Epigenetic Identification of *Paired Box Gene 5* as a Functional Tumor Suppressor Associated with Poor Prognosis in Patients with Gastric Cancer

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ABSTRACT

Background & Aims: DNA methylation induced tumor suppressor gene silencing plays an important role in carcinogenesis. By using methylation-sensitive representational difference analysis, we identified *paired box gene 5* (*PAX5*) being methylated in human cancer. *PAX5* locates at human chromosome 9p13.2 and encodes a 391 amino acids transcription factor. However, the role of *PAX5* in gastric cancer is still unclear. Hence, we analyzed its epigenetic inactivation, biological functions, and clinical implications in gastric cancer.

Methods: Methylation status of *PAX5* promoter in gastric cancer cell lines and clinical samples was evaluated by methylation specific polymerase chain reaction (MSP) and bisulfite genomic sequencing (BGS). The effects of PAX5 re-expression in cancer cell lines were determined in proliferation, cell cycle, apoptosis, migration and invasion assays. Its *in vivo* tumorigenicity was investigated by injecting cancer cells with PAX5 expression vector subcutaneously into the dorsal flank of nude mice. Chromosome Immunoprecipitation (ChIP) and cDNA expression array were performed to reveal the molecular mechanism of the biological function of *PAX5*.

Results: *PAX5* was silenced or down-regulated in seven out of eight of gastric cancer cell lines examined. A significant down-regulation was also detected in paired gastric tumors compared with their adjacent non-cancer tissues ($n = 18$, $P = 0.0196$). In contrast, *PAX5* is broadly expressed in all kinds of normal adult and fetal tissues. The gene expression of *PAX5* in the gastric cancer cell line is closely linked to the promoter hypermethylation status. In addition, the expression levels could be restored by exposure to demethylating agents 5-aza-2'-deoxycytidine.

Re-expression of PAX5 in AGS, BGC823 and HCT116 cancer cells reduced colony formation ($P < 0.01$) and cell viability ($P < 0.05$), arrested cell cycle in G0/G1 phase ($P = 0.0055$), induced cell apoptosis ($P < 0.05$), repressed cell migration and invasion ($P = 0.0218$) *in vitro*. It also inhibited tumor growth in nude mice ($P < 0.05$). The molecular basis of its function were investigated by cDNA expression array and demonstrated that ectopic expression of *PAX5* up-regulated tumor suppressor gene *P53*, anti-proliferation gene *P21*, pro-apoptosis gene *BAX*, anti-invasion gene *MTSS1* and *TIMP1*; and down-regulated anti-apoptosis gene *BCL2*, cell cycle regulator *cyclinD1*, migration related gene *MET* and *MMP1*. ChIP assay indicated that *P53* and *MET* are direct transcriptional target of PAX5. Moreover, *PAX5* hypermethylation was detected in 90% (145 of 161) of primary gastric cancers compared with 16% (3 of 19) of non-cancer tissues ($P < 0.0001$). After a median follow-up period of 15.4 months, multivariate analysis revealed that gastric cancer patients with *PAX5* methylation had a significant poor overall survival compared with the unmethylated cases ($P = 0.0201$).

Conclusions: Our results demonstrated that *PAX5* promoter methylation directly mediates its transcriptional silence and commonly occurs in gastric cancer. *PAX5* gene can act as a functional tumor suppressor in gastric carcinogenesis by playing an important role in suppression of cell proliferation, migration, invasion, and induction of cell apoptosis. Detection of methylated *PAX5* may be utilized as a biomarker for the prognosis of gastric cancer patients.

摘 要

研究背景及目的：DNA 甲基化誘導的抑癌基因沉默在癌症發生過程中扮演重要的角色。通過檢測癌症中 DNA 甲基化水平的差異，我們找到了配對盒基因 5（*paired box gene 5*，*PAX5*）。*PAX5* 基因位於 9 號染色體（9p13.2），編碼一個含有 391 個氨基酸的轉錄因子。然而，*PAX5* 基因在胃癌中的作用還不清楚。因此，我們研究了這個基因在胃癌中的表觀遺傳機理、生物學功能以及臨床檢測方面的應用。

方法：甲基化特異的聚合酶鏈式反應（MSP）和亞硫酸氫盐測序法（BGS）被用於檢測 *PAX5* 基因在胃癌細胞系和臨床樣本中的甲基化狀況。我們在 *PAX5* 基因沉默的癌細胞系中重新表達 PAX5 蛋白，并研究該蛋白在細胞增殖、細胞週期、凋亡、轉移和侵入方面的作用。給裸鼠皮下注射 PAX5 表達的癌細胞的實驗用於證實該蛋白在體內的抑癌性。該蛋白相關功能的分子機理的研究則採用染色質免疫共沉澱（ChIP）和 cDNA 表達陣列進行分析。

結果：在 87.5%（7/8）的胃癌細胞系中，*PAX5* 基因表達發生了沉默或下調。同時，PAX5 的病人胃癌組織中的表達顯著低於配對的旁臨非癌組織的表達（$P = 0.0196$）。與此相對的是，*PAX5* 基因在正常成人和嬰兒的各種組織中廣泛表達。有趣的是，*PAX5* 基因表達狀況與基因啟動子區域的超甲基化的關係密切。不僅如此，經過去甲基化處理的細胞系中，PAX5 的表達得到了恢復。我們在癌細胞系 AGS、BGC823 和 HCT116 中表達 PAX5 蛋白。實驗證明 PAX5 的表達可以在體外抑制細胞群落的生成（$P < 0.01$）以及細胞活性（$P < 0.05$），可以阻礙細胞週期的由 G1 期進入 S 期（$P = 0.0055$），可以誘導細胞凋亡的發生（$P < 0.05$），阻止癌細胞轉移和侵入（$P = 0.0218$）。裸鼠體內試驗實驗還表明，

PAX5 可以抑制腫瘤的生長（$P < 0.05$）。通過 cDNA 表達分析，與 *PAX5* 失活的細胞相比，我們檢測到了抑癌基因 *P53*、細胞增殖抑制基因 *P21*、促凋亡基因 *BAX*、抗侵入基因 *MTSS1* 和 *TIMP1* 基因的上調，以及抗凋亡基因 *BCL2*、細胞週期調控基因 *cyclinD1*、癌細胞轉移相關基因 *MET* 和 *MMP1* 基因的下調。這些基因共同參與了上述抑癌作用的過程。并且，PAX5 蛋白能够直接调控 *P53* 和 *MET* 基因的表达。此外，我們還檢測了 *PAX5* 基因在臨床樣本中的甲基化頻率，161 個胃癌組織樣本中有 145 個檢測到了甲基化（90%），而在 19 個正常胃組織中，只有 3 個檢測到了甲基化（16%），兩組樣品甲基化差異顯著（$P < 0.0001$）。經過平均 15.4 個月的隨訪，所得的病人資料被用於多元分析。分析顯示 *PAX5* 未被甲基化胃癌病人的生存時間顯著長于 *PAX5* 被甲基化的胃癌病人（$P = 0.0201$）。

結論：上述的實驗結果證實了 *PAX5* 基因啟動子區域的甲基化直接導致了該基因表達的下調，而這種下調在胃癌病人中廣泛存在。功能分析也顯示 *PAX5* 基因可以抑制細胞增殖、轉移、侵入并誘導細胞凋亡，從而抑制胃癌。因此，檢測 *PAX5* 基因的甲基化可以被應用於胃癌患者的預後。

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PUBLICATIONS

Conference Abstracts:

Xiaoxing Li, Kin F Cheung, Junhong Zhao, Xiaowei Ma, Minnie YY Go, Eagle SH Chu, Alfred SL Cheng, Qian Tao, Joseph JY Sung, Hsiang-fu Kung, Jun Yu, Epigenetic Identification of Paired Box Gene 5 as a Functional Tumor Suppressor Associated with Poor Prognosis in Patients with Gastric Cancer. Digestive Disease Week 2010 Abstract, submitted

Chunming Ding, **Xiaoxing Li**, Grace Wong, Larry H Lai, Joseph J Sung, Molecular Analysis of Stomach Microbial Diversity in Non-*Helicobacter pylori* Gastritis and Normal Patients. Digestive Disease Week 2008 Abstract, San Diego, California, USA, 2008.

Journal Articles:

Xiao-Xing Li, Grace Lai-Hung Wong, Ka-Fai To, Vincent Wai-Sun Wong, Larry Hin Lai, Dorothy Kai-Lai Chow, James Yun-Wong Lau, Joseph Jao-Yiu Sung, Chunming Ding, Bacterial Microbiota profiling in Gastritis without *Helicobacter pylori* infection or Non-Steroidal Anti-Inflammatory Drug Use. PLoS One, 2009, 4(11): e7985.

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5-Aza:	5-aza-2'-deoxycytidine
AJCC:	American Joint Committee
AN2:	Type 2 aniridia
ANOVA:	Analysis of variance
AP-2:	Activating protein 2
ATCC:	American Type Culture Collection
AUC:	Area under the ROC curve
BAX:	BCL2-associated X protein
BCL2:	B-cell lymphoma-2
BGS:	Bisulfite genomic sequencing
B.M.:	bone marrow
BNIP3:	B-cell lymphoma-2/adenovirus E1B 19 kD-interacting protein 3
BRCT:	Breast cancer C-terminal
BSA:	bovine serum albumin
BSAP:	B-cell specific transcription factor
Cag A:	Cytotoxin-assisted gene A
CCND1:	Cyclin D1
CD19:	B-lymphocyte antigen CD19
CDK:	Cyclin-dependent kinases
CGI:	CpG island
ChIP:	Chromosome immunoprecipitation
CHNG2:	Congenital hypothyroidism non-goitrous type 2
CNS:	Central nervous system
COBRA:	Combined bisulfite restriction analysis
COX-2:	Cyclooxygenase-2
CRC:	Colorectal cancer
CT:	Computed tomography
CTF:	CCAAT-binding transcription factor

DAB:	Diaminobenzidine
DAPK:	Death-associated protein kinase
DMEM:	Dulbecco's Modified Eagle's Medium
DNMT:	DNA methyl-transferases
EBV:	Epstein-Barr virus
ECM:	Extracellular matrix
E. coli:	*Escherichia coli*
EUS:	Endoscopic ultrasonography
Eya4:	Eyes absent homolog 4
FBS:	Fetal bovine serum
FITC:	Fluorescein isothiocyanate
FKHR:	Forkhead in rhabdomyosarcoma
FLT3:	Fms-related tyrosine kinase 3
GBX1:	Gastrulation brain homeobox 1
GC:	Gastric cancer
GFP:	Green fluorescent protein
HEPES:	4-(2-hydroxyerhyl)piperazine-1-erhanesulfonic acid
hMSH2:	Human mutS homolog 2
HSIL/SCC:	High-grade lesions/squamous cell carcinomas
H. pylori:	*Helicobacter pylori*
ID-4:	Inhibitor of DNA binding 4
IL-1:	Interleukin-1
ILK:	Integrin linked kinase
IRF8:	Interferon regulatory factor 8
LB:	Luria-Bertani
LIMS2:	LIM-and-senescent-cell-antigen-like-domains-2
LMX1A:	LIM homeobox transcription factor 1 alpha
L.N.:	Lymph node
MBP:	Methylcytosine-binding protein

MET (c-MET):	Mesenchymal-epithelial transition factor
MGMT:	O-6-methylguanine-DNA methyltransferase
MIM:	Missing in metastasis
MMP1:	Matrix metalloproteinases 1
Mox1:	Mesenchyme homeobox protein 1
MRI:	Magnetic Resonance Imaging
MSP:	Methylation specific polymerase chain reaction
MTS:	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
MTSS1:	Metastasis suppressor 1
ORF:	Open reading frame
P16 (CDKN2A):	Cyclin-dependent kinase inhibitor 2A
P21 (CDKN1A):	Cyclin-dependent kinase inhibitor 1A
P53 (TP53):	Tumor protein 53
PAX:	Paired box
PAX5:	Paired box gene 5
PBS:	Phosphate buffered saline
PCI:	Phenol/chloroform/isoamyl alcohol solution
PCR:	Polymerase chain reaction
PI:	Propidium iodide
PPARγ:	Peroxisome proliferator activated receptor gamma
PTIP:	PAX interacting (with transcription-activation domain) protein
PVDF:	Polyvinylidene difluoride
qPCR:	Real-time quantitative polymerase chain reaction
RASSF1A:	Ras association domain family protein 1
ROC:	Receiver Operating Characteristic
RT-PCR:	Reverse transcription polymerase chain reaction
RUT:	Rapid urease test
Runx3:	Runt-related transcription factor 3
SAM:	S-adenosylmethionine

SD:	Standard deviation
SDS-PAGE:	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SF/HGF:	Scatter factor or hepatocyte growth factor
SFRP:	Secreted frizzled receptor protein
S.M.:	Skeletal muscle
SOCS3:	Suppressor of cytokine signaling 3
SOX1:	Sex determining region Y box 1
SYK:	Spleen tyrosine kinase
TBS/T:	Tris-buffered saline and 0.1% Tween 20
TBX18:	T-box protein 18
TIMP1:	Tissue inhibitors of metalloproteinase 1
TNF-α:	Tumor necrosis factor alpha
TNM:	Tumor, node and metastasis
TSS:	Transcription starting site
TUNEL:	Terminal deoxynucleotidyl transferase-mediated dUTP digoxigenin nick end labeling
UCSC:	University of California Santa Cruz Genome Bioinformatics
WHO:	World Health Organization
WS:	Waardenburg's syndrome
WT1:	Wilms' tumor suppressor gene 1

CHAPTER 1 Introduction

1.1 General Introduction

Gastric cancer (GC) is one of the most common cancers and leading cause of cancer-related death worldwide. GC is often asymptomatic or causes only nonspecific symptoms in its early stages. The tumor takes several years to progress to advanced stage before being discovered, so majority of GCs are diagnosed at a quite advanced stage, when the tumors are usually invasive and hard to treat. Because the main therapy for GC is surgical resection, early detection method for GC could help the doctors to improve clinical outcome of this disease.

DNA methylation biomarkers are suitable to be used as gastric diagnosis markers. They relate to key aspects of tumor biology and can be measured reliably on routinely available patient specimens. It has become evident that the epigenetic modification that leads to abnormal transcription regulation commonly occurs in cancers (P. A. Jones & Baylin, 2002). Therefore, methylation status is being actively investigated as a biomarker for cancer (Eads, Lord et al., 2001; Soria, Rodriguez et al., 2002).

Up to now, majority of genes regulated by epigenetics have not been well characterized. By using methylation-sensitive representational difference analysis, we identified *paired box 5* (*PAX5*), a gene that encodes a *paired box* transcription factor, in human cancer. This study will mainly focus on the biological function and clinical application of *PAX5* in GC.

1.2 Gastric Cancer

1.2.1 Over view of gastric cancer

Stomach, a part of human digestive system, is located in the upper abdomen region between esophagus and duodenum. Gastric cancer, medically called gastric malignant neoplasm, is a class of diseases in which a group of stomach cells display uncontrolled growth, invasion and sometimes metastasis to other locations in the body via lymph or blood.

Most (85% - 95%) cases of GCs are adenocarcinomas that occur in gastric mucosa. There are two major types of GCs which are noncardia and proximal cardia. In the noncardia GC, about 40% of cases develop in antrum and other 40% in body of stomach; in the cardia GC, approximately 15% of cases develop in the upper part. Other cases of GC develop in more than one part of the stomach.

GC is a cancer that generally metastasizes to other organs of the body before the symptoms occur. This is one of the main reasons for its poor prognosis. GC may have following signs and symptoms: indigestion, heartburn, loss of appetite in the early stage, pain in the upper abdomen, nausea and vomiting, diarrhea or constipation, bloating of the stomach after meals, weight loss, and vomiting blood or containing blood in the stool in the late stage. Over time, untreated stomach cancer will invade surrounding tissues such as the peritoneum, the lungs, or the liver.

1.2.2 Epidemiology of gastric cancer

According to the most recent available estimates, GC is the fourth most common cancer worldwide, with 934 000 cases per year (Parkin, Bray et al., 2005). Its incidence shows wide geographical variation. Figure 1.1 shows a map of the incidence rates of GC in males, standardized to the world population.

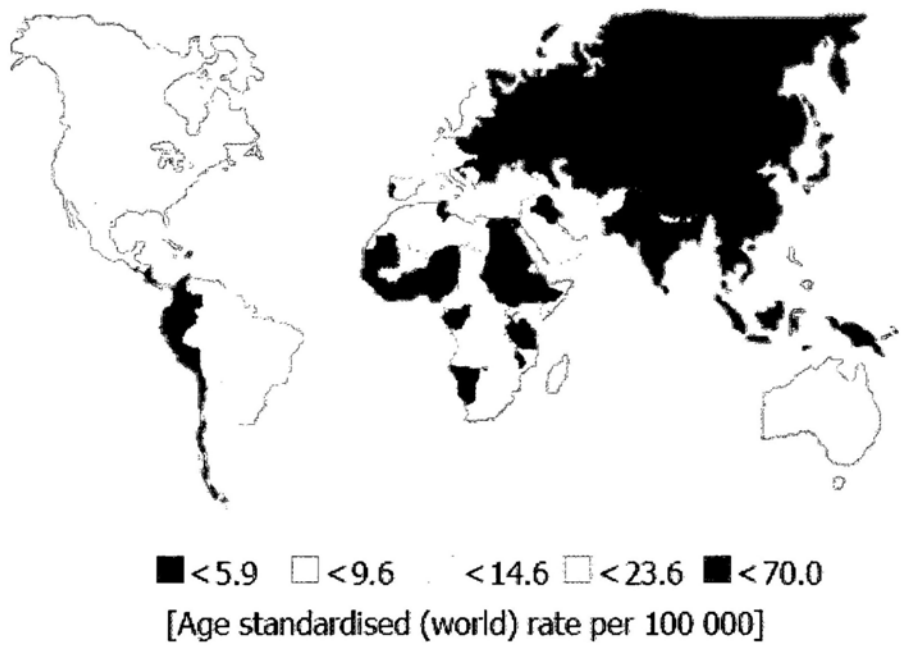


Figure 1.1 Incidence of GC in males. (Diagram adopted from Parkin (2004))

As one of the most common cancers, the incidence rates of GC show wide geographical (Figure 1.1). Around two-thirds of GC cases occur in the developing countries (Forman & Burley, 2006). As shown in Figure 1.1, the very high incidence areas (> 70 cases per 100,000 males) include East Asia, Eastern Europe and Western of South America, whereas the low incidence region includes North America, Western Europe and Australia.

GC is the commonest incident cancer in west pacific region including China, Japan,

Korea and Australia (Figure 1.2), and the second leading cause of cancer related deaths in this region. Additionally, it is the one of the commonest cancers and second leading cause of cancer death in China, which is the country with the most population (Table 1.1). According to the statistic of World Health Organization (WHO), the number of GC patient in China account for 42% of the total GC patient amount.

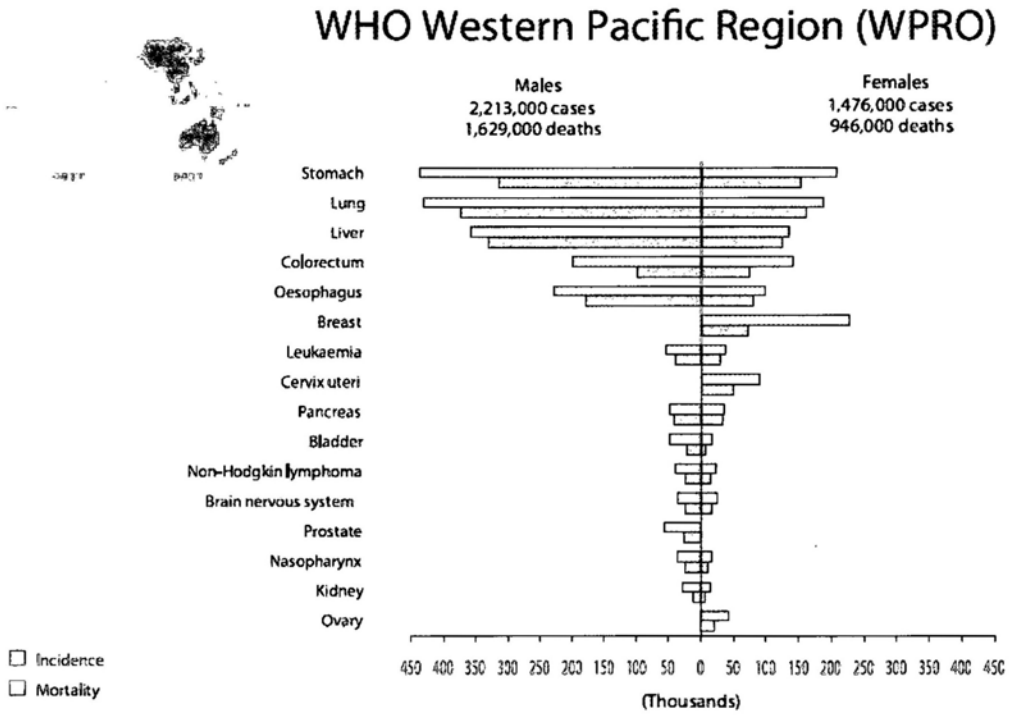


Figure 1.2 Cancer incidence and mortality in western pacific region estimated by WHO. (from World Cancer Reprot 2008)

Table 1.1 Cancer cases of commonest cancers in China projections for 2006.

(From website: www.excel-china.com)

Top Cancers in China in 2006

Incidence and Mortality rates

NO.	Cancer	Incidence (/100,000)	Mortality rate (/100,000)
1	Lung cancer	Male:49; female: 29.9	32
2	Gastric cancer	Male: 55.7; female: 21	20
3	Colorectal cancer	Male: 37.2; female: 33.5	5.4
4	Liver cancer	23.7	NA
5	Cervical cancer	20	3.25
6	Esophageal cancer	13	14.59
7	Breast cancer	11.61	2.61
8	Pancreatic cancer	5.1	About 5.1
9	Bladder cancer	Male: 4.02; female: 0.93	1.01
10	Brain cancer	4~9	About 4~9
11	Lymphoma	2	1.5
12	Nasopharyngeal cancer	2-3,~ 30-50	1.88
13	Leukemia	4.7	2.53
14	Ovarian cancer	4.39	NA

Note: The statistic data above was collected from various sources that differed slightly and are compilations.

www.excel-china.com

In the gender group, GC is generally more common in males than females (Table 1.1). It is reported that the GC risk in males is 2 times higher compared with females (Crew & Neugut, 2006). GC has been reported as the third commonest cancer (after lung and prostate cancers) in male and the fifth commonest cancer (after breast, cervix, large bowel and lung cancers) in female all over the world; it is also the second cause of cancer deaths in male and the fourth in female (Parkin et al., 2005). However, GC is commonest cancer in male in China.

The incidence of GC increases with age progressively. The incidence is highest around 60 to 75 years old and relatively low before 45 years old. GC appears 10 to 15 years later onset of the intestinal type in female patients than in males. In addition, the intestinal type GC is relatively rare in females than in males. However, after the menopause, the incidence of GC in female begins to rise rapidly since the estrogens may act as protect effect against GC (Sipponen & Correa, 2002).

1.2.3 Gastric cancer pathology

According to the classification set up by Lauren, there are two histopathological types in GC, one is intestinal and other one is diffuse type. The ratio for each type is intestinal type 50%, diffuse type 35%, and others are mixed type (Sengupta, Saha et al., 1992).

The intestinal type usually happens in distal stomach and follows a prolonged precancerous phase in patients with atrophic gastritis and intestinal metaplasia. In this type of GC, tumor cells develop irregular tubular structures, multiple lumens and reduced stroma. According to glandular structure, cellular pleomorphism and mucosecretion, adenocarcinoma can be divided into three grades of differentiation: well, moderate and poorly differentiate. In the clinical application, using the intestinal type brings a better prognosis compared with the diffuse type.

The development of GC is a multistage progression of mucosal lesions from gastritis to GC. Some situation may be precancerous and may lead to raised risk of stomach cancer. They include: chronic gastritis, atrophic gastritis and pernicious anemia.

Staging is a way of judging the development of the tumor in a patient. The staging process bases on the tumor and the range to which it has spread to other tissues. The most common staging system for stomach carcinoma is set up by American Joint Committee (AJCC) in 2006 which is called TNM (tumor, node and metastasis) staging system:

- Stage 0: GC has just begun to affect the mucosal layer without invasion to other layer. The survival rate is greater than 90% (The 5-year survival rates are from the introduction of Layke and Lopez (2004)).

