the BMP6, association between methylation status of the *BMP6* and clinicopathological, and association between expression of the *BMP6* and clinicpphatological parameters in CRC. MSP method was used for examining the methylation status of the *BMP6* promoter region. Furthermore, IHC method was used for examining the expression of the BMP6.

The major findings of this study were summarized and shown below:

1. BMP6 methylation was detected in 34 of 85 cases (40%). There was no statistically significant association between BMP6 hypermethylation and clinicopathological parameters of the CRCs studied.

2. Tumors with an AS of 6 or less had a 50% incidence of hypermethylation, compared to 30% of tumor with higher score. IS score of more than 3 was significantly correlated with lower methylation status. Although an inverse correlation between hypermethylation status and protein expression can be impressed, the correlation did not reached a statistically significant level.

3. There was no statistically significant association between BMP6 expression and clinicopathological parameters of the CRCs studied.

4. Two-year progress-free survival (2Y-PFS) in CRC stage I-II (100%) was significantly higher than those of stage III-IV (63%) (p-value < 0.01). However, 2-Y PFS in CRC with BMP-6 hypermethylation (74%) was not different from those without hypermethylation (76%) (p-value 0.63).

In conclusion, our study showed for the first time that the promoter of the *BMP6* gene was methylated in a subset of CRC. The methylation of *BMP6* tended to be inversely correlated with its protein expression. Although there was no correlation between the methylation status and clinicopathological data, the study observed a tendency that early T stage tumor had higher BMP6 immunoreactivity. This may suggest tumor suppressor role of the gene. Further study that includes more samples may provide a clearer picture regarding this correlation.

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