- Stage I: Tumor invades other part of the stomach wall. Up to six nearby lymph nodes may be involved. The survival rate ranges from 58% to 78%.

- Stage II: Tumor invades the lamina proparia or submucosa and migrates to seven to fifteen regional lymph nodes. Or, tumor invades muscularis propria or subserosa and migrates to up to six regional lymph nodes. Or, tumor invades visceral peritoneum without invasion of adjacent structures. The survival rate is approximate 34%.

- Stage III: Tumor invades all tissue layers of stomach and migrates to seven to fifteen regional lymph nodes. Or, tumor penetrates visceral peritoneum without invasion of adjacent structures and metastases up to six regional lymph nodes. Or, tumor invades adjacent tissues. The survival rate is around 20%.

- Stage IV: Cancer affects nearby organs and tissues. Or, cancer may even have been carried through the lymph system to distant parts of the body. The survival rate is less than 7%.

1.2.4 Risk factors for gastric cancer

There are several risk factors for the development of GC. Researchers and doctors would like to know more information about the causes for stomach cancer. For

instance, a higher risk of GC has been observed among people living in Japan. However, the reason of this increased risk is still unclear. The major risk factors for stomach cancer include the following:

- Dietary factors

Many studies including some case–control ones, tried to find the relationship between dietary factors and the development of stomach cancer. People who eat large amounts of foods preserved with nitrates, nitrites and other substances, such as through smoking, salting or pickling have an adverse effect which are potentially carcinogenic (H. J. Kim, Chang et al., 2002; S. A. Lee, Kang et al., 2003; Tsugane, Sasazuki et al., 2004). In contrast, it seems that diets rich in fruits, vegetables, and other food with plentiful vitamin C provide a protective effect and are associated with the reduced GC risk (Steinmetz & Potter, 1996; Plummer, Vivas et al., 2007)). Heavy alcohol use may also increase GC risk, reporting by case-control studies (Bagnardi, Blangiardo et al., 2001).

- *Helicobacter pylori* infection

Helicobacter pylori (*H. pylori*) isolated by Marshall and Warren (1984), is a gram-negative bacteria responsible for the gastric mucosa. It is one of the most common infections with an estimated prevalence of about 50% all over the world and nearly 90% in developing countries. Infection of the stomach by *H. pylori* is a common cause of gastric ulcers. Numerous cohort and case control studies has demonstrated that increased risk of developing GC has been enhanced by *H. pylori*

infection (Moayyedi, Axon et al., 2002). It has been reported that developing countries with a high prevalence of *H. pylori* infection have high incidence rate of GC; prevalence of *H. pylori* infection declined in many developed countries also decreased GC possibility. For example, China and Japan which have the high incidence also have the high prevalence of *H. pylori* infection (Prinz, Schwendy et al., 2006). Additionally, it is also believed that this is mainly a cohort effect, with the high prevalence of infection in birth cohorts (Webb, Knight et al., 1994). The prevalence of *H. pylori* infection is also associated with socioeconomic factors such as educational and living level, also poor healthy condition and overcrowding because its infection can be spread by oral ingestion (Goodman & Correa, 2000; Olmos, Rios et al., 2000). Although *H. pylori* always habitats in stomach mucosa, resulting in chronic gastritis, there may be no symptom in many cases.

Being an important cause of stomach cancer (Olmos et al., 2000), *H. pylori* infection has be classified as a type I carcinogen in human beings (World Health Organization and the International Agency for Research on Cancer Consensus group, 1994). *H. pylori* infection is a sequential multi-step process of gastric carcinogenesis suggested to be induced through the long term chronic gastric inflammation (Naumann & Crabtree, 2004) and drives the procedure of gastric lesions of chronic gastritis, gastric atrophy, intestinal metaplasia, dysplasia and gastric carcinoma (Figure 1.3) (Correa, 1996).

There are several cofactors in host which determine the individual susceptibility of *H. pylori*. One of the cofactors is genetic polymorphisms in human being. The

genetic polymorphisms include the pro-inflammatory cytokine *interleukin-1* (*IL-1*), *interleukin-10* (*IL-10*) and *tumor necrosis factor alpha* (*TNF-α*). People with both high risk genotypes and *H. pylori* infection would result in increased risk of non-cardia cancer (El-Omar, Rabkin et al., 2003). The cytotoxin-assisted gene A (Cag A), which is another cofactor, increases the risk for the development of gastric carcinoma when compared to cases with negative Cag A (Blaser, Perez-Perez et al., 1995; Parsonnet, Friedman et al., 1997; Huang, Zheng et al., 2003).

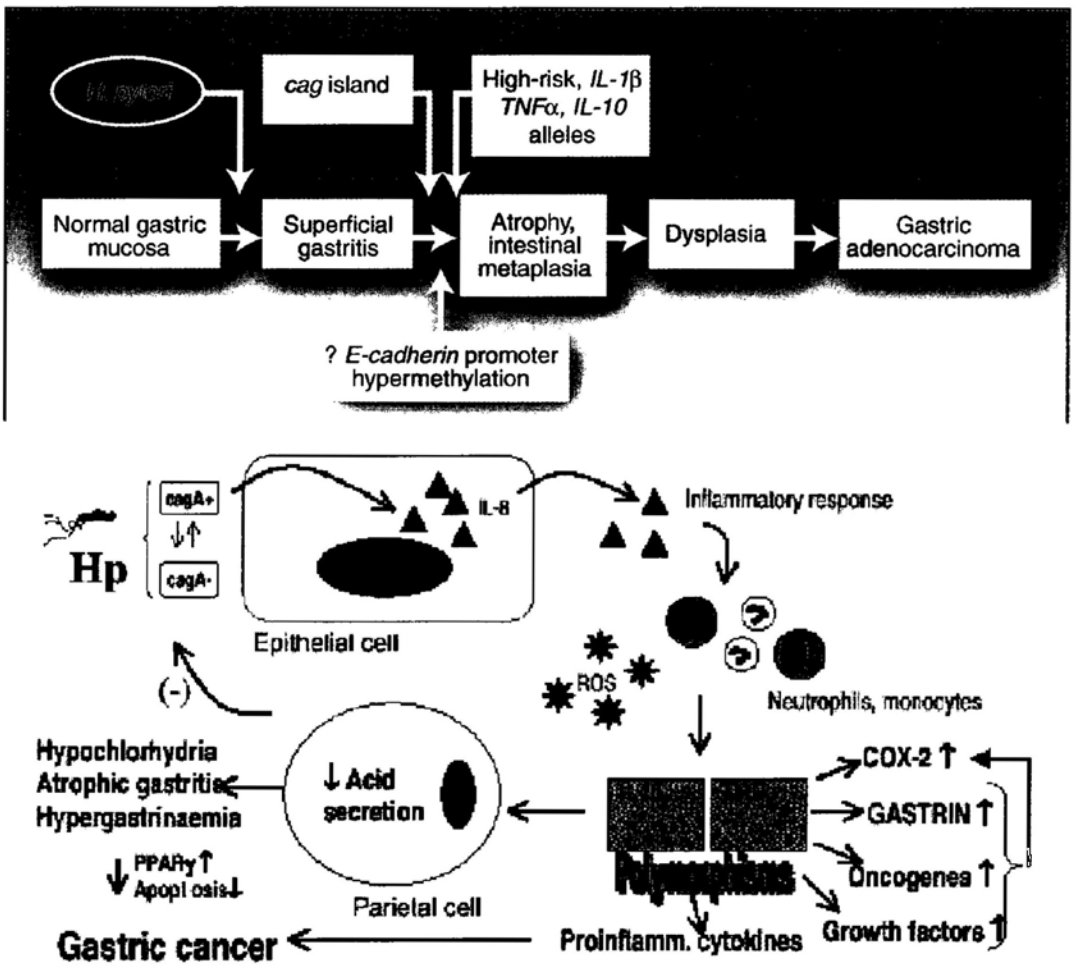


Figure 1.3 *H. pylori* infection leads to GC. (Adapted from Peek , Schneider et al. (2006) and Konturek (2003))

- Cigarette smoking

According to the American Cancer Society, smoking causes about one-third of all cancer deaths in the USA. Besides, population-based studies in both Europe and Asia have revealed a significant dose-dependent association between smoking and increased GC risk (Koizumi, Tsubono et al., 2004).

- *Epstein-Barr virus* infection

Epstein-Barr virus, which frequently referred to as EBV, affects approximately 10% of GC all over the world (Mladenova & Pellicano, 2003; Fukayama, Hino et al., 2008). To date, the role of EBV in the EBV-associated gastric carcinoma remains limited. It is still not known whether EBV infection is the cause or consequence of GC (K. Takada, 2000).

- Others

There are some other risk factors attributable to the GC, including age (Hisamichi, Sasaki et al., 1979), gender (Sipponen, Kekki et al., 1988), environmental exposures such as mineral dusts, nitrogen oxides (Cocco, Palli et al., 1994), medical condition such as pernicious anemia, prior gastric surgery for benign condition (Hsing, Hansson et al., 1993).

1.2.5 Diagnosis for stomach cancer

Up to 80% of GC patients during the early stages are asymptomatic, so the initial diagnosis is usually delayed. Patient with the symptoms like indigestion, heartburn, loss of appetite, which are suspicious for early stage of GC, should be examined with diagnosis. The steps of diagnosis of patient with symptoms suspicious for GC have been summarized by Layke and Lopez in 2004 (Figure 1.4).

- Double-contrast barium swallow

First, a double-contrast barium swallow, which is cost-effective and noninvasive, should be carried out to provide preliminary information that may help the physician determine the situation of gastric lesion. However, barium swallow may provide indeterminate results, so further diagnostic method is necessary.

- Endoscopy

Endoscopy is the diagnostic imaging procedure in gastric carcinoma. It is diagnostic test with highly sensitivity and specificity, especially when combined with endoscopic biopsy test. Histological analysis can help to determine the pathological situation of the tumor tissue.

- Computed Tomography (CT) Scan

After the initial diagnosis of GC, further evaluation is necessary to determine treatment options. CT can provide a series of detailed cross-sectional images and display the situation of stomach very clear. It can also show the cancer metastasis

and help to determine the stage of cancer.

- Endoscopic ultrasonography (EUS)

EUS is a technique that leads to more accurate GC staging. Using EUS, high-frequency sound-waves can help us to detect the invasion depth of carcinoma and find the involved local lymph nodes.

- Magnetic Resonance Imaging (MRI)

MRI is a scanning method that uses strong magnets and radio waves to detect possible problems in patient's stomach. The signal can be translated into detailed figures to see more information than that could be observed through a CT scan.

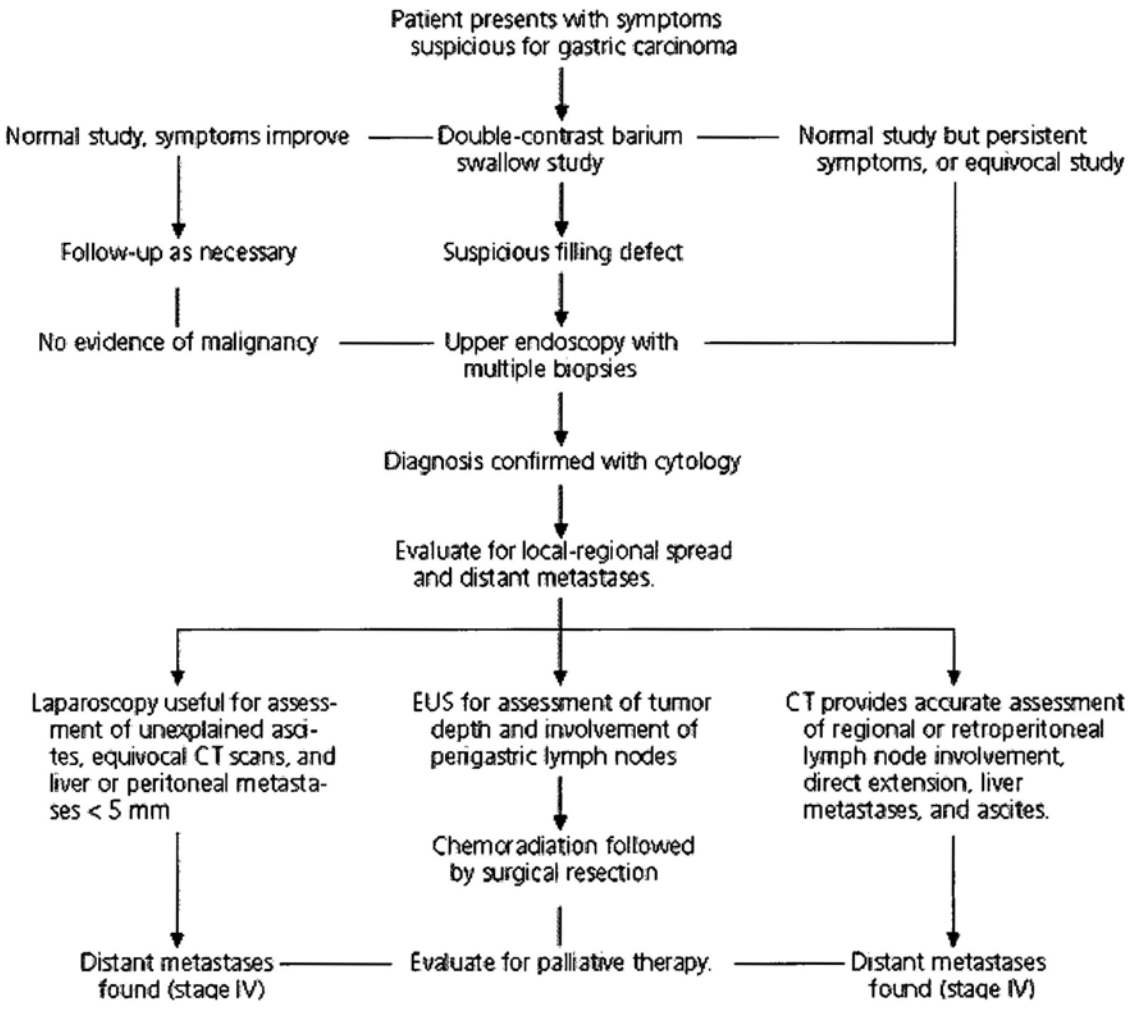


Figure 1.4 Diagnostic work-up for patient with symptoms suspicious for GC.

This figure comes from the study of Layke and Lopez in 2004. (CT = computed tomography; EUS = endoscopic ultrasonography)

1.2.6 Treatment of stomach cancer

The choice of the treatment of gastric carcinoma depends mainly on the place and size of the tumor, the stage of GC, and personal general health (Table 1.2). Treatment for stomach cancer may involve gastrectomy, chemotherapy, and radiation therapy. Many patients may have more than one type of treatments.

- Surgery

Gastrectomy including subtotal gastrectomy and total gastrectomy is a kind of surgeries, which removes the GC and part or total of the stomach. Gastrectomy is usually done in earlier stages of stomach cancer. According to the location and extent of the tumor, the surgeons will determine which gastrectomy may be best for the patients. If the location of tumors is limited, the surgeons will remove part of stomach that contains tumor. If the location of tumors has been extended, or if the biopsy reveals as diffuse type, whole stomach, nearby lymph nodes, and other tissues where the tumor has spread to will be cut.

- Chemotherapy

Chemo-therapeutic regimen is a method which uses drugs to inhibit cancer cell growth or even help to kill cancer cells. Chemotherapy may be administered in pill form taken through oral cavity, injected through a vein, or placed directly into the focus of tumor. This treatment may be recommended at any stage of cancer, but usually it is chosen when the GC has spread. Additionally, chemotherapy also may be used to relieve the discomfort and pain caused by GC.

- Radiotherapy

Radiation therapy, which includes external-beam and internal-beam radiation therapy, is a treatment using high-energy radiation to kill or shrink cancer cells. External-beam radiation therapy delivers the radiation through the outside of the body; if the radioactive materials are delivered to the region of tumor cells by catheters, needles or wires, it is called internal-beam radiation therapy. This treatment will be applied if cancer remains after surgery.

Table 1.2 Treatment for GC according to different stages.

Stage	Treatment options
0	- Gastrectomy with lymphadenectomy
I	- Distal subtotal gastrectomy (if the lesion is not in the fundus or at the cardioesophageal junction) - Proximal subtotal gastrectomy or total gastrectomy, both with distal esophagectomy (if the lesion involves the cardia) - Total gastrectomy (if the tumor involves the stomach diffusely or arise in the body of the stomach and extends to within 6 cm of the cardia or distal antrum) - Postoperative chemoradiation therapy in patients with node-positive (T1 N1) and muscle-invasive (T2 N0) disease - Neoadjuvant chemoradiation therapy
II	- Distal subtotal gastrectomy (if the lesion is not in the fundus or at the cardioesophageal junction) - Proximal subtotal gastrectomy or total gastrectomy (if the lesion involves the cardia) - Total gastrectomy (if the tumor involves the stomach diffusely or arises in the body of the stomach and extends to within 6 cm of the cardia) - Postoperative chemoradiation therapy - Neoadjuvant chemoradiation therapy
III	- Radical surgery. Curative resection procedures are confined to patients who at the time of surgical exploration do not have extensive nodal involvement. - Postoperative chemoradiation therapy - Neoadjuvant chemoradiation therapy
IV	*Patients with no metastases (M0)* - Radical surgery if possible, followed by postoperative chemoradiation - Neoadjuvant chemoradiation therapy *Patients with distant metastases (M1)* - Palliative chemotherapy - Endoscopic laser therapy or endoluminal stent placement may be helpful in patients whose tumors have occluded the gastric inlet. - Palliative radiation therapy may alleviate bleeding, pain, and obstruction. - Palliative resection should be reserved for use in patients with continued bleeding or obstruction.

(Adapted from Layke and Lopez's study (2004))

1.3 DNA Methylation

1.3.1 General introduction of epigenetics

Epigenetics refers to heritable DNA or chromatin changes in gene regulation without a change in the underlying DNA sequence or the sequence of the proteins associated with DNA. Epigenetics plays an essential role in cellular differentiation and development. The epigenome refers to the overall epigenetic state of a cell while the epigenetic code refers to epigenetic features such as DNA methylation and histone modifications that associate with different phenotypes in different cells.

It is increasingly evident that epigenetic events take part in normal cell physiology procedure. More information about the role of epigenetics can help us to understand the pathogenesis of human diseases, especially the mechanism of cancers. Numerous studies have indicated that epigenetic changes were involved in transcriptional regulation (Contente, Kenyon et al., 1999; Grummt & Pikaard, 2003). During tumorigenesis, the epigenetic usually present as the abnormal transcriptional inhibition in tumor suppressor genes. The most studied of epigenetic modification is that of DNA methylation, which occurs primarily in CpG and is often altered in cancer cells. This section will mainly discuss DNA hypermethylation as an essential determinant in carcinogenesis, especially in GC.

1.3.2 Cytosine methylation and CpG island

Methylation is a form of alkylation with a methyl group, replacing a hydrogen atom (Figure 1.5). This procedure is catalyzed by DNA methyl-transferases (DNMT) which transfer methyl groups from S-adenosylmethionine (SAM) to cytosine residues in CpG sites (Januchowski, Prokop et al., 2004).

DNA methylation in vertebrates usually occurs at the CpG site. CpG site represents 5% to 10% in human genome. In DNA from human normal healthy tissues, about 3.5%–4% of cytosines are methylated (Ehrlich, Gama-Sosa et al., 1982; Ehrlich, Jiang et al., 2002). The methylated cytosine residues in the human genomic DNA are not distributed evenly (Antequera & Bird, 1993). The regions of the DNA that have a higher frequency of CpG sites are named as CpG islands, which are usually more than 500 bp with a C + G frequency greater or equal to 55% and a ratio of CpG observed/expected ratio greater than 0.65 (Takai & Jones, 2002). They are commonly unmethylated and heavily acetylated to make the chromatin structure suitable for binding of transcriptional regulators. Usually, CpG islands are located nearby the regions of promoter and exon 1 of genes (Antequera, 2003). More than 50% genes in human genome have CpG islands overlapping the transcription start sites (Y. Wang & Leung, 2004). Thus, the presence of a CpG island can be utilized in the prediction and annotation of genes.

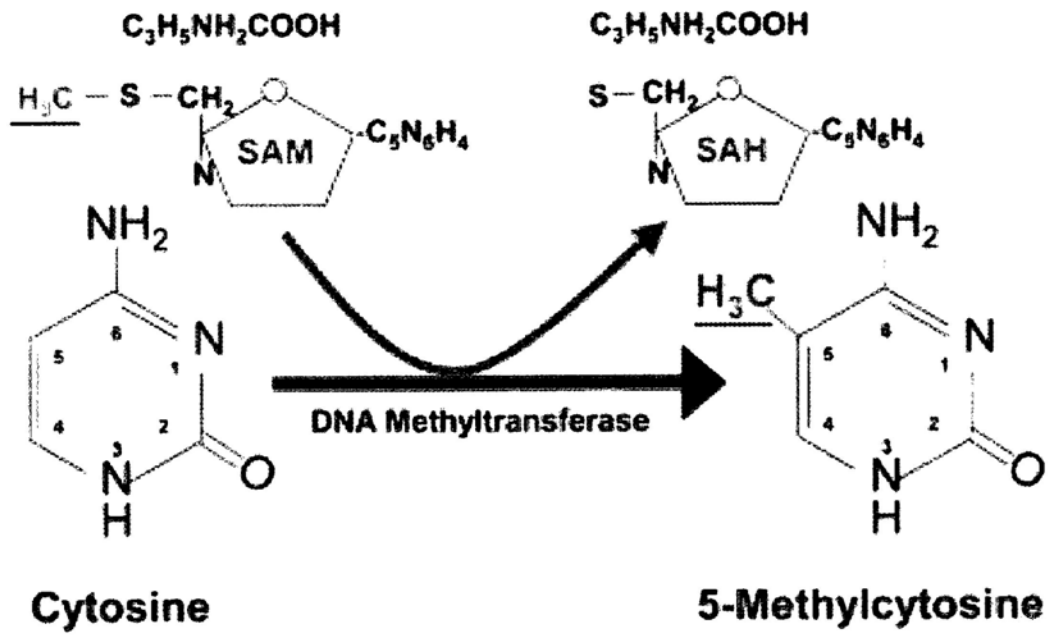


Figure 1.5 Conversion of cytosine to 5'-methyl-cytosine. (Adopt from Attwood and Yung et al. (2002))

1.3.3 DNA methylation dependent gene silencing

The relationship of DNA methylation and transcriptional repression in human cell has been recognized by many researchers; however, the mechanism of transcriptional suppression regulated by DNA methylation is still controversial. Distinct mechanisms have been suggested to express the methylation dependent transcriptional silencing.

DNA methylation may affect certain transcription factors interacted with their target promoter sequences (Figure 1.6A). There are two possible pathways. The transcription activators specifically recognize unmethylated site in the promoter sequence may be blocked by the promoter methylation (Figure 1.6B); or the methylcytosine-binding proteins (MBPs) binding to CpG island within a promoter region blocks access of transcription activators to their binding sites (Figure 1.6C). Numerous evidences have demonstrated that methylated CpG island can reduce the binding affinities of transcription factors such as E2F, activating protein 2 (AP-2) and c-Myc (Momparler, 2003).

However, not all transcription factors have CpG sites within their binding motif. DNA methylation does not affect the activity of *Sp1* and *CCAAT-binding transcription factor* (*CTF*) too much (Tate & Bird, 1993). Hence, decreased transcription factors binding ability alone can not completely explain the numerous biological phenomena associated with methylation dependant transcriptional repression. MBPs bound to CpG sites can form complexes with the histone

deacetylases and the co-repressors. These complexes may lead to histone deacetylation and chromatin structure changes which cause transcriptional inactivation (Figure 1.6D).

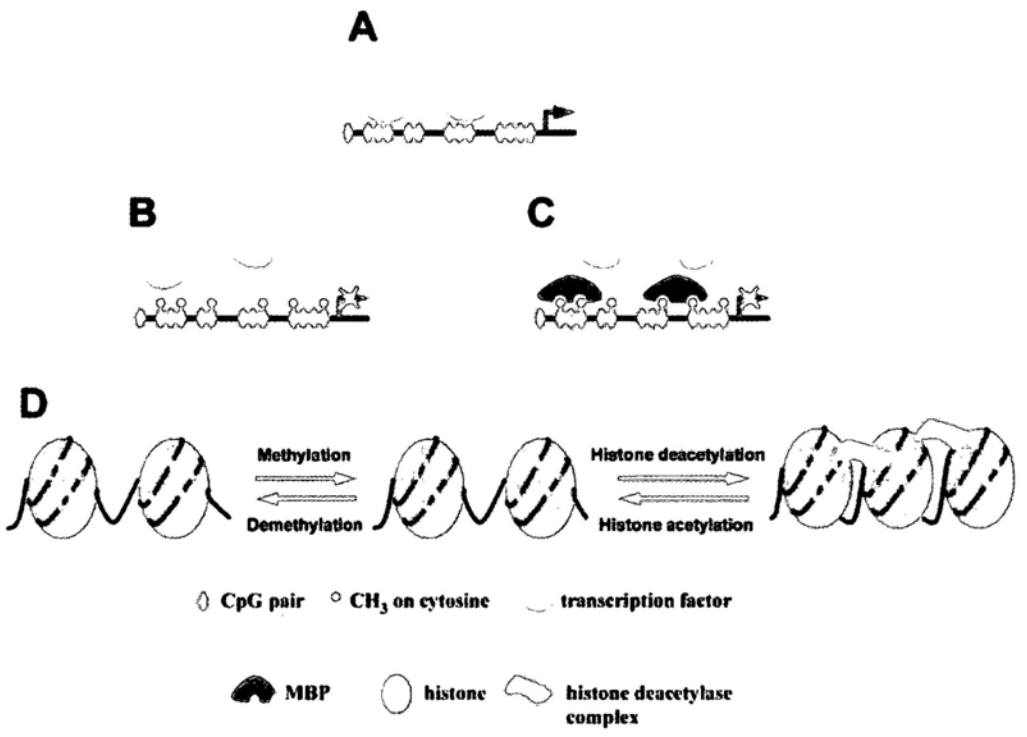


Figure 1.6 Mechanisms by which DNA methylation represses gene transcription. (Adopt from Attwood and Yung et al. (2002))

1.3.4 Method for DNA methylation study

Since the usage of bisulfite conversion, many laboratories have improved the procedure, new methods, and numerous research kits to study the DNA methylation status. However, the core principal of these methods has not been changed: convert a C residue to a U residue. In our study of *PAX5*, we perform the techniques such as methylation specific PCR (MSP) and bisulfite genomic sequencing (BGS). Figure 1.7 shows the strategy of method selection for methylation study. In this part, some common methods for DNA methylation study will be introduced.

First of all, to pick out the interested candidate genes from thousands of human genes, large scale methylation detection method should be used, such as *in vitro* DNA methylation assay or large scale sequencing. *In vitro* methylation sensitive restriction assay is mainly performed with the restriction enzyme *SssI* CpG methyltransferase, which recruits SAM (S-adenosylmethionine) as methyl donor (Schmitt, Oakeley et al., 1997). Following the enzyme digestion, southern detection or PCR amplification is performed to find the specific genes whose promoters are methylated. The limitation is that this method could only detect methylation differences at loci where the enzymes could recognize. New generation sequencing technology provides a high throughput method for analyzing genome-wide methylation. DNA library-based sequencing could detect methylation status at each CpG site.

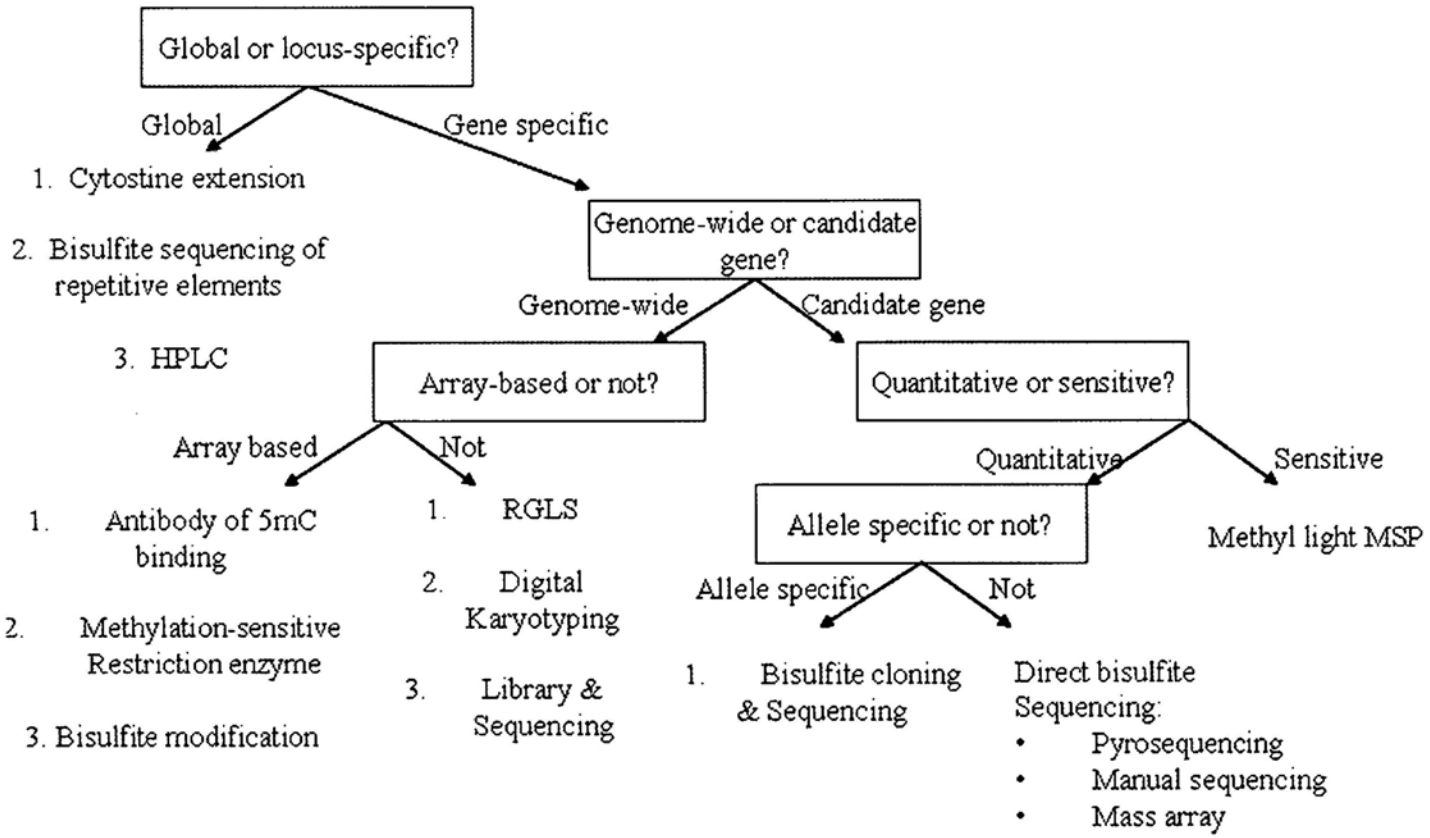


Figure 1.7 Flow chart for DNA methylation investigation method selection. (Shen & Waterland, 2007)

After the candidate methylated genes are found, other methods can be employed to study the specific genes, such as sensitive method, MSP; semi-quantitative method, combined bisulfite restriction analysis (COBRA); and quantitative method, BGS. All these methods mentioned above are based on genomic DNA bisulfite treatment.

By using methylation or unmethylation primers, MSP is performed to detect methylation status of specific CpG site (Herman, Graff et al., 1996). It provides a sensitive, easy, fast and cost-effective method, which is suitable for the clinical application. This method is widely used in imprinting analysis and diagnosis marker development (Kosaki, McGinniss et al., 1997; Kubota, Das et al., 1997).

COBRA can detect DNA methylation levels at specific loci in small amounts of DNA (Xiong & Laird, 1997). Post bisulfite modification, the region of interest is amplified by normal PCR using the primers without CpG site. The PCR products containing both methylated and unmethylated sequences are digested by the enzyme *BstUI*, which cleaves the motif 'CCGG'. After digestion, the fragments are analyzed by gel electrophoresis. The methylation level is defined by relative intensity of the methylated and unmethylated band. Using this method, it is not likely to generate false positives results, since it bases on the cleavage of restriction enzyme specific recognize the methylated sites. However, like all the methods based on specific enzyme digestion, the limitation is COBRA could only detect methylation level at loci where the enzymes recognized.

The bisulfite sequencing can be divided into bisulfite cloning and sequencing or direct bisulfite sequencing (Frommer, McDonald et al., 1992). The same as COBRA, normal PCR is performed using non-CpG primers. Then the PCR amplicon can be sequenced directly to provide a strand-specific percentage for the population of molecules or can be cloned into a plasmid and sequenced to get maps of methylation from single DNA molecules.

1.3.5 Methylation and cancer

DNA methylation has been recognized as a major mechanism for tumor development. Generally speaking, normal cell follows conserved methylation profiles. Both hypermethylation and hypomethylation can contribute to carcinogenesis. Gene-specific hypermethylation can affect many different types of cancers and is often mediated through the silencing of tumor suppressors (Ehrlich et al., 1982). Hypomethylation can affect genomic instability that is frequently observed in cancer (Ehrlich et al., 2002), activation of oncogenes, or loss of imprinting (Rollins, Haghighi et al., 2006). When aberrant DNA methylation, either hypermethylation or hypomethylation, is introduced into cells, related genes are dysregulated, resulting to malignant transformation.

Hypomethylation in cancer genome influences both single-copy genes and repeat sequences. Great variability of hypomethylation may apply not only to different types of cancers, but also to the same type of cancer. Genomic hypomethylation may lead to genomic rearrangements or oncogene activation that can cause the initiation or development of cancer. Compared with hypermethylation, the role of hypomethylation is still less characterized.

The role of DNA hypermethylation in gene expression down-regulation can often be understood in terms of a biological function involved in cancer development, such as silencing of tumor suppressor genes. For example, it has been reported that about 420 genes are transcriptional suppressed by DNA hypermethylation in

AGS cell line (Ushijima, Nakajima et al., 2006). Silencing or suppression of genes through hypermethylation can lead to many of the hallmarks of cancer including inhibition of apoptosis, enhancement of cell proliferation, insensitivity to antigrowth signals, sustained angiogenesis, limitless replicative potential, and tissue migration and metastasis (Hanahan & Weinberg, 2000). Hypermethylation may provide targets of cancer therapy. In the following sections, we will mainly discuss the hypermethylation which more associates with our study.

- Apoptosis

The internal and external cellular environment is always carefully monitored and any abnormalities can trigger a cascade of events leading to the programmed cell death, which is also called apoptosis. Large number of proteins may be involved in apoptotic pathways.

There are two major different pathways, which lead to apoptosis, mitochondrial- and caspase-mediated pathways. These two pathways can function independently but also interact with each other (Hanahan et al., 2000). B-cell lymphoma-2 (BCL-2)/adenovirus E1B 19 kD-interacting protein 3 (BNIP3) is a member of the BCL-2 protein family, which is linked to apoptosis. Activated BNIP3 is localized to the mitochondria, resulting in hypoxia-induced cell death (Regula, Ens et al., 2002). Hypermethylation-induced transcriptional silencing of BNIP3 allows cancer cells to avoid cell death induced by hypoxia (Murai, Toyota et al., 2005).

Multiple caspase pathways, all of which cause a dismantling of cellular and

organelle membranes, have been discovered. Caspase-8 can activate the caspase pathway and trigger the apoptosis process (Thornberry & Lazebnik, 1998). Inactivation of caspase-8 hypermethylation in its control region will contribute to apoptosis suppression (Martinez, Setien et al., 2007).

- Cell cycle regulation

Cell cycle machinery includes five sequential phases, which are G0, G1, S, G2 and M phases. The ability of cancer cells proliferating at rates much greater than normal cells is based on their propensity to bypass cell cycle checkpoints faster. The non-dividing cells always stay in the state G0, which is a quiescent phase. Two crucial regulatory molecules, cyclins and cyclin-dependent kinases (CDKs), can determine the cell cycle procedure.

The protein named p16INK4a is a well-known tumor suppressor binding to and inhibiting CDK4 and 6. Binding of p16INK4a prevents combination of cyclin D and CDK complex and inhibits the cells enter S phase of cell cycle (Rocco & Sidransky, 2001). Hypermethylation dependant inactivation of p16INK4a in cancer cells may induce high rate of proliferation, which is a major contributor for tumorigenesis (Attri, Srinivasan et al., 2005; Hou, Ji et al., 2005; Lima, Leal et al., 2008).

- Tissue Invasion and Metastasis

Cell-cell adhesion, which is essential for establishment of tissues, is generally reduced in cancers. One of the major signals of malignant tumors is the loss of

cellular adhesiveness, allowing cells to move and colonize in other tissues. Dysregulation of cytoskeletal signaling proteins may also induce invasion and metastasis. It has been estimated that the migration causes 90% of human cancer deaths (Sporn, 1996).

Cadherins and the catenins protein play an essential role for maintenance of cell -cell junctions. Down-regulation of E-cadherin expression through DNA hypermethylation has been reported in a study by Yoshiura et al. (Yoshiura, Kanai et al., 1995). The *E-cadherin* gene also showed promoter region hypermethylation in hepatocellular carcinomas (Yoshiura et al., 1995). It has been illustrated that loss function of E-cadherin was significantly associated with tumor invasion in GC ($P < 0.05$) (Chen, Chu et al., 2003).

Integrin linked kinase (ILK), which can interact with the protein named LIM-and-senescent-cell-antigen-like-domains-2 (LIMS2), has been reported as a crucial protein in adhesion by forming complexes in extracellular matrix (ECM) sites (Wu & Dedhar, 2001; Wu, 2004; Legate, Montanez et al., 2006). Recently, GC cell lines have shown methylation associated silencing of LIMS2. Thus, silencing of *LIMS2* expression by methylation may play a key role in invasion and migration in GC (S. K. Kim, Jang et al., 2006).

1.3.6 DNA methylation induced by *H. pylori* infection

Many studies have demonstrated that *H. pylori* infection is strongly related with GC. Moreover, there are numerous evidences that *H. pylori* infection is concerned to the precancerous stomach lesions such as gastritis, intestinal metaplasia and dysplasia (Correa, 1995; Arif & Syed, 2007; Trajkov, Stardelova et al., 2007). However, the mechanism of aberrant DNA methylation induced by *H. pylori* is still not clear. It has been proposed that *H. pylori* infection may influence DNA methyltransferase or inflammatory mediators (Nardone & Compare, 2008).

To investigate the relationship between *H. pylori* infection and the methylation level, 154 volunteers with or without *H. pylori* infection was recruited, and their methylation status at 8 different loci were evaluated. The methylation levels in *H. pylori*-infected people were dozens of folds higher than those in *H. pylori*-negative individuals (Maekita, Nakazawa et al., 2006), suggesting that *H. pylori* infection induced hypermethylation in gastric mucosa. Additionally, a large number of evidences have proofed that many tumor suppressor genes are epigenetic silenced by DNA methylation associated with *H. pylori* infection. For instance, methylation of genes including *Cyclooxygenase-2* (*COX-2*), *p16*, *E-cadherin*, *runt-related transcription factor 3* (*Runx3*) was significantly higher in *H. pylori* positive individuals compared with *H. pylori* negative individuals (Akhtar, Cheng et al., 2001; A. O. Chan, Lam et al., 2003; Miyazaki, Murayama et al., 2007; Kaise, Yamasaki et al., 2008). These findings suggest that *H. pylori*

infection may induce DNA methylation in the progress of GC.

1.3.7 Clinical applications of DNA methylation

The study DNA methylation would increase the knowledge of transcriptional gene regulation and have a major impact in development of cancer screening, diagnostics, prognosis and treatment.

In gastrointestinal cancer screening, traditional method such as fecal occult blood test is not sensitive enough, resulting in large number of false negative results. Methylation markers based on molecular technique will be more sensitive to detect tumor samples. In addition, it can be applied as a noninvasive detection method from free DNA in plasma, body fluids and stool. However, the greatest challenge for clinical application of methylation biomarkers is to identify methylation of which specific genes are associated with a particular cancer. Many potential methylation biomarkers in tumor tissues have been discovered, such as *BCL2* (Friedrich, Weisenberger et al., 2004) and *Death-associated protein kinase* (*DAPK*) (M. W. Chan, Chan et al., 2002) in bladder cancer; *Ras association domain family protein 1* (*RASSF1A*) (Lindsey, Lusher et al., 2004) in brain cancer; *O-6-methylguanine-DNA methyltransferase* (*MGMT*) (Esteller, Hamilton et al., 1999) and *Human mutS homolog 2* (*hMSH2*) (Zhang, Fu et al., 2006) in colon cancer; *E-cadherin* (A. O. Chan et al., 2003) in GC. However, variation of the sensitivity and specificity of these biomarkers has been observed. Early stage detection of tumors before they spread to an incurable stage is one of the greatest

challenges in oncology. The most important thing for the markers before its clinical application is control study to standardize the assay. Hence, more comprehensive methylation profiling in large sample size is needed to validate the application of these biomarkers in clinical cancer.

Prognosis of cancer broadly stands for the estimation of the possible course and prediction of future outcome for a cancer patient. For a molecule prognostic biomarker to be clinically applicable, it must be high specific and sensitive. It will be more ideal, if the marker is detectable in specimens obtained through non-invasive or minimally invasive procedures. Aberrant methylation markers are also promising for using as a prognosis tool. A number of genes, which are hypermethylation in cancer and associated with poor prognosis, have been reported, such as *p16* (*CDNK2A*, *Cyclin-dependent kinase inhibitor 2A*) (Kawamoto, Enokida et al., 2006) in bladder cancer; *cyclin D2* (*CCND2*) (Sakuma, Akahira et al., 2007) in ovarian cancer; *suppressor of cytokine signaling 3* (*SOCS3*) (B. He, You et al., 2003) in lung cancer; *Spleen tyrosine kinase* (*SYK*) (Yuan, Wang et al., 2006) in liver cancer; *inhibitor of DNA binding 4* (*ID4*) (Umetani, Takeuchi et al., 2004) and *interferon regulatory factor 8* (*IRF8*) (D. Yang, Thangaraju et al., 2007) in colon cancer; and *MGMT* (Park, Han et al., 2001) in GC.

Compared with using single marker at a time, approaches that allow the simultaneous analysis of multiple DNA methylation markers are probably to provide a more accurate and reliable diagnosis or prognosis result. For instance,

DNA methylation of 7 genes, including *secreted frizzled receptor proteins 1, 2, 4, 5* (*SFRP1, 2, 4, 5*), *paired box gene 1* (*PAX1*), *Sex determining region Y box 1* (*SOX1*) and *LIM homeobox transcription factor 1 alpha* (*LMX1A*) has been used as a biomarker panel for screening and prognostic prediction of ovarian cancer (Su, Lai et al., 2009).

Multiple treatment approaches have been developed over the past decade to treat carcinoma by DNA demethylation. Most of the therapies try to cure tumor by reducing DNA methylation level through inhibition of the enzyme DNMTs. After incorporating into DNA, methylation inhibitors can form irreversible covalent bonds with DNMTs (L. Zhou, Cheng et al., 2002). The DNMTs will remain bind to the DNA, losing the ability of methylation and preventing them from carrying out methylation elsewhere. DNMT inhibitors such as azacytidine, zebularine and decitabine have all shown an ability to decrease methylation (Kaminskas, Farrell et al., 2005). Researchers are now trying to develop more superior delivery and handling methods to increase the efficiency of the demethylating drugs.

1.4 *PAX* Genes

1.4.1 *PAX* gene family

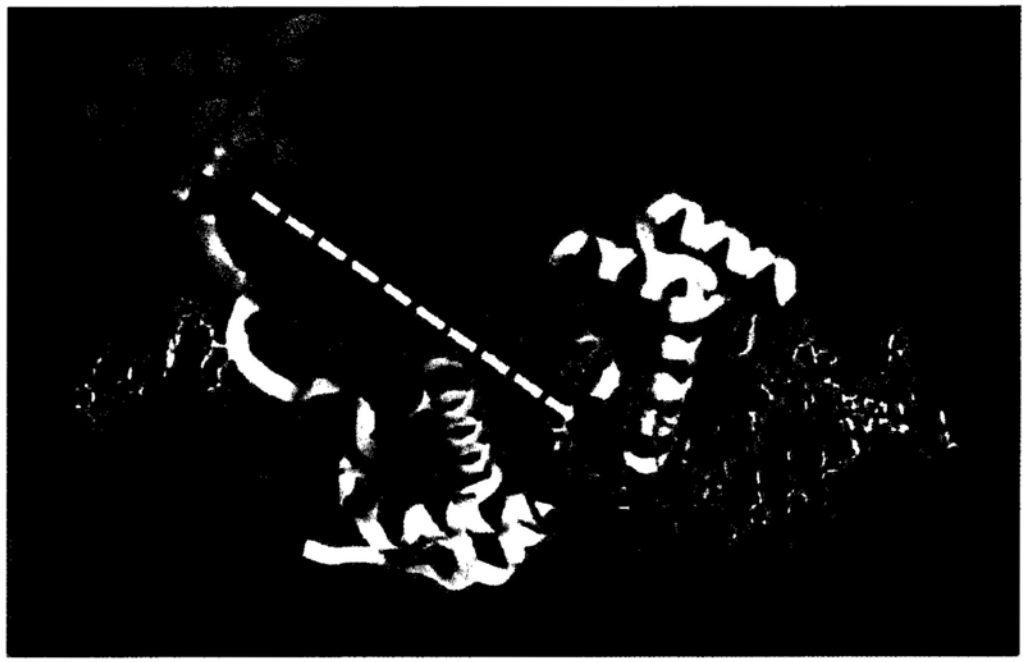
PAX (paired box) gene family is a highly conserved transcription factor family belonging to the helix turn helix class. The paired box domain is a DNA-binding domain of about 128 aa, which was originally described in the Drosophila segmentation gene paired (Bopp, Burri et al., 1986; Frigerio, Burri et al., 1986). A paired box domain has two subdomains (PAI and RED) which consist of three alpha-helixes respectively (Figure 1.8). In addition, some PAX proteins comprise a paired type homeo domain and a conserved octapeptide.

In higher vertebrates including human beings, nine members of the PAX family have been isolated which are classified into four subgroups based on various aspects of similarity, with orthologues present in worms, flies, frogs, fish and birds. Subgroup I without homeo domain includes PAX1 and PAX9; subgroup II with octapeptide and partial homeo domain includes PAX2, PAX5, and PAX8; subgroup III with both completed octapeptide and homeo domain includes PAX3 and PAX7; subgroup IV with no octapeptide region includes PAX4 and PAX6.

The DNA binding activity of PAX is mainly confirmed by the third helix of the PAI domain in the major groove whereas the first and second helixes are antiparallel (Figure 1.8). Additionally the RED and homeo domain can be involved in DNA binding function, so various combinations of DNA binding are possible (Jun & Desplan, 1996). The conserved DNA binding site of PAX proteins

has been characterized by Czerny et al. (1993). The octapeptide region and homeo domain are also reported as protein-protein interaction domain. The octapeptide region is known as a repression domain(Eberhard, Jimenez et al., 2000), while the homeo domain is reputed as a activation domain (Lechner, Levitan et al., 2000).

Usually, the expression of genes in PAX gene family is temporally and spatially restricted during development of central nervous system (CNS) and various other organs (Jostes, Walther et al., 1990; Walther & Gruss, 1991). The PAXs play key roles in cell fate, early organogenesis and so on (Adams, Dorfler et al., 1992; Chamberlin, Palmer et al., 1997; Mastick, Davis et al., 1997). Thus it is important to know more information about the regulatory targets of PAXs to gain a better insight in biological development.



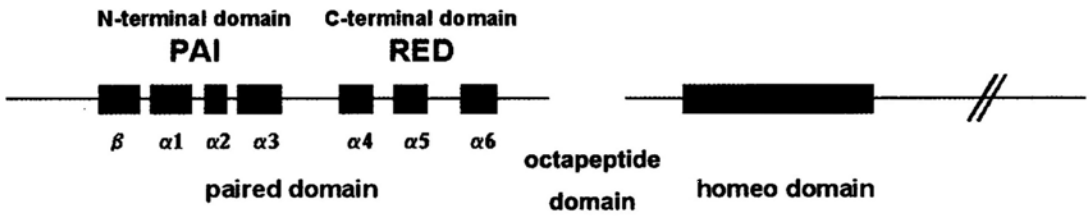


Figure 1.8 Structure of PAX protein. (This figure is modified by figures of Bopp (1986) and Jun (1996))

1.4.2 Members of *PAX* gene family

PAX1 gene is mapped to human chromosome 20p11.2 (Schnittger, Rao et al., 1992). This gene may play a role in the formation of segmented structures in the embryo. In mouse, *Pax1* mutations produce the phenotype, which was characterized by vertebral malformations along the entire rostro-caudal axis (Wilm, Dahl et al., 1998; Giampietro, Raggio et al., 2005). The *Pax1* mutant phenotype was also featured by vertebral segmentation defects reminiscent of the human disorder Klippel-Feil syndrome (McGaughran, Oates et al., 2003). It is also reported that Mesenchyme homeobox protein 1 (Mox1) proteins shown to be critical in axial skeleton development during embryogenesis can physically interact with PAX1 (Stamataki, Kastrinaki et al., 2001).

PAX2 gene locating on human chromosome 10q24.3 was found to be expressed in developing excretory system (Dressler, Deutsch et al., 1990). During human development *PAX2* has a spatially restricted expression along the compartmental boundaries of the neural tube, and within developing eye, ear and kidneys (Terzic, Muller et al., 1998). A single nucleotide deletion in exon five of the *PAX2* gene results optic nerve colobomas, renal anomalies and vesicoureteral reflux (Sanyanusin, Schimmenti et al., 1995) It has been demonstrated that the *PAX2* gene is required for producing a protein that is involved in the formation of the eye, ear, brain and CNS, kidney, and genital tract (Favor, Sandulache et al., 1996; Torres, Gomez-Pardo et al., 1996; Eccles, He et al., 2002). As a transcription

factor, PAX2 was identified as a direct regulator of *Brn1* gene involved in mid-hindbrain development (Bouchard, Grote et al., 2005).

The *PAX3*, which transcribes a 479 amino acid protein in humans, locates on chromosome 2q36.1. PAX3 is expressed during early neurogenesis (Mansouri & Gruss, 1998). During embryonic development, the *PAX3* gene is activated in neural crest cells, which migrate from the developing spinal cord to specific regions in the embryo (Kioussi, Gross et al., 1995; Lacosta, Muniesa et al., 2005; Otto, Schmidt et al., 2006). As a transcription regulator, the protein made by the *PAX3* gene directs the activity of other genes that signal neural crest cells to form specialized cell types or tissues such as limb muscles, craniofacial bones, nerve tissues and melanocytes, which produce the pigment melanin (Watanabe, Takeda et al., 1998; Bondurand, Pingault et al., 2000; Mayanil, George et al., 2001). In addition, PAX3 protein can physically interact with T-box protein 18 (TBX18), and cooperatively regulate the gene expression necessary for maintaining anterior-posterior somite polarity (Farin, Mansouri et al., 2008). *Pax3* mutants are the cause of Waardenburg's syndrome (WS), a disease that is an autosomal dominant combination of deafness and pigmentary disturbance (Baldwin, Lipsky et al., 1994; Pierpont, Doolan et al., 1994). Additionally, loss of function of *PAX3* disrupts development of certain muscles and craniofacial bones, producing the limb and facial features that are unique to WS types I and III (Hoth, Milunsky et al., 1993; Tekin, Bodurtha et al., 2001).

PAX4 gene encodes a 350 aa protein and locates on 7q32.1 (Tamura, Izumikawa et

al., 1994). This protein plays an key role in the differentiation and development of pancreatic islet-β cells (Sosa-Pineda, Chowdhury et al., 1997). PAX4 can act as a transcriptional suppressor that binds to a promoter element of glucagon, insulin and somatostatin (Smith, Ee et al., 1999; Petersen, Jorgensen et al., 2000; Sosa-Pineda, 2004; J. Wang, Elghazi et al., 2004). *Pax4* mutations are associated with diabetes mellitus type 1 and type 2 (Shimajiri, Sanke et al., 2001; Holm, Rydlander et al., 2004; Mauvais-Jarvis, Smith et al., 2004).

Protein PAX5, also known as B-cell specific transcription factor (BSAP) plays an important role in early stage of B-cell differentiation as well as neural development and spermatogenesis (Barberis, Widenhorn et al., 1990; Adams et al., 1992; Nutt, Urbanek et al., 1997). The *PAX5* gene is mapped to chromosome 9p13 region, which is involved in t(9;14)(p13;q32) translocations occurring in small lymphocytic lymphomas of the plasmacytoid subtype and in derived large-cell lymphomas(Iida, Rao et al., 1996; H. Ohno, Ueda et al., 2000). This translocation induces the potent Emu enhancer of the IgH gene into close proximity of the *PAX5* gene promoters, suggesting that the deregulation of *PAX5* gene transcription contributes to the pathogenesis of lymphomas (Busslinger, Klix et al., 1996). A transcript variant arising as a consequence of alternative promoter usage has been described (Busslinger et al., 1996). The transcription regulation target gene of PAX5, such as *CD19*, was characterized by several studies (Kozmik, Wang et al., 1992; Kanteti, Nallasura et al., 2009).

The *PAX6* gene is on chromosome 11p13. PAX6 protein is a transcription factor

that can regulate genes involved in the formation and the development of the eyes, nose, brain, CNS, and the pancreas (Quinn, West et al., 1996; Ericson, Rashbass et al., 1997; St-Onge, Sosa-Pineda et al., 1997). It competes in binding to promoter elements in the glucagon, insulin and somatostatin with PAX4 (Petersen et al., 2000). It has been reported many times that defects of *PAX6* gene may lead to type 2 aniridia (AN2), which is a bilateral panocular disorder featured by complete or partial lack of iris, fovea and abnormalities of the lens and anterior chamber (Jordan, Hanson et al., 1992; A. Davis & Cowell, 1993; Neethirajan, Hanson et al., 2003; Song, Liu et al., 2005). Abnormal *PAX6* gene expression is also associated with other diseases such as keratitis, ectopia pupillae, foveal hypoplasia and Gillespie syndrome (Glaser, Ton et al., 1994; Mirzayans, Pearce et al., 1995; Azuma, Nishina et al., 1996; Hanson, Churchill et al., 1999).

PAX7, also known as *Hup1*, seats on chromosome 1p36.1 (Schafer & Mattei, 1993). In mice, this gene mainly expressed during the development of nervous and muscular system (Jostes et al., 1990). The specific function of this gene has not been well characterized. It may be required by maintenance of a subpopulation of superior collicular neurons (Thompson, Zembrzycki et al., 2008). DNA-protein interaction assay shows that PAX7 can directly bind to the promoter region of genes such as *gastrulation brain homeobox 1* (*GBX1*) and *eyes absent homolog 4* (*Eya4*) (White & Ziman, 2008). Additionally, it is reported that fusion of PAX7 to Forkhead in rhabdomyosarcoma (FKHR) by the variant t(1;13)(p36;q14) translocation may cause alveolar rhabdomyosarcoma (R. J. Davis, D'Cruz et al.,

1994).

The gene encoded PAX8 protein maps to chromosome 2q13. During embryonic development, the *PAX8* gene expression was found in developing excretory system and the thyroid gland (Plachov, Chowdhury et al., 1990). Following birth, the PAX8 protein regulates several genes involved in the production of thyroid hormones (M. Ohno, Zannini et al., 1999; Pasca di Magliano, Di Lauro et al., 2000). Defects in *PAX8* gene can cause congenital hypothyroidism non-goitrous type 2 (CHNG2) (Macchia, Lapi et al., 1998; Lanzerath, Bettendorf et al., 2006)

The gene encoded PAX9, which shared high similarity with PAX1 protein, locates on chromosome 14q13.3. In mouse embryos, expression of Pax9 was found in the pharyngeal pouches and their derivatives, the head, the developing vertebral column, the limbs and the tail (Neubuser, Koseki et al., 1995). It is also involved in the development of tooth (Peters, Neubuser et al., 1998). *PAX9* mutation is a cause of oligodontia (Stockton, Das et al., 2000; Frazier-Bowers, Guo et al., 2002).

1.4.3 PAXs and cancer

PAX gene family is quite small with only 9 members in human beings, but the PAXs are developmentally crucial in all kinds of tissues. So the PAXs may play essential role in promotion or prevention in the control mechanisms of cell malignant growth. This is supported by the fact that expression of *PAXs* is dysregulated in several different types of tumors, although the roles for *PAX* genes in cancer are not clearly understood.

Many reports have shown that *PAX* gene might associate with carcinogenesis (W. Wang, Kumar et al., 1998; Baumann Kubetzko, Di Paolo et al., 2004; Q. Wang, Fang et al., 2008). The *PAX* genes frequently express in human cancer cells, and are required for the cell survival (Muratovska, Zhou et al., 2003). It is reported that apoptosis was induced by down-regulation of *PAX* gene expression in cancer cells (Bernasconi, Remppis et al., 1996; Buttiglieri, Deregibus et al., 2004). According to Figure 1.9, expression of several *PAX* genes (*PAX3, 6, 7, 8, 9*) was detected in wide range of cancer types. This may indicated that some of the *PAX* genes take a role of oncogene in the cancer tissues.

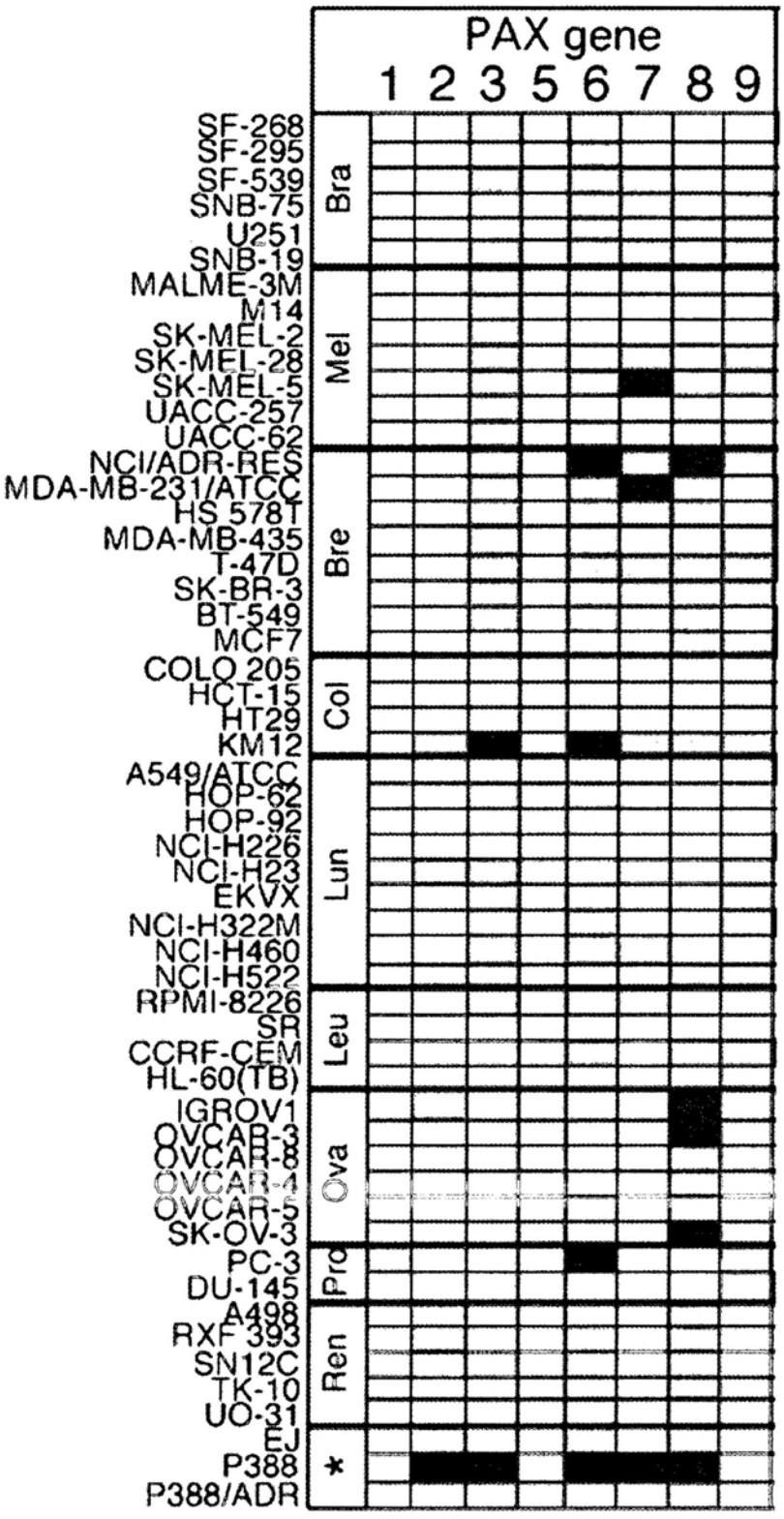


Figure 1.9 Detection of *PAX* gene mRNA in 54 cancer cell lines. The mRNA level of eight *PAX* genes (*PAX1 - 3* and *PAX5 - 9*) was analysed by qPCR in 54 cell lines (named on the left side of the figure) (Muratovska et al., 2003).

In the past studies, it seems that there are some associations between the structure and the role of PAXs in carcinogenesis. As mentioned before, PAXs in subgroups II and III all have an octapeptide region and at least a partial homeodomain (Figure 1.10); subgroup I PAX proteins contain only octapeptide region but no homeodomain; subgroup IV members possess only homeodomain but no octapeptide. Many proteins have been characterized as coordinator to interact with PAXs through octapeptide and homeodomain. For example, the homeodomain in PAX2 can interact with breast cancer C-terminal (BRCT) domain in PAX interacting protein (PTIP) protein, which is involved in DNA repair/recombination or cell cycle control (Lechner et al., 2000). Further more, Grg4 protein and Pax5 protein can interact via two separate domains: the Q and SP regions of Grg4, and the octapeptide motif and homeodomain of Pax5 (Eberhard et al., 2000). So the researchers supposed that both octapeptide and homeodomain may be very important to be involved in a specific network related to carcinogenesis (Robson, He et al., 2006). Subgroups I and IV members in tumorigenesis may link neutral contributions to cancer.

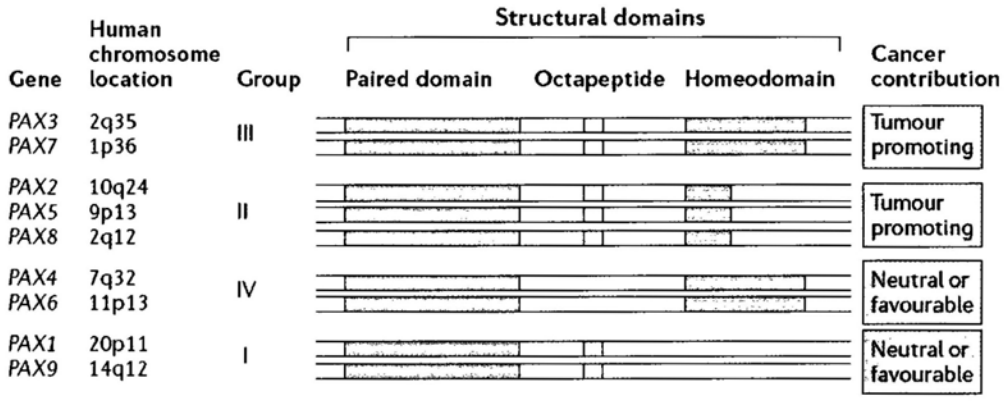


Figure 1.10 Relationship between *PAX* gene subgroup and cancer contribution. (Robson et al., 2006)

PAX genes from subgroup II, *PAX2* and *PAX8*, often co-express during development (Bouchard, Souabni et al., 2002). More understanding of the coordinate roles of *PAX* genes in cancer and development is required. Tumor-associated expression of *PAX2* and/or *PAX8* has been observed in carcinomas of the kidney (Eccles, Yun et al., 1995; Narlis, Grote et al., 2007), prostate (Khoubehi, Kessling et al., 2001; Bose, Gibson et al., 2009), breast (Silberstein, Dressler et al., 2002) and ovary (Muratovska et al., 2003). *PAX2* gene can negatively regulate the expression of the host defense peptide human beta defensin-1 in prostate cancer (Bose et al., 2009). Increase expression of *PAX2* gene can directly induce the up-regulation of The *Wilms' tumor suppressor gene*, *wt1* (Dehbi, Ghahremani et al., 1996). *PAX8* can also mediate the activation of the *wt1 tumor suppressor gene* (Dehbi & Pelletier, 1996). The *PAX8* gene is sometimes involved in the formation of thyroid tumors. In some of these neoplasms, the *PAX8* gene on chromosome 2 is fused with the *peroxisome*

proliferator activated receptor gamma (*PPARγ*) gene on chromosome 3. It is likely that the fused gene disrupts the normal control of cell division or triggers new cell activities that promote tumor formation (Kroll, Sarraf et al., 2000). Transcriptional activation of the *BCL2* apoptosis suppressor gene can be induced by the PAX8 protein (Hewitt, Hamada et al., 1997). It is also reported that another PAX gene from subgroup II, *PAX5* (*BSAP*), was involved in cancer process as an oncogene, such as astrocytomas (Stuart, Kioussi et al., 1995), neuroblastoma (Baumann Kubetzko et al., 2004), medulloblastoma (Kozmik, Sure et al., 1995), lymphomas (Cook, Aguilera et al., 2004; Souabni, Jochum et al., 2007) and lung cancer (Kanteti et al., 2009). *PAX5* gene is also involved in several chromosome translocations events that fuse PAX5 with other proteins in carcinogenesis (Cazzaniga, Daniotti et al., 2001; Bousquet, Broccardo et al., 2007; Nebral, Konig et al., 2007).

The members of subgroup III, PAX3 and PAX7, take part in the procedure of stem cell self renewal (Yablonka-Reuveni, Day et al., 2008; Pawlikowski, Lee et al., 2009). Changes in the activity of the *PAX3* gene are associated with some cases alveolar rhabdomyosarcoma that occur mainly in adolescents and young adults (Barr, Galili et al., 1993; Galili, Davis et al., 1993). The fusion event that *PAX*3 on chromosome 2 or *PAX7* gene on chromosome 1 fused with the *FKHR* gene on chromosome 13 may enhance changes that can lead to cancer (Shapiro, Sublett et al., 1993; R. J. Davis et al., 1994; Bennicelli, Fredericks et al., 1995). It has been reported that patients with metastatic alveolar rhabdomyosarcoma who are

positive for the PAX7–FKHR fusion protein had an overall 4-year survival rate of 75%, compared with 8% in the PAX3–FKHR group (Sorensen, Lynch et al., 2002), suggesting that different outcome for patient survival is depended on which *PAX* gene is involved.

The subgroups I and IV are associated with cancer to a relative low degree. There are only few reports about their role in cancer. *PAX9* was thought as an oncogene in lung cancer (Kendall, Liu et al., 2007; Hsu, Acharya et al., 2009).

Majority of reports considered *PAX* genes involved in tumorigenesis as malignant oncogene, but part of the PAXs may have the potential to function as tumor suppressor in some studies. For instance, *PAX6* gene may have a tumor-suppression function in human glioblastoma (Y. H. Zhou, Wu et al., 2005) and prostate cancer (Shyr, Tsai et al., 2009). PAX4 protein can act as a tumor suppressor in human melanoma (Hata, Hamada et al., 2008).

Paradoxically, all *PAX* genes play similar role in developmental promotion, but do not show common functions in cancer processes. The case for the anti-oncogenic activity PAXs is not yet strong enough.

1.4.4 PAX markers and prognosis

There are more examples of PAXs in favorable prognosis in cancer. High levels of *PAX6* expression associate with improved prognosis in malignant astrocytic gliomas, whereas low levels correlate with unfavorable outcomes (Y. H. Zhou, Tan et al., 2003). As mentioned above, a tumor suppression function for PAX6 in glioblastoma has been reported (Y. H. Zhou et al., 2005). In line with this study, there are several reports suggest that *PAX6* is subject to methylation in some human tumors (Salem, Markl et al., 2000; Hellwinkel, Kedia et al., 2008). Although silencing of *PAX6* is not the result of the non-promoter methylations (Salem et al., 2000), it has been suggested that exonic CpG islands (CGIs) are more easily methylated than promoter CGIs, and that methylation can be spread to other islands, including promoter CGIs (Nguyen, Liang et al., 2001). Other evidence indicated methylation dependent silencing of *PAX6* in some breast cancer cell lines and primary tumors (Ballestar, Paz et al., 2003).

As mentioned above, potential of *PAX4* as a tumor suppression gene has been demonstrated by Hata et al. in 2008. Additionally, there is report about epigenetic dependent gene aberrant expression of *PAX4* gene in hematologic malignancies (Li, Nagai et al., 2006), suggesting that *PAX4* may have the potential to utilize as a biomarker for tumor.

A panel of methylation markers including *PAX1* was set up in cervical cancer (Lai, Lin et al., 2008). In that study, the specificity for high-grade lesions/squamous cell

carcinomas (HSIL/SCC) screening could reach 99% by using *PAX1* methylation marker alone. *PAX1* acts as a prognosis marker in ovarian tumor is also reported recently (Su et al., 2009).

In conclusion, the function of *PAX* genes in human cancer and their roles as diagnosis marker are very exciting research areas and promises to deliver many new insights into mechanism of cancer and the clinical knowledge. However, much further work is needed to translate the study result into clinical application.

CHAPTER 2 Material and Method

2.1 Human Gastric Specimens

2.1.1 Tissue samples

There are three cohorts of human samples involved in this study: 1) Normal gastric mucosa biopsies for methylation status profiling were from the Endoscopy Center of Prince of Wales Hospital. 2) GC tissues and their corresponding adjacent non-cancerous tissues, which were at least 5 cm away from the tumor edge, were obtained from GC patients during endoscopy. These samples were used in the *PAX5* gene expression level comparison. 3) Paraffin embedded GC samples also for checking methylation status were from Guangzhou Zhong Shan Hospital, China. The GC samples were staged according to the American Joint Committee on Cancer TNM System. All fresh tissues were snap frozen in liquid nitrogen and then stored at -80 ^{o}C until further processing.

To evaluate the methylation status and the clinical significance of *PAX5* in GC patients, 161 GC specimens and 19 normal gastric biopsies were used for the assay of BGS. GC group included 107 male and 54 female, with average age 56.8±12.6, and normal group included 7 male and 12 female, with average age 51.9±17.2. Other clinicopathologic features such as *Helicobacter pylori* (*H. pylori*) infection, TNM stages and differentiation status were also determined. By using rapid urease test (RUT), 29 patients were found infected by *H. pylori*, and 70 patients were *H. pylori* infection negative. The patient number in TNM stage I, II, III and IV were 20, 23, 49 and 52, respectively. There were 94 patients with low

differentiation GC, and 43 patients had developed moderate or high differentiation GC. The patients' information was provided by the Guangzhou Zhong Shan Hospital. Some of the information was not complete. Informed consent was given to all the patients and controls, and the study protocol was approved by the Clinical Research Ethics Committee of the Chinese University of Hong Kong.

2.1.2 Tumor cell line

Sixteen tumor cell lines from gastrointestinal tract were used, including 8 GC cell line (AGS, BGC823, Kato III, MKN28, MKN45, N87, SNU1 and SNU16) and 8 Colorectal cancer (CRC) cell line (Caco2, DLD1, HCT116, HT29, LoVo, LS180, SW480 and SW620) were purchased from the ATCC (American Type Culture Collection, Manassas, VA, USA). All the GC cell lines and 3 CRC cell line (LoVo, LS180 and SW480) were cultured in RPMI 1640 medium (Sigma-Aldrich, St Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich). The Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich) with 10% FBS was used to culture HT29, SW620 and Caco2. The cultivation of cell line HCT116 was performed by using McCoy's 5a medium (Sigma-Aldrich) with 10% FBS. All these cell lines were incubated in an incubator with 95% air and 5% CO2 at 37 ^{o}C. Culture media were renewed every two to four days. Cells were split at 1:3~1:4 ratio using 0.25% Trypsin-EDTA solution (Invitrogen, Carlsbad, CA, USA).

2.2 Bioinformatics Analysis of *PAX5* Gene

The online database of University of California Santa Cruz Genome Bioinformatics (UCSC) (http://genome.ucsc.edu/) was used to obtain the related information about *PAX5* gene.

CpG islands in the *PAX5* gene promoter region were predicted by CpG Island Searcher (http://cpgislands.usc.edu/) (Takai & Jones, 2003). CpG islands are defined as DNA region greater than 500 bp with GC content above 55% and an observed/expected CpG ratio above 0.65 (Takai et al., 2002).

2.3 Gene Expression Analysis

2.3.1 RNA isolation

Total RNA was isolated using Quizol reagent (Qiagen, Valencia, CA, USA). First, about 5-10 × 10^6 cells or 30 mg tissue was homogenized in 1 mL Qiazol reagent and incubated at room temperature for 10 min. For each sample, 0.2 mL Chloroform was added. The mixture should be shaken vigorously for 15 sec and placed at room temperature for another 3 min. Samples were centrifuged at 12,000 g for 20 min at 4 °C and separated into two layers. The upper aqueous phase containing RNA was transferred to a new tube, mixed with 0.7 ml isopropanol, incubated at room temperature for 10 min and then centrifuged at 12,000 g for 10 min at 4 °C. After discarding the supernatant, the RNA pellet was washed twice with 1 mL 75% ethanol; air dried for 5 min and re-dissolved the RNA with

RNase-free H_2O. Contamination of DNA was eliminated by the RNase-free DNaseI digestion (GE Healthcare, Buckinghamshire, England). The quality and quantity of total RNA were determined by measuring absorbance at 260nm/280nm using NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE, USA). The purified RNA was store at -80 °C until using.

2.3.2 cDNA synthesis

MultiScribe Reverse Transcriptase Kit (Applied Biosystems, Foster City, CA, USA) was used to synthesize cDNA. The reaction mixture contained 1 × Reverse Transcriptase buffer, 1 × dNTP, 1 × random primer (supplied by kit), 2.5 U/μL reverse transcriptase, 1 U/μL RNase inhibitor and 2 μg total RNA. The mixture was incubated at 25 °C for 10 min, then 37 °C for 120 min, then 85 °C 5 min to inactivate the enzymes. The cDNA was stored at -80 °C until other application.

2.3.3 Semiquantitative reverse transcription PCR (RT-PCR)

Semiquantitative RT-PCR was performed in a total volume of 25 μL reaction containing GeneAmp 1 × PCR Buffer II (Applied Biosystems), 2.5 mM $MgCl_2$, 200 μM each of dNTP, 200 nM each of primers, 0.5 U of AmpliTaq Gold DNA polymerase (Applied Biosystems) and 30~50 ng cDNA. The PCR program started with an initial denaturation at 95 °C for 10 min, followed by 32–35 cycles (94 °C for 30 sec, 58 °C for 30 sec, and 72 °C for 30 sec) of amplification, with a final extension at 72 °C for 10 min. The PCR bands were visualized under ultraviolet

light and photographed. The expression of the target gene was normalized by the expression of house keeping gene *ß–actin*, which served as an internal control. All primers used to amplify the transcripts are listed in Table 2.1.

2.3.4 Real-time quantitative PCR (qPCR)

For the real-time quantitative RT-PCR, the *PAX5* expression was determined using the ABI PRISM 7500 Sequence Detection System (Applied Biosystems). The qPCR was performed according to the protocol of the SyberGreen Master Mix (Applied Biosystems) in a total volume of 25 µL reaction containing 1X SyberGreen Master Mix, 100 nmol/L primers and 30 ng cDNA template. The qPCR condition was 95 °C for 10 min, then 40 cycles of 95 °C for 15 sec, 58~60 °C (according to the annealing temperature of the primers) for 40 sec, 72 °C for 30 sec. The gene expression data was analyzed using the relative quantification $2^{-\Delta\Delta C_T}$ method (Livak & Schmittgen, 2001).

2.3.5 mRNA expression array

Gene expression profiles in GC cell line with or without PAX5 protein were analyzed by the Cancer Pathway Finder PCR Array systems (SABiosciences, Frederick, MD, USA). This array system can detect 84 genes representative of the six biological pathways involved in transformation and tumorigenesis (http://www.sabiosciences.com). Real-time PCR using ABI PRISM 7500 Sequence Detection System (Applied Biosystems) was performed according the

protocol. Simply mix the cDNA template with the appropriate ready-to-use PCR master mix (supplied by kit), aliquot 25 μL to each well of the 96-well plate, and then run the real-time PCR cycling program: 1) 95 °C for 10 min, 2) 40 cycles of 95 °C for 15 sec and 60 °C for 1 min. The expression results were analyzed by web based PCR Array Data Analysis Software according to the instruction (http://www.sabiosciences.com/pcrarraydataanalysis.php). Gene expression up-regulation or down-regulation with fold-changes of 1.5 times was considered to be of biological significance.

2.3.6 Protein extraction

Protein was prepared by using CytoBuster Protein Extraction Reagent (Merck Chemicals, Nottingham, UK). The cells were pelleted at 3000 g for 10 min. Then the pellet was resuspended in CytoBuster using 100 μL per 10^6 cells. The mixture was incubated at room temperature for 5 min. Then centrifuge the tube for 10 min at 4 °C at 15,000 g and transfer the supernatant to a fresh tube.

2.3.7 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blot

Forty micrograms of protein were separated by 5% upper gel and 10% lower gel. After SDS-PAGE, the protein was transfer to an equilibrated polyvinylidene difluoride (PVDF) membrane (Amersham Biosciences, Buckinghamshire, UK) by semi-dry machine at 15 V for 40 min. The membrane was blocked in 5% non-fat

milk dissolved by TBS/T solution (Tris-buffered saline (Invitrogen) and 0.1% Tween 20 (Sigma-Aldrich)) at room temperature for 1 hr with shaking. After blocking, the membrane was incubated in primary antibody diluted in 5% non-fat milk at 4 °C overnight with shaking. After incubation with the secondary antibody at room temperature for 1 hr, the proteins were detected by enhanced chemiluminescence (ECL, Amersham Corporation, Arlington. Heights, IL, USA).

2.4 DNA Methylation Analysis

2.4.1 Genomic DNA extraction

Genomic DNA from GC cell lines and tissue samples were isolated by using DNA mini kit (Qiagen) according to the kit protocol. About 25 mg samples were lysed in 180 μL of QIAamp ATL buffer and 20 μL of proteinase K in a 1.5 mL microcentrifuge tubes for 1 hour at 56 °C. Four microliter of RNase A (100 mg/ml, QIAgen) was added and mixed by pulse-vortexing for 15 s followed by 2 min incubation at room temperature. Then 200 μL of AL buffer was added to the lysate and samples were incubated for 10 min at 70 °C. After adding 200 μL of absolute ethanol, the solution was mixed by pulse-vortexing for 15 s. Then lysates were purified over a QIAamp column as specified by the manufacturer. The genomic DNA was diluted in 200 μL DNase-free H_2O. The quality and quantity of DNA were determined by measuring absorbance at 260nm/280nm using NanoDrop ND-1000 (NanoDrop).

2.4.2 Sodium bisulfite conversion

The genomic DNA was modified by sodium metabisulfite as description by Tao et al. (2002). Briefly, 5 μg genomic DNA in 30 μL TE buffer (Sigma-Aldrich) was mixed with 3.3 μL of 3 mM NaOH to a final concentration of 0.3 mM and incubate at 37 °C for 15 min. Denatured DNA was mixed with 333 μL of bisulfite solution and treated in darkness for 4 hr at 55 °C. The bisulfite solution was prepared as 2.4 M sodium metabisulfite (pH 5.0–5.2) (Sigma-Aldrich) and 0.5 mM hydroquinone (Sigma-Aldrich). The treated DNA was desalted and purified using the Qiaex II kit (Qiagen) according to the protocol supplied by the kit. DNA was then treated with 0.3 M NaOH at 37 °C for 15 min and precipitated with 3 M ammonium acetate and 3 volumes of ethanol. Recovered DNA was dissolved in 100μL TE buffer (pH 8.0) and stored at -20 °C.

2.4.3 Demethylation treatment using 5-aza-2'-deoxycytidine

Cells were seeded at a density of $1x10^5$/100-mm dishes and grew for 24 hr. Cells were then treated with 2 μM 5-aza-2'-deoxycytidine (5-Aza) (Sigma-Aldrich) for 5 days. The 5-Aza was replenished every day. The gene expression of *PAX5* was evaluated using semiquantitative RT-PCR.

2.4.4 Methylation specific PCR (MSP)

Methylation specific and unmethylation specific primers were designed to assess methylation status in the GC and CRC cell lines. The mixture for PCR contained

1 × PCR Buffer II (Applied Biosystems), 2 mM $MgCl_2$, 200 μM each of dNTP, 600 nM each of primers, 0.5 U of AmpliTaq Gold DNA polymerase (Applied Biosystems) and 20 ng bisulfite treated DNA. The PCR program was 95 °C for 10 min, followed by 38 cycles (94 °C for 30 sec, 60 °C for 30 sec, and 72 °C for 30 sec) of amplification, with a final extension at 72 °C for 5 min. Bands of MSP were observed under ultraviolet light and photographed.

2.4.5 Bisulfite genomic sequencing (BGS)

Direct BGS with PCR product was used to profile methylation status in normal and GC samples. Sequencing was performed using the BigDye Terminator Cycle Sequencing kit version 1.0 (Applied Biosystems). Briefly, 10-μL mixture, including 2 μL of BigDye-Terminator Ready Reaction Mix, 3.2 pmol specific primers and 10 ng of PCR product were added for sequencing reaction as follows: 25 cycles of 96 °C, 30 sec, 50 °C, 15 sec; 60 °C, 4 min. A 60-μL mixture of 10 μL of sodium acetate (5 M, pH 5.2) and 50 μL of absolute ethanol were added into each reaction product. After storage at -80 °C for 20 min, the mixture was centrifuged at 3,700 rpm for 30 min at 4 °C. The supernatant was discarded and the pellets were washed once with 100 μL of 75% ethanol. Finally, the dried pellets were dissolved in 10 μL of Hi-Di Formamide (Applied Biosystems). After denaturation at 95 °C for 5 min, the sequencing solution was kept on ice for 2 min and then analyzed by the ABI 3100 Genetic analyzer (Applied Biosystems). Sequences were analyzed by using SeqScape software (Applied Biosystems).

Methylation percentage of each CpG site was calculated according this formula: Methylation% = H_C/ (H_C + H_T) × 100%, (H_C = Height of peak C, H_T = Height of peak T).

2.5 Biological Function Analysis

2.5.1 Cloning of *PAX5* and construction of expression vector

The full-length cDNA of *PAX5* gene expression vector was generated by PCR-cloning. Total RNA from human stomach (Ambion, Austin, TX, USA) was reverse transcribed into cDNA. Sequence corresponding to the open reading frame (ORF) of *PAX5* was amplified by PCR. PCR product was subcloned into the pCDNA3.1 TOPO TA expression vector according to the manufacturer's guideline (Invitrogen). Briefly, 1 μL PCR product was ligated into the 0.5 μL TOPO vector in a total volume of 2.5 μL containing 240 mM NaCl and 12 mM $MgCl_2$. The mixed reaction was incubated for 30 min at room temperature before heat shock transformation.

The heat-shock transformation was performed using JM109 chemically competent *Escherichia coli* (*E. coli*) cell (Promega, Madison, WI, USA). The JM109 competent cells (50 μL) were thawed on ice, and 2 μL of ligation product was added into the cells. After incubation on ice for 20 min, the cells were heated shock for 45 sec at 42 °C without shaking in a water bath and then immediately transferred the tube on ice for 5 min. S.O.C. medium (250 μL) was added to the cells and the tube was shaken at 220 rpm at 37 °C for 1 hr in a shaking incubator. After

incubation, 150 μL cells were spread on Luria-Bertani (LB) agar plates containing 0.1 mg/ml ampicillin and incubated overnight at 37 °C.

Bacterial colonies were identified by PCR. Positive colonies were cultured in LB medium with 0.1 mg/ml ampicillin. Insert DNA was checked by sequencing. Plasmids with non-mutation target gene were isolated using HiSpeed Plasmid Maxi Kit (Qiagen). Briefly, bacterial were cultured in 1 mL LB medium containing 0.1 mg/ml ampicillin at 37 °C overnight with shaking at 250 rpm. Then, 0.5 mL bacterial culture was further inoculated into 100 mL LB medium containing 0.1 mg/ml ampicillin and grew at 37 °C for 16 hr with shaking at 250 rpm. Bacterial pellet was harvested by centrifugation at 6000 g for 15 min at 4 °C. The pellet was resuspended in 10 mL Buffer P1. Bacterial protein, chromosomal and plasmid DNA were denatured with 10 mL Buffer P2. The tube was inverted upside down for six times and then placed at room temperature for 5 min. The mixture was neutralized with 10 mL Buffer P3, followed by incubation at room temperature for 10 min. Debris within the cell lysate was cleared by filtrating with the QIAfilter cartridge. The filtrated lysate was applied to the resin column supplied in the kit through gravity flow, and plasmid DNA was bound to the resin column. The column was washed with 30 mL QC buffer by gravity flow to remove all contaminants during plasmid preparations and carbohydrates from bacterial strains. Plasmid DNA was eluted with 15 mL Buffer QF. DNA was precipitated by isopropanol precipitation and the DNA pellet was washed with 70% ethanol. The DNA pellet was air-dried and dissolved with 1 mL DNase-free H_2O.

2.5.2 *PAX5* gene transfection

Cells were seeded at $1 \times 10^4 \sim 2.5 \times 10^4$ cells on a 24-well plate without antibiotics for about 24 hr till the cell density reached about 90% confluent. Cells were then transfected with 0.8 μg *PAX5* and control vector (pCDNA3.1) respectively using Lipofectamine 2000 (Invitrogen). Lipofectamine 2000 (2.0 μL) diluted in 50 μL Opti-MEM (Invitrogen) was incubate at room temperature for 5 min. Then, plasmid DNA diluted in 50 μL Opti-MEM was combined with the Lipofectamine mixture. After 24 ~ 48 hr incubation at 37 °C in a 5% CO_2 incubator, cells were harvested for testing of transgenic expression. For stable cell lines, cells were passaged at a 1:10 ratio into fresh growth medium with proper concentration of neomycin (G418) (Invitrogen). Stable transfection cells were harvested after 14-21 days of selection for functional assays.

2.5.3 Colony formation assay

Two days after transfection, cells were subsequently split at 1:20 ratio on six-well plates with RPMI1640 in 10% FBS containing 500 μg/mL neomycin (G418). After 14-18 days of selection, cells were fixed with 70% ethanol for 10 min and stained with 0.5% crystal violet solution for 10 min. Colony with more than 50 cells per colony was counted. The experiment was conducted in three independent triplicates.

2.5.4 Cell viability assay

MTS assay, which is the short form of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium assay, was performed using CellTiter 96 AQ_{ueous} One Solution Cell Proliferation Assay kit (Promega). Transfected cells were trypsinized and counted. Cells were diluted to 5,000 cells per 100 μL in RPMI1640 medium. For each well in the 96-well plate, 100 μL of cells were seeded. The plate was incubated at 37 °C in a 5% CO_2 incubator. After 48 hr, 20 μL MTS reagent was added into the culture medium. The culture was incubated at 37 °C in a CO_2 incubator for 30 min to 2 hr. Absorbance of the samples was measured at 490 nm 48 hr post transfection. This experiment was replicated three times.

2.5.5 Wound healing assay

The wound healing assay allows the study of cell migration. Briefly, trypsinized cells with a concentration of 5×10^5 cells per well were seeded in a 6-well plate in complete medium. The cells were incubated at 37 °C in a 5% CO_2 incubator for 24 hr. After removing the culture medium from cells, 3 scratch wounds across each well were made by using a very fine pipette tip. The loosely held cells were removed by washing with 1× Phosphate buffered saline (PBS) twice. The starvation medium with half concentration FBS was added in each well. Images of the wounds were taken at 0 hr, 24 hr and 48 hr. This assay was replicated twice.

2.5.6 Invasion assay

Matrigel invasion assay was performed using the 24-well matrigel biocoated invasion chamber (BD Biosciences, Bedford, MA, USA) according to the protocol of the kit. Stable cells with *PAX5* gene or control vector pCDNA3.1 were used in this assay. For each matrigel transwell, 0.5 mL of cell suspension with 2.5 $\times 10^4$ cells was added. As chemoattractant, 0.75 mL of culture medium containing 10% FBS and 0.1% bovine serum albumin (BSA) was added in the lower chamber. The chambers were incubated for 24 hr in an incubator at 37 ^{o}C with 5% CO_2 atmosphere. Non-invading cells were removed from the upper surface of the membrane. Cells that invaded through the matrigel membrane were stained using 0.5% crystal violet solution. The number of invaded cells was count under a microscopy. Data were collected and analyzed from three independent assays.

2.5.7 Cell cycle analysis and annexin V apoptosis assay

Propidium iodide (PI) is an intercalating agent and a fluorescent molecule that can be used to stain DNA. This dye is excluded by viable cells but can penetrate cell membranes of dying or dead cells. Cells staining with PI are usually analyzed by flow cytometry to evaluate cell viability or DNA content in cell cycle analysis. For cell cycle analysis, the cells were harvested and washed twice by 1× PBS buffer. Cold 70% ethanol was used to fix the cell at 4 ^{o}C overnight. The fixed cell was wash twice by 1× PBS. PI staining solution (50 μg/mL PI and 100 μg/mL RNase A in 1× PBS buffer) was added to the cells and mixed well. The mixture

was placed at 4 °C for 30 min until analyzed by flow cytometry. About 20,000 cells were counted, and the results were analyzed by ModFit 3.0 software (Verity Software House, Topsham, ME, USA).

Annexin V is a protein which could bind the cell membrane after apoptosis have occurred and before membrane integrity has been lost. The proportion of apoptotic cells was evaluated using Annexin V and PI double staining. Briefly, the cells washed with 1 × PBS was resuspended in 100 μL ice-cold annexin-binding buffer (10 mM HEPES, 140 mM NaCl and 2.5 mM CaCl2, pH 7.4) containing 5 μL Annexin V conjugated with Alexa Fluor 488 (Invitrogen) and 2 μL PI staining (50 μg/mL) (BD Pharmingen, San Jose, CA, USA). After incubation for 15 min at room temperature, cells were mixed with additional 400 μL of ice-cold annexin-binding buffer and analyzed using flow cytometry.

2.5.8 *In vivo* tumorigenicity

BGC823 cells (1×10^6 cells in 0.1 mL PBS) transfected with pCDNA3.1-*PAX5* or pCDNA3.1 only were injected subcutaneously into the dorsal left flank of 5-week-old male Balb/c nude mice, separately. After tumors were visible, the tumor size was measured every 2 days until 3 weeks. Tumor volume (mm^3) was estimated by measuring the longest and shortest diameter of the tumor and calculating as follows: volume = $(\text{shortest diameter})^2 \times (\text{longest diameter}) \times 0.5$. Care of animals and all experimental procedures were approved by the Animal Ethics Committee of the Chinese University of Hong Kong. After 3 weeks, the

mice were sacrificed, and the tumors were weighed and fixed in formalin for histological analysis.

2.5.9 Chromatin Immunoprecipitation (ChIP) for PAX5 protein

ChIP is a method for finding the location of DNA binding sites on the genome for a particular protein of interest. This technique provides a figure of the protein-DNA interactions that occur inside the nucleus of cells or tissues. In this study, Red ChIP Kit (The Transcription Factor Chromatin Immunoprecipitation Kit from Diagenode, Belgium) was used to determine the downstream target of PAX5 protein.

ChIP analysis was performed according to the manufacturer's protocol. Briefly, for each assay, 1×10^6 AGS cells with PAX5 expression was fixed by cross-linking buffer mixing 30 µL Buffer A with 70 µL of 37% formaldehyde (w/v) and 1 mL PBS for 10 min at room temperature. Glycine (100 µL 0.125 M) was added in, and the mixture was incubated for 5 min at room temperature to stop the cross-linking. Cells were scraped from the culture flask after adding 150 µL Buffer B. Ice-cold Buffer C was added to resuspend the pellet and mixture was incubated for 10 min at 4 °C. Mixture of 1 µL Protease Inhibitor mix (P.I.) and 25 µL Buffer D was added for cell lysis. Samples were sonicated for 12 cycles of [30 sec "ON" / 30 sec "OFF"] each. After shearing, the samples were centrifuged to remove debris. An aliquot of 100 µL sheared chromatin was kept as input sample. For immunoprecipitation, 30 µL of sheared chromatin was mixed with

IP-incubation mixture containing 6 μL BSA, 10 μL P.I., 60 μL Buffer E, 30 μL beads, 10 μL PAX5 antibody (0.2 μg/μL, SC-1974, Santa Cruz Biotechnology, California, USA) and 154 μL water. The tubes were incubated overnight at 4 °C on a rotating wheel. The DNA-protein-antibody complex was washed with wash buffer-1, twice; with wash buffer-2, once; with wash buffer-3, once; with wash buffer-4, twice, and eluted by adding 400 μL of Buffer F. The beads were precipitated by centrifugation. The supernatants corresponded to the DNA isolated by immunoprecipitation. Reverse cross-linking was performed by adding 5 M NaCl. The mixture was incubated for 4 hr at 65 °C. The pooled-down DNA and input DNA were purified using phenol/chloroform/isoamyl alcohol (PCI) solution (25:24:1). The DNA was stored in -20 °C until qPCR analysis.

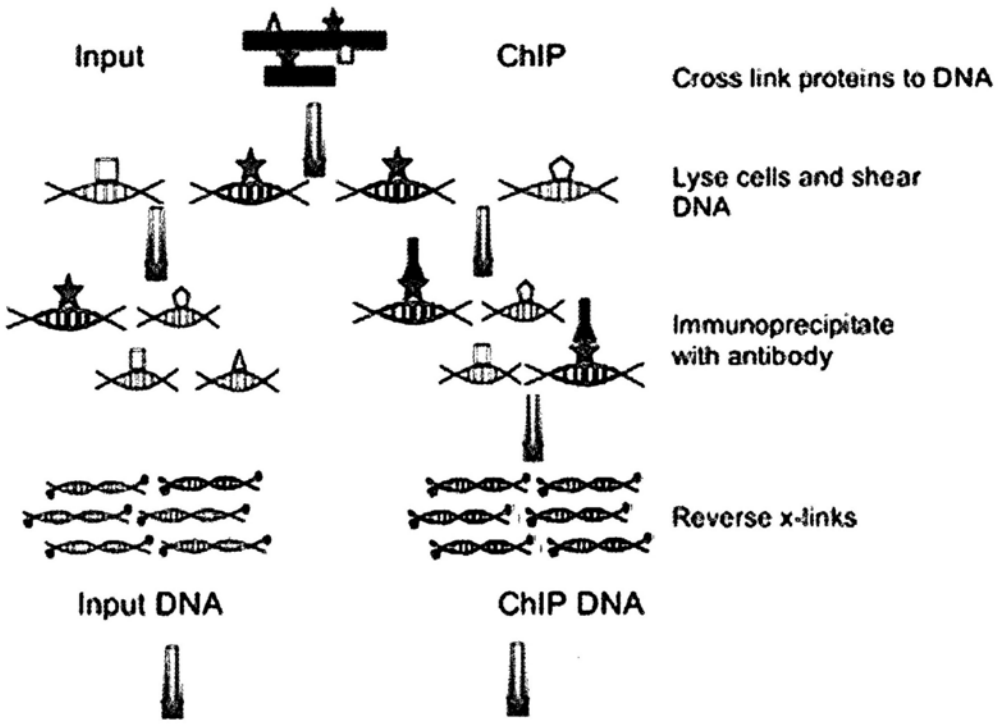


Figure 2.1 Flow chart for ChIP-PCR. This figure is modeled after the figure published online (http://www.nimr.mrc.ac.uk/DEVBIOL/mohun/cardiacdiff/) by Mohun's group.

2.6 Histologic Assay

2.6.1 Preparation of paraffin tissue sections

After the mice were sacrificed, the tumors were removed and fixed by formalin. Then the fixed tissues were embedded in paraffin wax. Paraffin blocks were cut into 4 - 6 micron thick, and floated on a water bath containing distilled water. The sections were picked up on slides. The slides were dried in drying oven or at room temperature overnight.

2.6.2 Immunostaining

Immunohistochemical staining was performed with Histostain-Plus Kits (Invitrogen). Briefly, paraffin sections were deparaffinized with xylene and rehydrated in a graded series of ethanol. Slides were submerged in Peroxidase Quenching Solution (3% hydrogen peroxide) for 10 min. Then the slides were handled with microwave epitope retrieval for 10 min, and washed 3 times with PBS for 2 min. Serum blocking solution was added in, and incubated for 10 min. It should not be rinsed at this step. Then the primary antibody was applied overnight at 4 °C. After that, the slides were rinsed 3 times with PBS for 2 min. The slides were incubated with enough biotinylated secondary antibody for 30 min, and were rinsed 3 times with PBS for 2 min. After rinsing, enzyme conjugate was applied in a humidified chamber for 30 min, followed with rinsing 3 times by PBS for 2 min. The color was developed in a diaminobenzidine (DAB) substrate

solution. Finally, the sections were counterstained with hematoxylin and mounted with histomount.

2.6.3 In situ DNA nick end labeling

Terminal deoxynucleotidyl transferase-mediated dUTP-digoxigenin nick end labeling (TUNEL) assay was performed with Dead End TM Colorimetric TUNEL System (Promega). Briefly, paraffin sections were dewaxed, rehydrated, rinsed with distilled water and washed in 1× PBS. Then the tissues were digested with 20 μg/mL proteinase K at room temperature for 25 min. Refixation of tissues were done with 10% buffered formalin in PBS. Following the application of an equilibration buffer, the sections were incubated in working strength TdT enzymes that contained Biotinylated Nucleotide Mix at 37 °C for 60 min. The reaction was stopped by the application of working strength stop/wash buffer. After washing, quenching of endogenous peroxidase was performed with 0.3% hydrogen peroxide for 5 min. Streptavidin HRP solution was applied in a humidified chamber for 30 min at room temperature. The color was developed in a DAB substrate solution. Sections were then counterstained with haematoxylin. Cell nuclei with brown signal were regarded as programmed death cells. The apoptosis cell ratio was calculated as percentage of positive cell in at least 1,000 cells.

2.7 Statistic Analysis

The difference of *PAX5* mRNA expression level between tumor and adjacent non-tumor primary gastric tissues was analyzed by paired t-test. Independent samples t-test was performed to analyze statistical significant difference between control and PAX5 over-expressed cells in colony formation, MTS assay, cell cycle, annexin V-PI double staining assay, TUNEL, invasion assay and tumor weight in nude mice. The chi-square test was employed for analysis of patient features. Receiver Operating Characteristic (ROC) curve was utilized to estimate the cut off value of the methylation percentage for determination of methylation status. Kaplan-Meier survival curve and log-rank test were used to evaluate overall survival data corresponding to *PAX5* methylation status. The difference in tumor growth rate between the 2 groups of mice stably transfected with PAX5 expression vector and control vector was determined by repeated measures analysis of variance (ANOVA). Data were considered statistically significant when P is less than 0.05; and very significant when P is less than 0.01.

Table 2.1 DNA sequences of primers used in this study.

Primer name	Sequence (5'-3')
RT-PCR	
PAX5RTF	GTCCATTCCATCAAGTCCTG
PAX5RTR	TTGCTGACACAACCATGGCT
Cloning	
PAX5CloneF	ATATaagcttGTCCATTCCATCAAGTCCTG
PAX5CloneR	ATATctcgagAGGGTCAGTGACGGTCATA
BGS	
PAX5aBGSF	gTTTTTTTAAAAGTATTTGTTTGGT
PAX5aBGSR	gCACCTTCTATTAAAACATAC
MSP	
PAX5MSPm1	AAATAAAAATTCGGTTTGCGTTC
PAX5MSPm2	AAACATACGCTTAAAAATCGCG
PAX5MSPu1	TAAAAATAAAAATTTGGTTTGTGTTT
PAX5MSPu2	TTAAAACATACACTTAAAAATCACA
CHIP-PCR	
β-tubulinF1	TTGATTCTGTCCTGGATGTGG
β-tubulinR1	TCAGACACTTTGGGTGAAGGC
MET-IPF1	TCCGCCTCTAACAATGAACTCC
MET-IPR1	AAGGTGAAACTTTCTAGGTGG
TP53-IPF	CACAATGCAGGATTCCTCCAA
TP53-IPR	CCTGGCACAAAGCTGGACA
MTSS1-IPF	AGACCAGCAGGCTCCTCTT
MTSS1-IPR	CGCCGCCTCCTTTTCACT
TIMP1-IPF	GTGGGTGATTGGATAGATTCT
TIMP1-IPR	CACCTCCACGTCAGTCACTA

CHAPTER 3 Result

3.1 Data Mining for *PAX5* Gene

3.1.1 *PAX5*

PAX5 is a member of *PAX* transcription factor gene family. Using University of California Santa Cruz Genome Bioinformatics (UCSC) database, we obtained the information that *PAX5* gene was located at chromosome 9p13.2 (Figure 3.1). Two transcript variants have been reported at this location (Busslinger et al., 1996). The second variant did not contain the paired box domain, which played an essential role in transcriptional activity, so we did not discuss it in this study. *PAX5* encodes a transcription factor which has 391 amino acids.

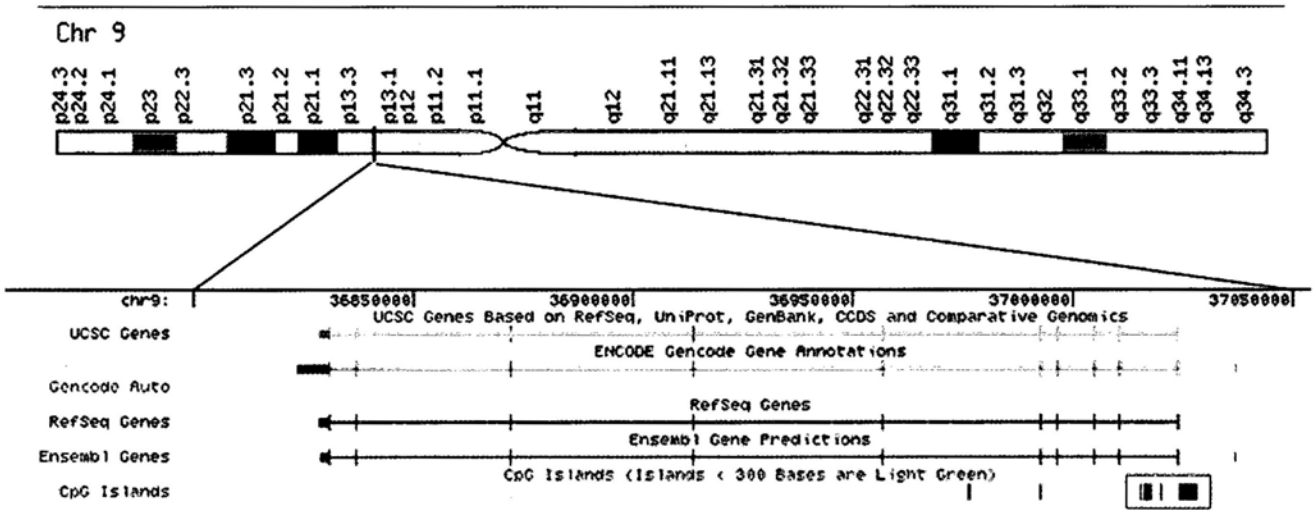


Figure 3.1 UCSC information about *PAX5* gene structure. Chromosomal location of *PAX5* is shown. CpG island is indicated with light green rectangle.

3.1.2 *PAX5* CpG island

Using CpG Island Searcher, we could identify a CpG island spanned the promoter region and the first exon: GC content, 59.4%; observed/expected CpG ratio, 0.885; 79 CpG sites in a 1014 bp region. We designed the MSP primers and BGS primers according to the CpG island analysis result (Figure 3.2 and Table 2.1).

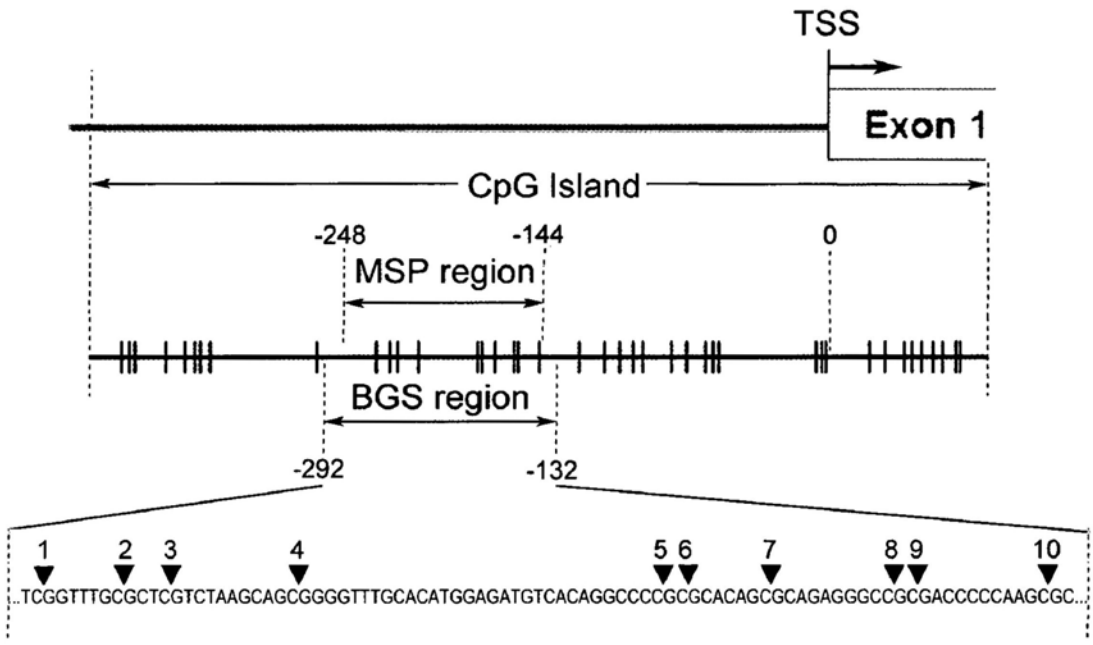


Figure 3.2 *PAX5* gene CpG island. The promoter region and partial of the first exon of PAX5 are shown. Transcription starting site is marked as TSS. The MSP region, BGS region and the 10 CpG sites within the BGS region are also presented.

3.2 *PAX5* Gene Expression

3.2.1 *PAX5* is expressed in most of human tissues

To determine the expression profile of *PAX5*, we examined the *PAX5* expression level in human normal adult and fetal tissues. Using semi-quantitative RT-PCR, *PAX5* was found to be really expressed in most of the human tissues and fetal tissues, especially in digestion organs like liver, esophagus, stomach, colon and rectum (Figure 3.3).

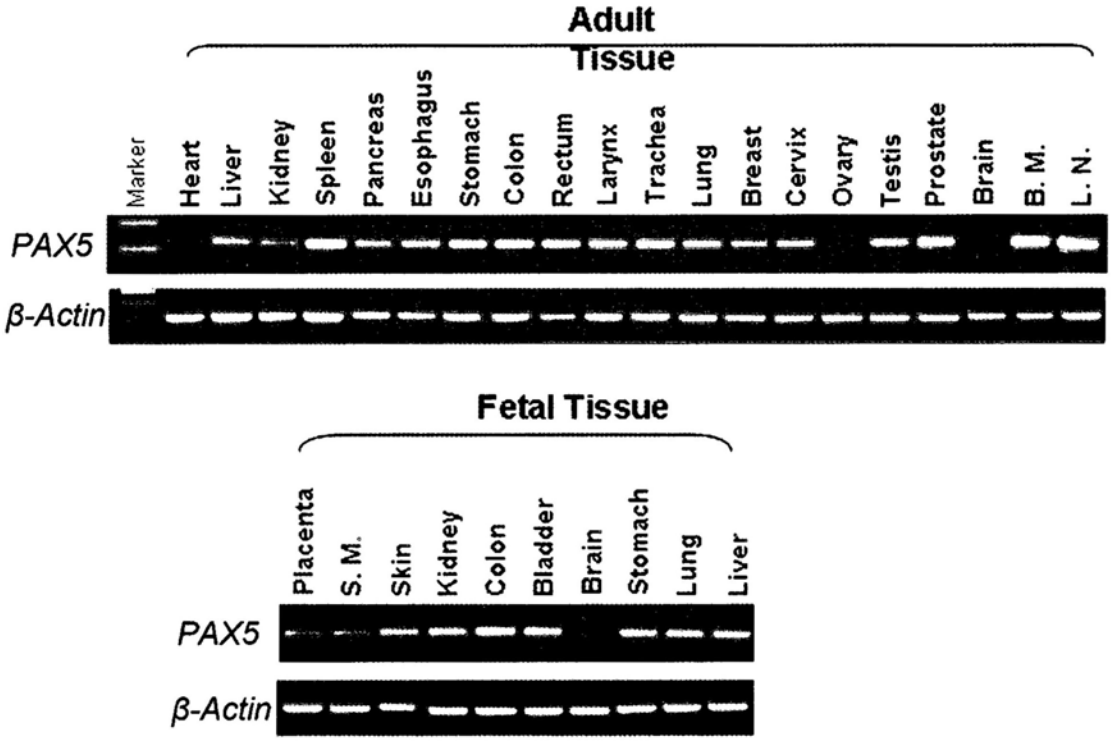


Figure 3.3 *PAX5* gene expression level in human tissues. Expression profile of *PAX5* in human normal adult and fetal tissues by semi-quantitative RT–PCR, with *β-Actin* as a control (S.M., skeletal muscle; B.M., bone marrow; L.N., lymph node).

3.2.2 *PAX5* is epigenetically suppressed in cancer cell lines

To characterize epigenetic effectors of *PAX5* in GC, we detected expression level and methylation status of *PAX5* gene in 8 GC cell lines and 8 CRC cell lines.

For GC cell lines, *PAX5* gene was silenced in 6 cell lines (KatoIII, MKN28, SNU1, SNU16, AGS and BGC823) and down-regulated in NCI87 (Figure 3.4A). *PAX5* transcription was silenced or down-regulated in 7/8 (87.5%) of GC cell lines. For CRC cell lines, loss of expression was detected in 2 cell lines (Caco2 and HCT116) and down-regulation was detected in 4 cell lines (HT29, Ls180, SW480 and SW620), with transcriptional silence or down-regulation were detected in 6/8 (75%) of CRC cell lines (Figure 3.4B).

To access whether the silence or down-regulation of *PAX5* could match the methylation status of the promoter region, MSP was performed using methylation specific primers and unmethylation specific primers. MSP amplicon covered the region of -248 bp ~ -144 bp relative to TSS. Full methylation was detected in 5 GC cell lines (KatoIII, MKN28, SNU1, AGS and BGC823), and partial methylation was found in 1 GC cell line (NCI87). The methylation status in GC cell lines matched the expression level well except for SNU16 cell line, in which *PAX5* was silenced but no methylation was detected. This may due to the reason that *PAX5* gene in SNU16 is silenced by other mechanisms such as histone modification or up-steam transcriptional regulation. Partial methylation was also detected in 6 CRC cell lines (Caco2, DLD1, HCT116, LoVo, LS180 and SW480)

which displayed silence or down-regulation of *PAX5* gene (Figure 3.4).

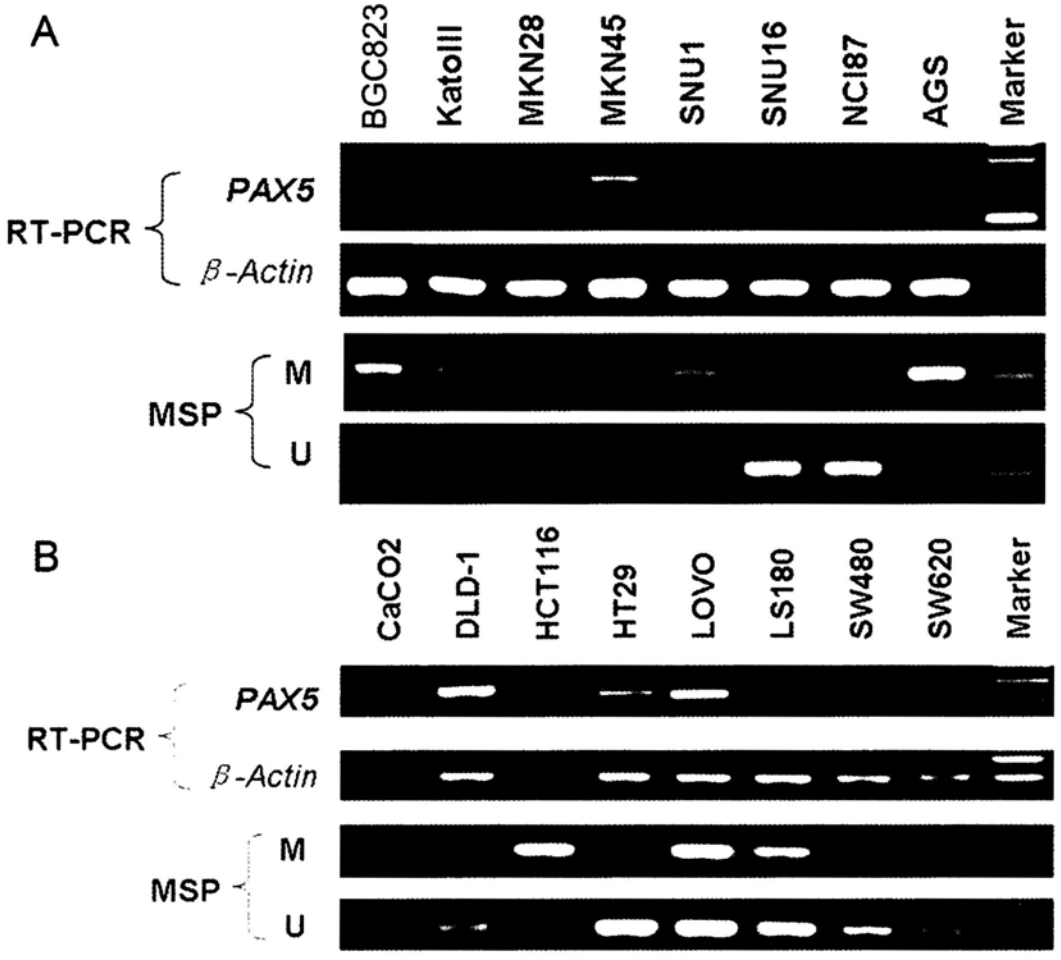


Figure 3.4 *PAX5* mRNA expression and promoter methylation in (A) GC cell lines (B) CRC cell lines. The mRNA expression of *PAX5* in cell lines was determined by RT-PCR. Amplification of *β-Actin* was performed as an internal control for RNA quality. MSP was performed to detect the methylation status. (M: bands amplified by methylation primers, U: bands amplified by unmethylation primers)

3.2.3 *PAX5* expression could be restored after demethylation treatment

To confirm that *PAX5* expression was repressed by promoter methylation, 5-Aza was used to pharmacologically interfere with promoter methylation in methylated cell lines of AGS, MKN28, KatoIII, HCT116, SW620 and SW480. As shown in Figure 3.5, 5-Aza treatment could restore expression of all these cell lines, conferring promoter methylation contributes to the epigenetic suppression of *PAX5* in GC and CRC cell lines.

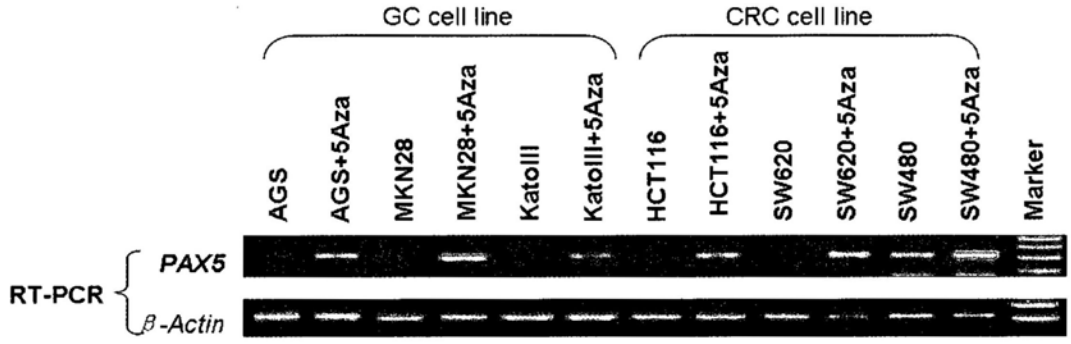


Figure 3.5 *PAX5* gene expression in 5-Aza demethylation treated GC and CRC cell lines. RT-PCR was performed to analyze *PAX5* expression in *PAX5* methylated cell lines treated with 5-Aza.

3.2.4 *PAX5* expression in paired cancer and adjacent normal samples

To evaluate the clinical significance of *PAX5* gene in primary tumors, we compared the mRNA expression level of *PAX5* in 18 pairs of GC biopsies and their adjacent non-tumor tissues using qPCR. Compared with the adjacent normal tissues, *PAX5* was significantly down-regulated in GC tissues (P = 0.0196) (Figure 3.6A). We also compared the expression level of *PAX5* in 18 pairs of primary CRC specimens and their adjacent normal tissues, the result did not show significant difference (P = 0.6830) (Figure 3.6B). This finding suggested that *PAX5* gene played a more important role in GC than in CRC.

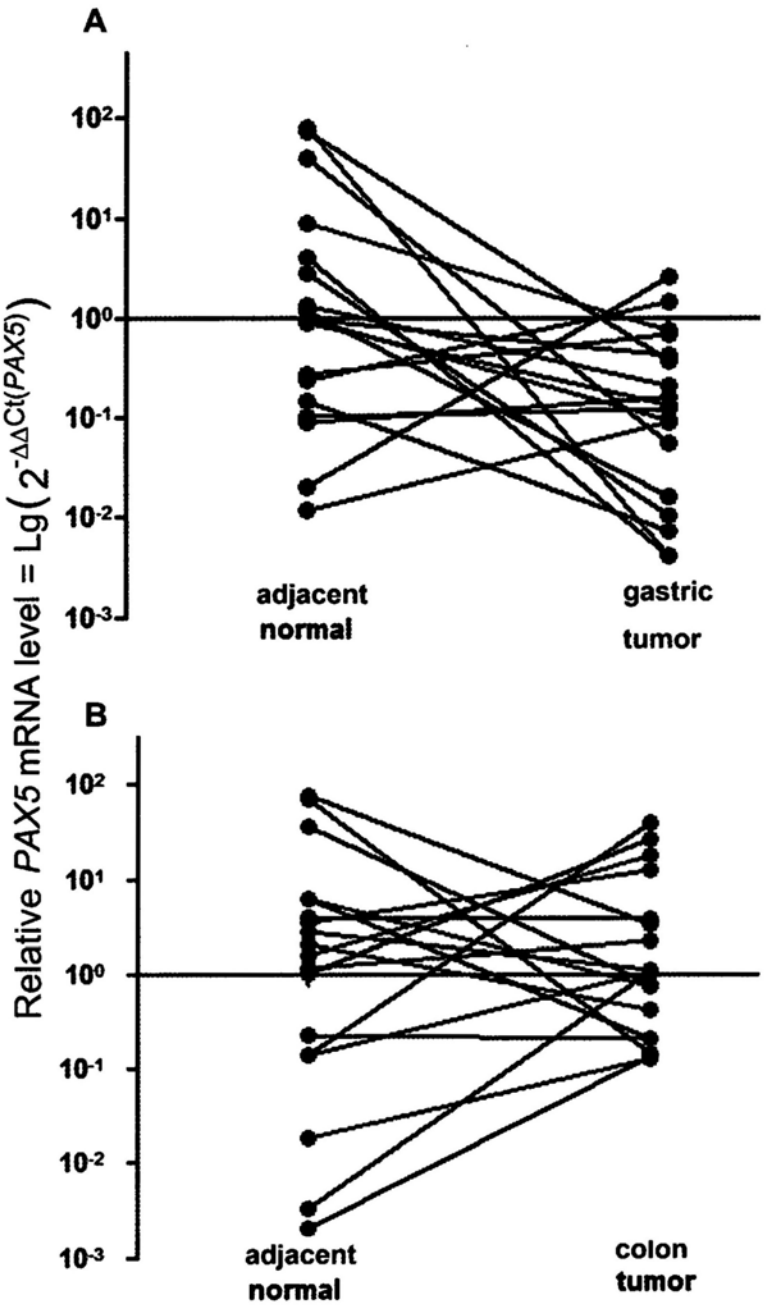


Figure 3.6 (A) *PAX5* gene relative expression level in paired GC samples and (B) in paired CRC samples. Relative mRNA level of *PAX5* was presented by $Lg(2^{-\Delta\Delta Ct(PAX5)})$. Totally, *PAX5* expression in 18 paired of GC and 18 paired of CRC with their corresponding adjacent non-cancerous tissues were quantified by qPCR. The dots for each paired samples were connected with a *line*. P values were calculated with paired T test.

3.3 Functional Assay

3.3.1 Transfection using vector with green fluorescent protein (GFP)

To check the transfection efficiency, pCDNA3.1-eGFP was transfected into cell lines. The transient transfection efficiency was about 40% ~ 50% for AGS, and >80% for HCT116 (Figure 3.7). In this regard, the transient transfection of HCT116 was used directly for functional assays. Stable transfection of *PAX5* had to be established by using 2 to 3 weeks of G418 selection for AGS due to the low transient transfection efficiency. BGC823 with stable *PAX5* transfection was used in the *in vivo* tumorigenicity assay for long-term action.

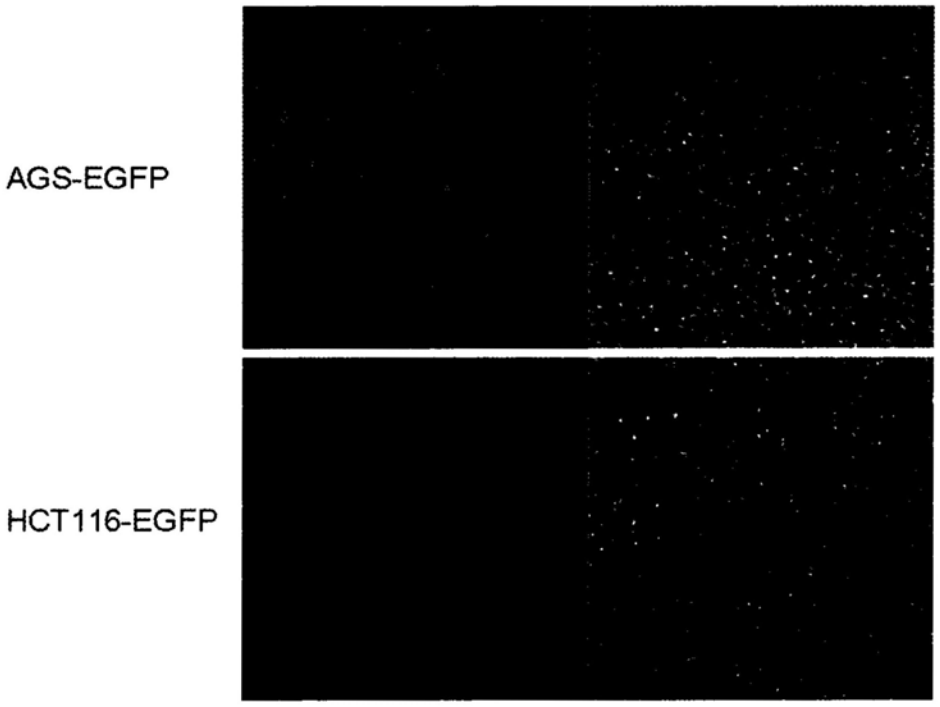


Figure 3.7 pCDNA3.1-eGFP transfected as a transfection efficiency control. For each cell line, the visual fields of the two pictures are the same.

3.3.2 *PAX5* was over-expressed in the transfected cell lines

Three cell lines (AGS, BGC823 and HCT116), whose *PAX5* gene was silenced and fully methylated, were chosen to perform the biological function assay. *PAX5* mRNA level was detected in the stable transfection cell lines AGS, BGC823 and transient transfection cell line HCT116 by using qPCR. As shown in Figure 3.8, cells transfected with pCDNA3.1-*PAX5* had thousands folds of more *PAX5* mRNA than cells transfected with empty vector (pCDNA3.1). In keeping with the enhanced mRNA level, protein expression of PAX5 was also up-regulated in *PAX5* transfected cell lines as determined by western blot, while no signal was detected in the cell lines transfected with empty vector (Figure 3.9).

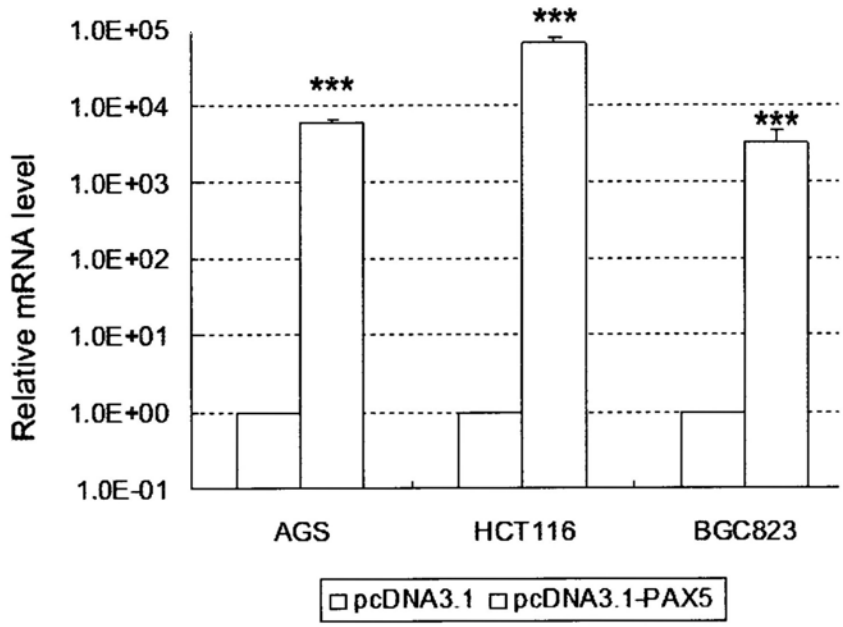


Figure 3.8 Relative *PAX5* mRNA level in transfected cell lines. The expression level in control cell line was defined as 1. The relative fold was calculated as $2^{(Ctcontrol - Ct_{PAX5})}$. (*** P value of t test is less than 0.0001)

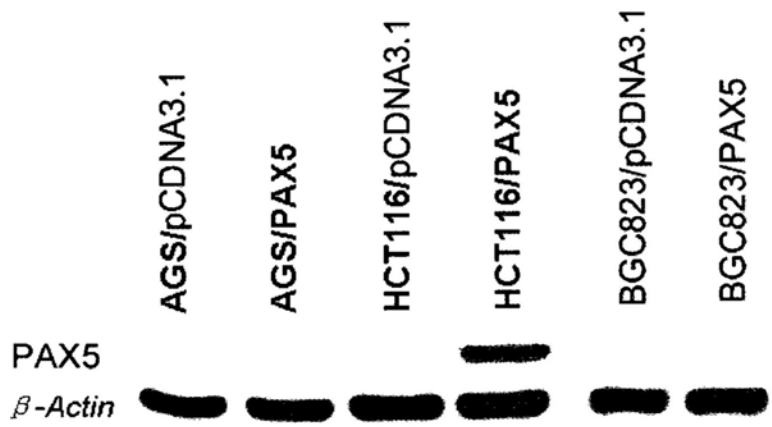


Figure 3.9 PAX5 protein expression in transfected cell lines. PAX5 protein was expressed in AGS, HCT116 and BGC823 cell lines. The internal control of this assay was β-Actin protein.

3.3.3 Inhibition of cell proliferation by PAX5

In vitro biological effects of PAX5 on cell growth in the PAX5 non-expressing cell lines (AGS, HCT116 and BGC823) were examined by cell viability assay and colony formation assay. MTS assay was used for measuring the cell viability. In the cell lines with *PAX5* over-expression, cell viabilities were significantly inhibited to 52% (AGS, $P = 0.0034$), 51% (HCT116, $P = 0.0277$) and 59% (BGC823, $P = 0.0066$), compared to control cells (100%) (Figure 3.10). In addition, after transfection of *PAX5*, cell growth was significantly suppressed to 41% (AGS, $P = 0.0028$), 2% (HCT116, $P < 0.0001$) and 42% (BGC823, $P = 0.0090$), compared with the control cells (100%) (Figure 3.11). Both colony formation and MTS assay solidly demonstrated that PAX5 could inhibit cell growth of GC cells *in vitro*.

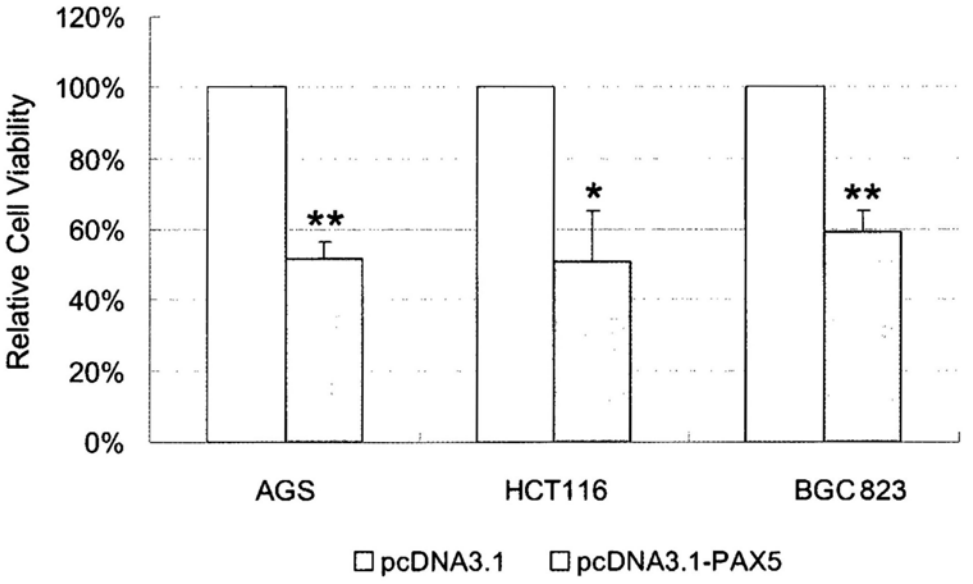


Figure 3.10 MTS assay on re-expression of PAX5. The relative cell viability is shown as mean of absorbance ± SD from three independent experiments. Histogram represents relative percentage of cell viability. (* P value of t test is less than 0.05; ** P value is less than 0.01.)

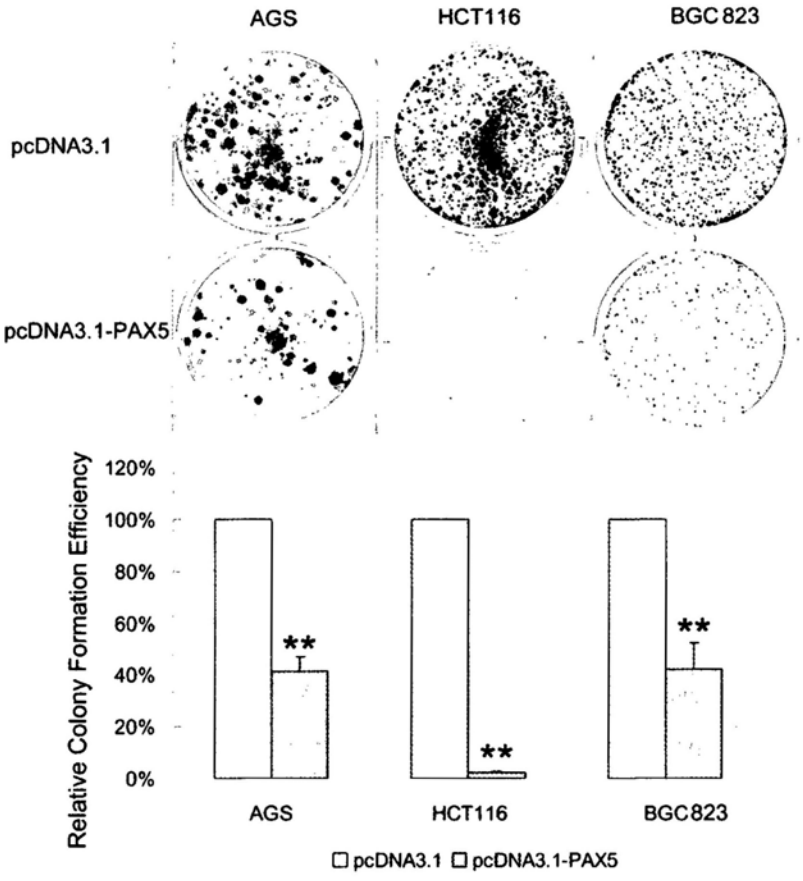


Figure 3.11 Colony formation assay on cell lines transfected with or without PAX5. The effect of PAX5 re-expression on tumor cell growth was assayed using colony formation assay. Quantitative analyses of colony numbers are shown as values of mean ± SD (standard deviation) from three independent experiments. Histogram represents relative percentage of number of colony. (** P value of t test is less than 0.01.)

3.3.4 PAX5 caused cell cycle arrest in G1 phase

To determine the influence of PAX5 on cell cycle distribution, both AGS/PAX5 and AGS/pCDNA3.1 was stained with PI. Cell numbers in different phases of cell cycle were counted by FACScan. The cell proportion in G1 phase of cells transfected with AGS/pCDNA3.1 was 49.2% ± 1.5%, while the proportion in G1 phase of cells transfected with AGS/PAX5 was 58.6% ± 2.6% (Figure 3.12A). The proportion in AGS/PAX5 was statistically significant higher compared with the control group (P = 0.0055) (Figure 3.12B).

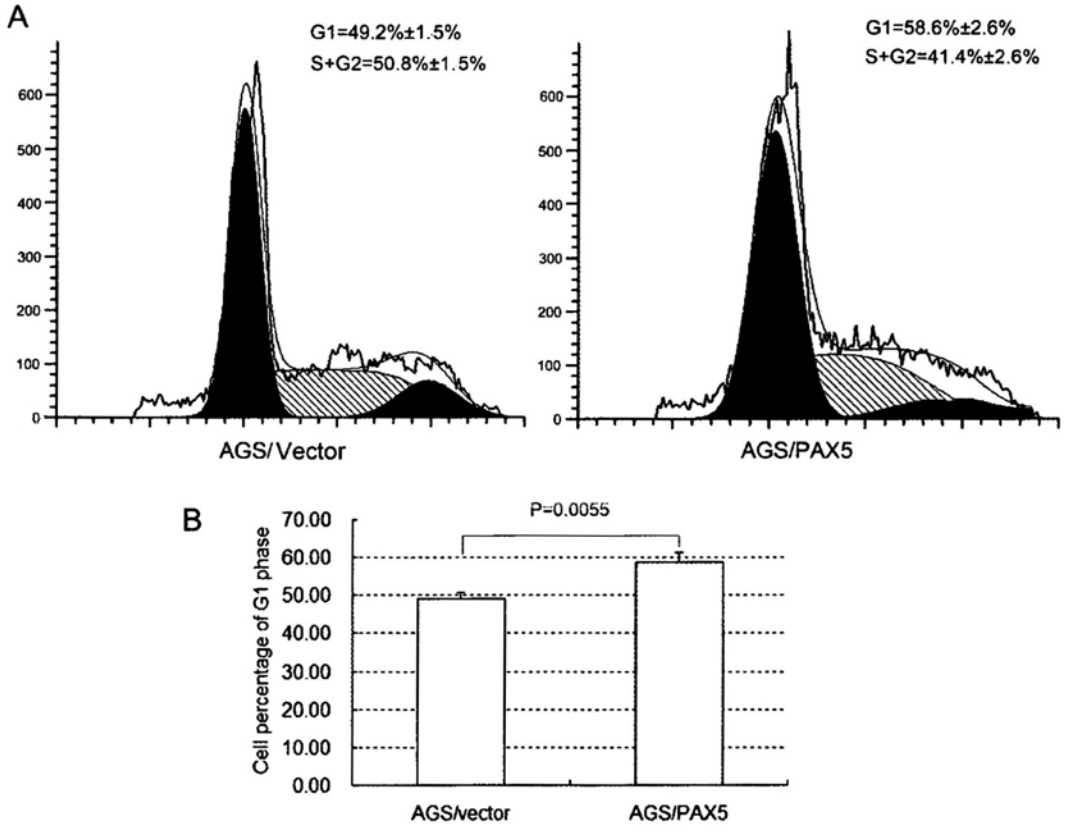


Figure 3.12 PAX5 caused cell cycle arrasted in G0G1 Phase. (A) Representative diagram of flow cytometry. (B) The overall result of the cell cycle analysis with mean ± SD was represented as histogram.

3.3.5 Induction of cell apoptosis by PAX5

To determine if the PAX5 mediated growth inhibition was the result of apoptosis,, cells apoptosis was determined by annexin-V-FITC/PI FACs analysis. Figure 3.13 demonstrated an increase in the numbers of early apoptotic cells (5.60% ± 0.75% *vs.* 2.87% ± 0.38%, P = 0.0316) in AGS stablely transfected with PAX5 in comparison to vector controls. The numbers of dead and late apoptotic cells did not show significant difference between these two groups (P > 0.10)

TUNEL staining was performed to validate the apoptosis induced by PAX5 in the cancer tissue from nude mice. Consistent with the result in annexin-V-FITC/PI FACs analysis, percentage of TUNEL-positive cell was higher in PAX5 expressed tissues compared with the vector controls (3.62% ± 1.12% *vs.* 1.54% ± 0.71%, P = 0.0080; Figure 3.14).

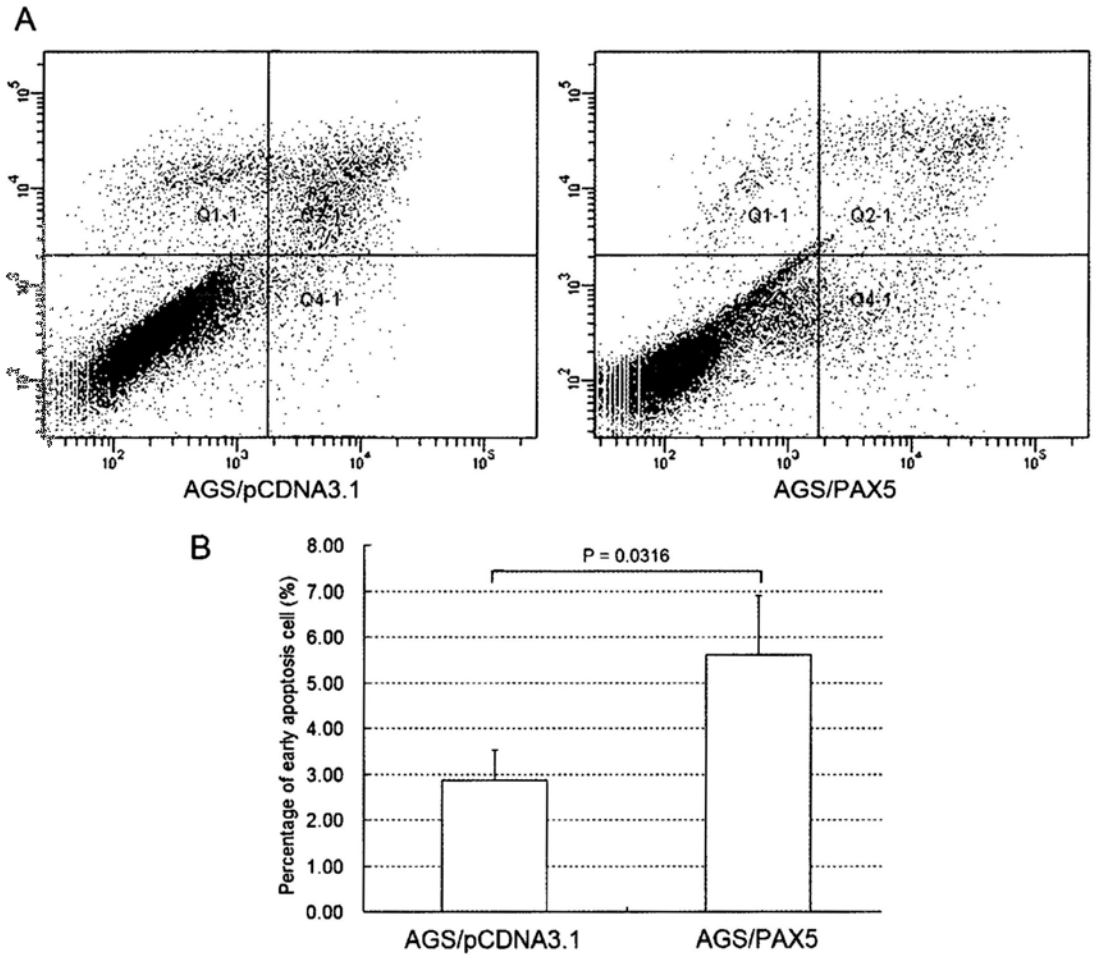


Figure 3.13 Flow cytometry with annexin V and PI double staining. (A) The staining result is shown in four quadrants: Q1 – PI stained only, dead cells; Q2 – both annexin V and PI stained, dead or late apoptosis cells; Q3 – no dye stained, alive cells; Q4 - annexin V stained only, early apoptosis cells. (B) The overall result of the assay is shown as histogram representing the percentage of early apoptosis cells.

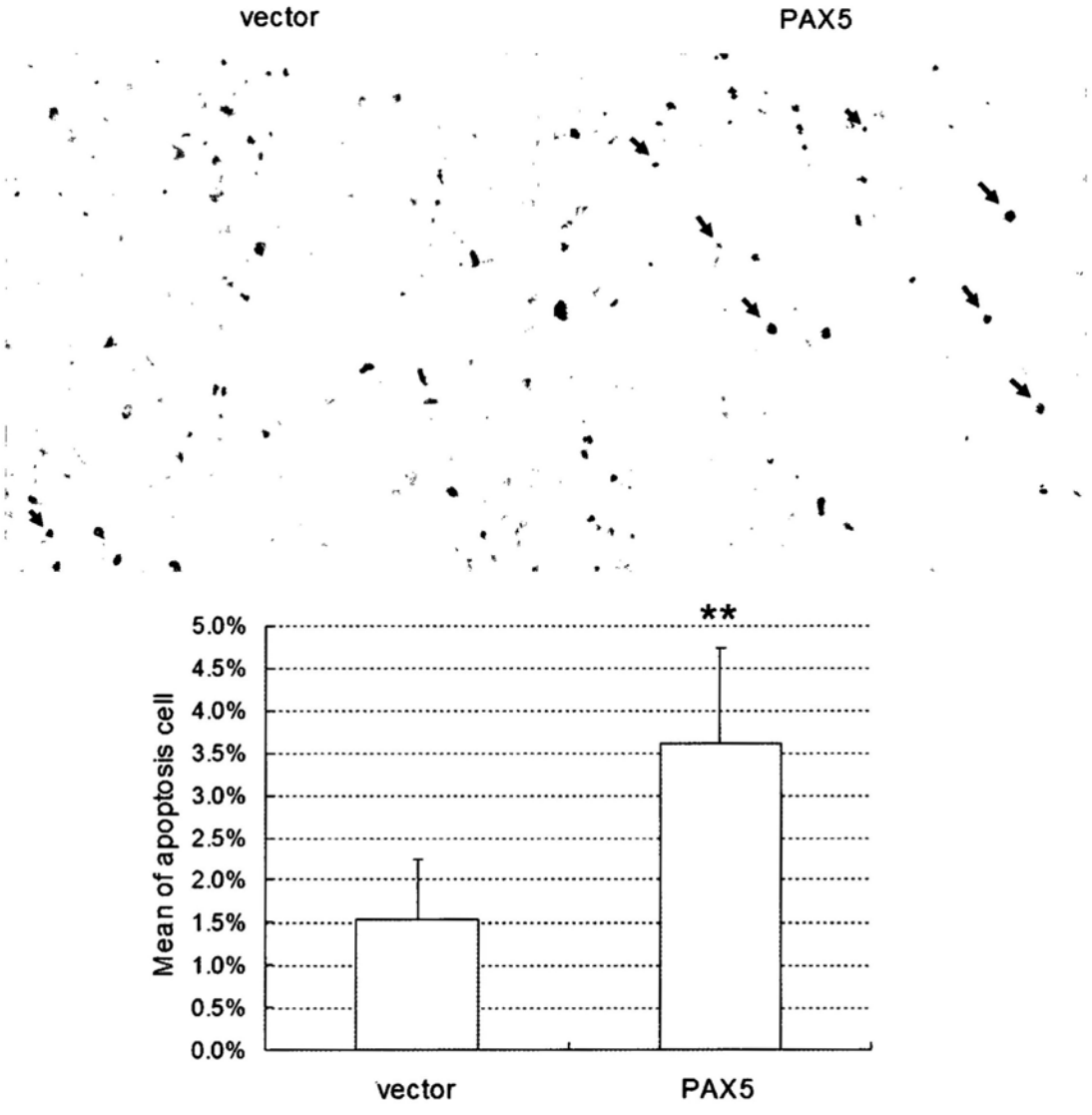


Figure 3.14 TUNEL staining of BGC823 cell in tumor tissues. The black arrows in the upper graphs show the apoptosis cell in brown. The lower histogram represents the overall result of the percentage of apoptosis cell with mean ± SD. (** P value of statistical significant is less than 0.01.)

3.3.6 PAX5 inhibits GC cell migration and invasion

To investigate the effect of *PAX5* in cancer cell metastasis and invasiveness, the monolayer wound-healing assay was performed. A delay in the closure of the wound gaps for AGS/PAX5 was observed, compared with control cell AGS/vector (Figure 3.15).

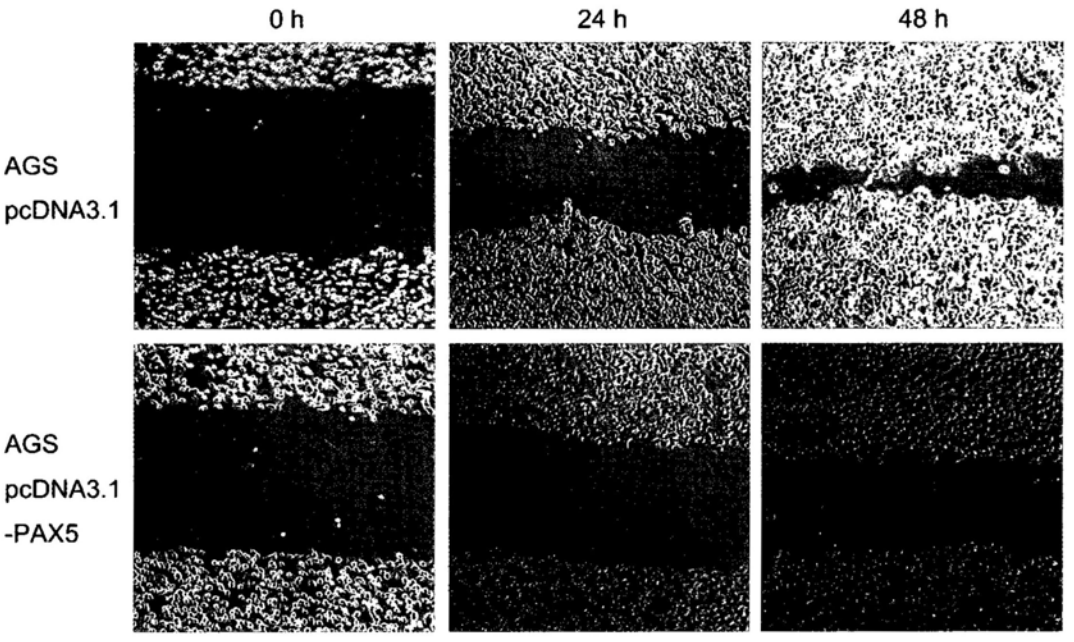


Figure 3.15 Wound healing assay. Photographs were taken at 0 h, 24 h and 48 h to determine the difference between AGS/vector and AGS/PAX5.

For the quantitative assessment of cell migration and invasion, we also performed the matrigel invasion assay. The invaded cell number in AGS with PAX5 expression was significantly lower than in control AGS without PAX5 expression ($P = 0.0218$) (Figure 3.16). The result of wound repair assay and matrigel assay suggested that *PAX5* gene could suppress the GC cell mobility and invasiveness.

AGS/pcDNA3.1 AGS/pcDNA3.1-PAX5

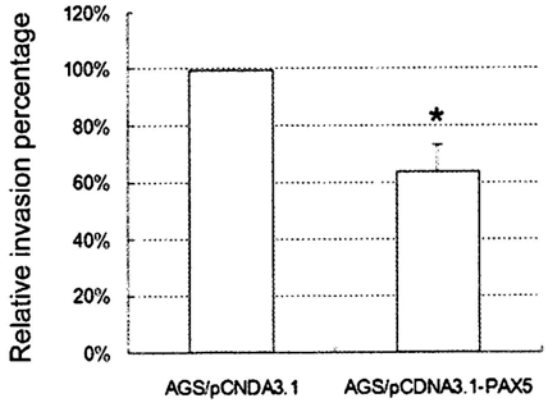


Figure 3.16 Invasion assay. Histogram represents relative percentage of invaded cells from three independent experiments. (* P value of t test is less than 0.05.)

3.3.7 *In vivo* tumor suppression

As mentioned previously, the hypermethylation induced suppression of *PAX5* expression in GC cell lines and tissues indicated that PAX5 might play an important role in inhibition of tumor growth. To approve this hypothesis, we randomly injected BGC823/PAX5 or control cell line BGC823/vector into the dorsal flank of nude mice to compare the tumor growth patterns *in vivo*. Twenty-two days after inoculation, the experiment was terminated and the mice were sacrificed (Figure 3.17A). The tumor growth curve in nude mice was shown in Figure 3.17B. The average tumor size was significantly lower in nude mice injected with BGC823/PAX5 as compared with the control mice injected with BGC823/vector ($P < 0.0001$). The tumors were isolated and weighed during harvesting. The tumor tissues from BGC823/PAX5 group was lighter than those from control group ($P = 0.0112$) (Figure 3.17C). Immunostaining using specific antibody was performed to confirm the existence of PAX5 protein expression in the tumor tissues from BGC823/PAX5 group and exhibited 20% - 30% PAX5 positive cells in this group (Figure 3.18). However, no PAX5 expression was observed in the tumors of the control group, proving consistent evidence that *PAX5* can inhibit the tumor growth in gastric carcinoma.

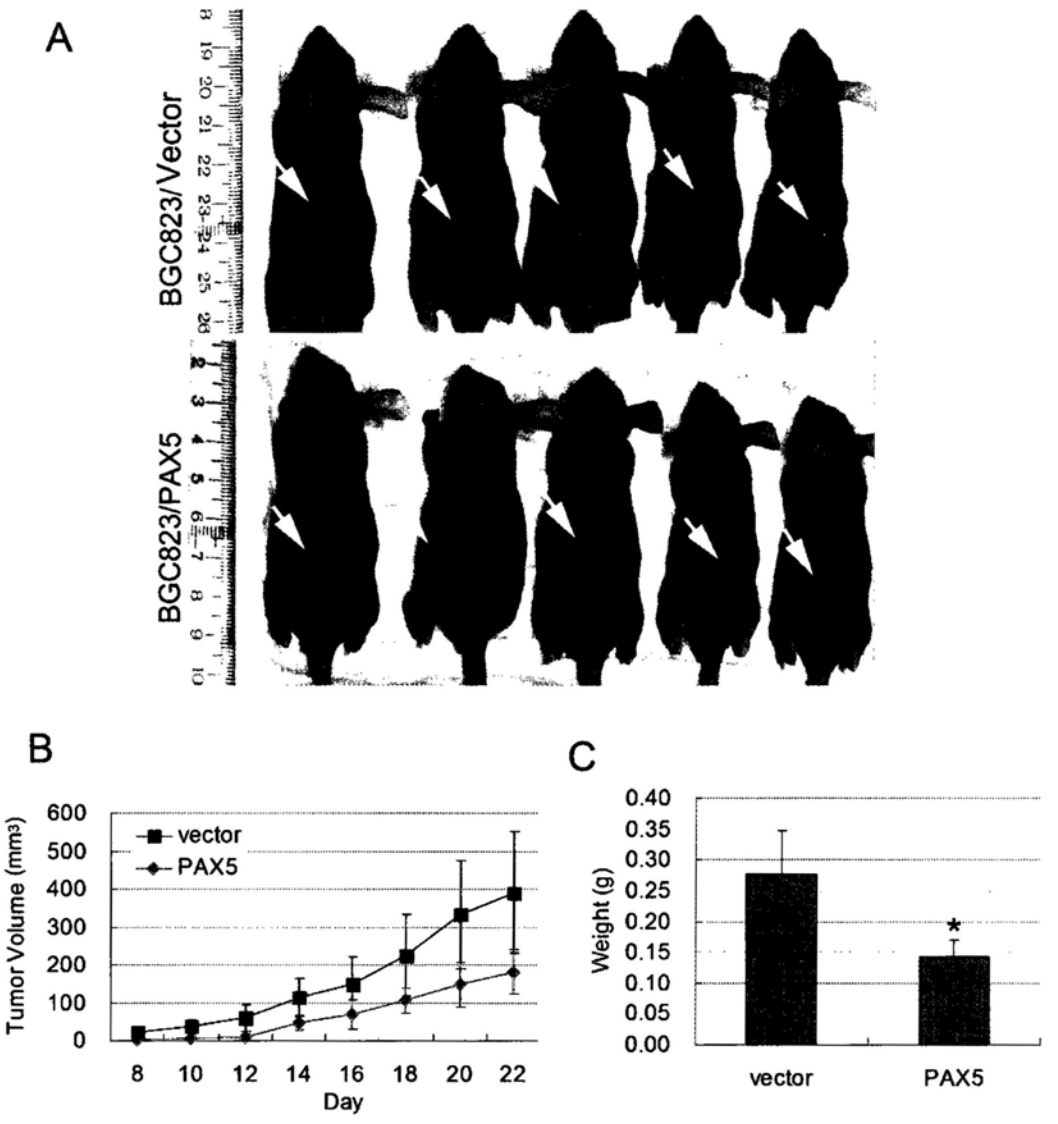


Figure 3.17 (A) Tumor growth in nude mice subcutaneously injected with BGC823/PAX5 or BGC823/vector. (B) Tumor volume curves were plotted against days after treatment. Statistical analysis using repeated-measures analysis of variance indicated the BGC823/PAX5 group showed a significantly slower tumor growth compared with the BGC823/vector group ($P < 0.0001$, repeated measures ANOVA) (n = 5/group). **(C) Average tumor weight.** Histogram represents mean of the tumor weight from the BGC823/PAX5 and BGC823/vector group. * P value of t test is less than 0.05.

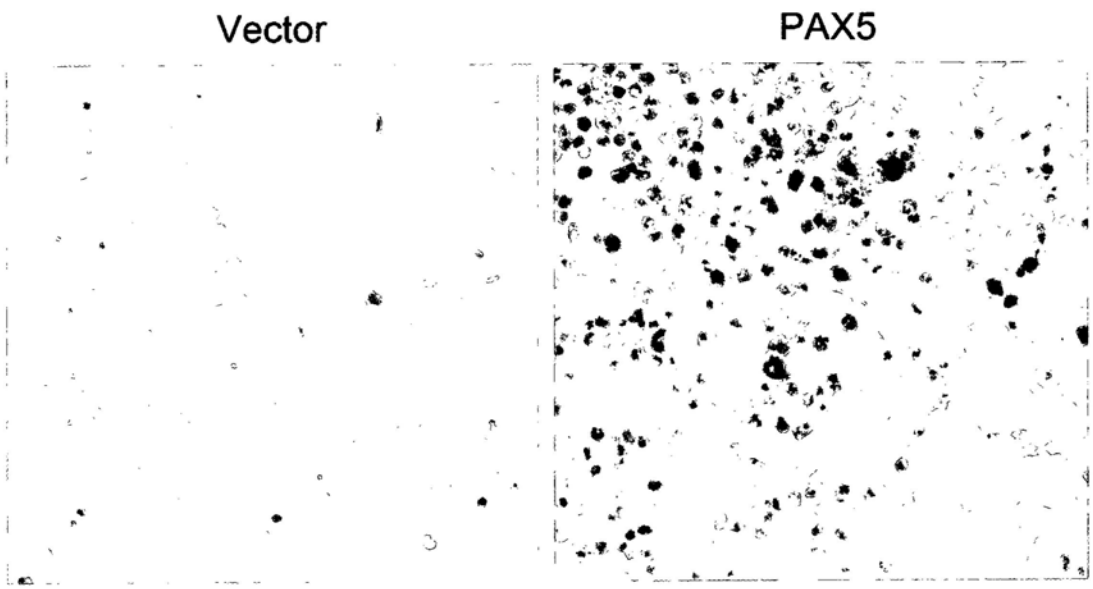


Figure 3.18 Immunostaining of PAX5 in xenograft tumor tissues in nude mice. Brown signals show the PAX5 protein expression.

3.3.8 Gene expression regulation of PAX5

As a member of PAX transcription factor family, *PAX5* can control the expression of target genes. Thus, we evaluated a molecular basis that PAX5 exerts the tumor-suppressor effect in GC through induction of apoptosis, control of cell growth, and inhibition of cell invasion and metastasis. By using cDNA super assay in AGS and HCT116 cell lines, we found some genes, which were involved in the tumorigenesis pathway, were up-regulated or down-regulated post PAX5 transfection. The genes with more than 1.5-fold change in both AGS and HCT116 cell line were listed in Table 3.1. Increased apoptosis ability by PAX5 might be associated with up-regulation of *Tumor protein 53* (*P53*), *BCL2-associated X protein* (*BAX*) and down-regulation of *BCL2*. Enhanced expression of P53 and P21 might lead to cell cycle arrest. In addition, inhibition of GC cell migration and invasion may result from the enhancement of *Metastasis suppressor 1* (*MTSS1*) and *Tissue inhibitors of metalloproteinase 1* (*TIMP1*) and depression of *Mesenchymal epithelial transition factor* (*MET*) and *Matrix metalloproteinases 1* (*MMP1*).

Table 3.1 Up or down regulation of PAX5 downstream genes detected both in AGS and HCT116 cell lines.

Gene Name	Up/Down Regulation		Function	Reference
	AGS	HCT116		
P53	2.8	3.0	Apoptosis and cell cycle	(Diller, Kassel et al., 1990; Shaw, Bovey et al., 1992)
P21	2.5	1.5	Cell cycle	(Zhang, Xiong et al., 1993; Clarke, Lotz et al., 1995)
BAX	2.5	7.3	Apoptosis	(Oltvai, Milliman et al., 1993)
BCL2	-2.4	-2.0	Apoptosis	(Naumovski & Cleary, 1994)
MET	-2.8	-2.4	Migration and invasion	(Jeffers, Rong et al., 1996)
MMP1	-10.6	-4.0	Migration and invasion	(Sauter, Rosenberger et al., 2008)
MTSS1	2.4	2.1	Migration and invasion	(Glassmann, Molly et al., 2007)
TIMP1	2.2	2.2	Migration and invasion	(Reed, Koike et al., 2003)

To confirm the result from cDNA array, protein expression of P53, P21 and Cyclin D1 related to the cell cycle arrest were evaluated by western blot (Figure 3.19). Consistent with the up-regulation of P53 and P21 mRNA, protein expression of P53 and P21 was also increased in the PAX5 transfected cell lines. PAX5 also enhanced protein expression of Cyclin D1, a key G1 phase regulator.

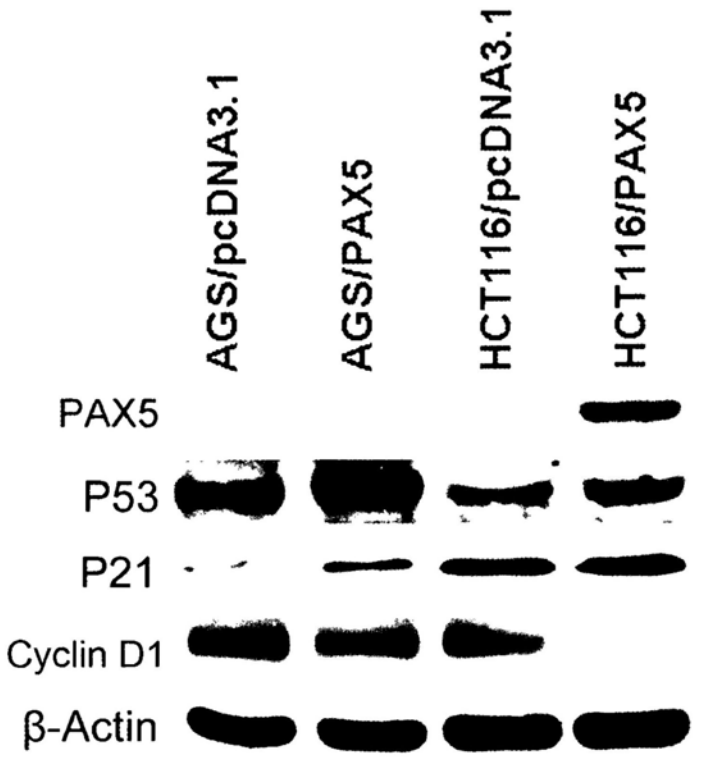


Figure 3.19 Protein expression of genes involved in the cell cycle regulation. Protein expression of PAX5, P53, P21 and Cyclin D1 in AGS and HCT116 cell lines with of without PAX5 transfection by western blot. β-Actin was used as internal control of this assay.

3.3.9 Binding of PAX5 protein to the promoter of *P53* and *MET*

As a nuclear transcription factor, PAX5 works through bind to this response element of the downstream targets. To determine whether PAX5 directly regulate the genes discovered in the cDNA array, we performed ChIP-qPCR assays. PCR product was generated using candidate gene promoter primers (Table 2.1) from ChIP or input samples in AGS/vector and AGS/PAX5 cells. As shown in Figure 3.20, more DNA levels from promoter region of *P53* and *MET* could be pulled down by PAX5 antibody in AGS/PAX5 cells compared with those in AGS/vector cells ($P < 0.01$). In contrast, promoters of *MTSS1* and *TIMP1* could not interact with PAX5 protein. This finding indicated that *P53* and *MET* genes are the direct targets of *PAX5* in the GC tumorigenesis.

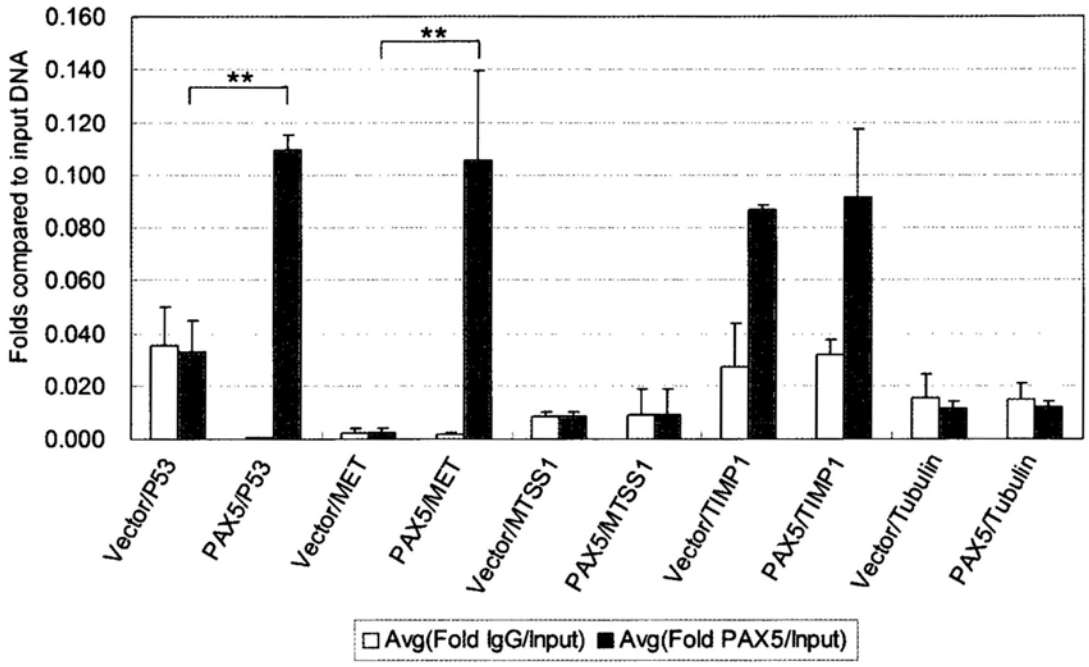


Figure 3.20 CHIP-qPCR result using DNA pulled by PAX5 antibody. Tubulin was used as an internal control of non-specific binding. (** P value of t test is less than 0.01.)

3.4 Methylation Status in GC Patients

The expression of *PAX5* was significant different in primary tumor and their adjacent non-tumor tissues, and showed correlation with methylation status in cancer cell lines. It can be postulated that the *PAX5* methylation level might be utilized as a molecule marker for GC.

3.4.1 Methylation status at each CpG site in the BGS region

BGS was performed to evaluate methylation status at each CpG site within the promoter region (Figure 3.2). Representative results of BGS had been shown in Figure 3.21. Full methylation (methylation 100%) shows only C peak at the CpG site, while unmethylation (methylation 0%) present only T peak. If there were both C and T peak at the CpG site, this situation was defined as partial methylation. Percentage of partial methylation was roughly calculated by the following formula: Methylation % = $H_C/ (H_C + H_T) \times 100\%$

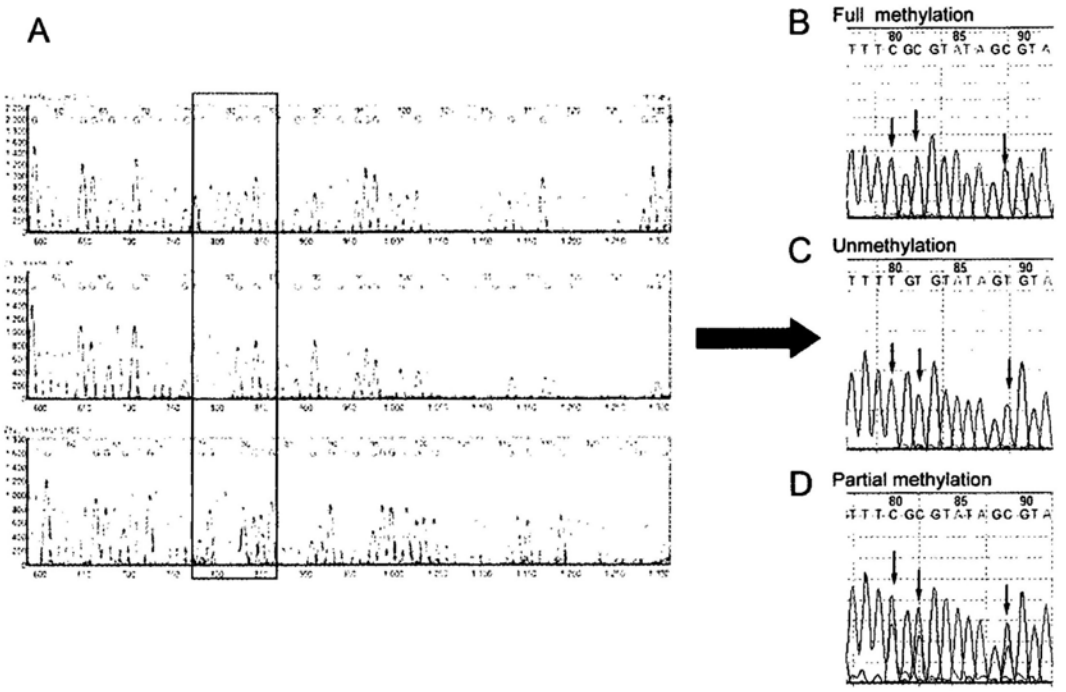


Figure 3.21 (A) Representative results of BGS. Red arrows mark the position of base C in each CpG site. Peak of C was indicated in blue color, while peak of T was shown in red color. **(B) Full methylation (C) Unmethylation (D) Partial methylation.**

Table 3.2 Methylation percentage of *PAX5* in GC cell lines.

	Methylation percentage at each CpG site (%)										
GC cell	1	2	3	4	5	6	7	8	9	10	Average
AGS	100	100	100	100	100	100	100	100	100	100	100.0
MKN28	80	80	80	100	90	90	100	100	100	100	92.0
MKN45	0	0	10	10	10	10	10	10	10	10	8.0

BGS was performed on 3 GC cell line (AGS, MKN28 and MKN45). Average methylation ratios of AGS, MKN28 and MKN45 were revealed to be 100%, 92% and 8%, respectively (Table 3.2). This completely consistent with the results from MSP assay that demonstrated full methylation in AGS, MKN28 and no methylation in MKN45

BGS was also performed on 19 normal gastric biopsies and 161 GC specimens. The average methylation ratios in normal gastric tissues were ranged from 0% to 39% (Table 3.3). There were 3 samples with methylation percentage more than 10% (15, 27, 39%). The average methylation ratios in GC tissues altered from 0% to 100% (Table 3.4).

Table 3.3 Methylation percentage of *PAX5* in normal gastric tissues.

			Methylation percentage at each CpG site (%)										
Case No.	Gender	Age	1	2	3	4	5	6	7	8	9	10	Average
P009	M	64	0	0	0	0	0	0	0	0	0	0	0.0
P012	M	47	0	0	0	0	0	0	0	0	0	0	0.0
P024	F	19	0	0	0	0	0	0	0	0	0	0	0.0
P032	M	35	0	0	0	0	0	0	0	0	0	0	0.0
P039	M	56	0	0	0	0	0	0	0	0	0	0	0.0
P050	M	51	0	0	0	0	0	0	0	0	0	0	0.0
P059	F	67	0	0	0	10	10	20	0	10	20	0	7.0
P061	F	52	0	0	0	0	0	0	0	0	0	0	0.0
P065	F	46	10	10	10	10	0	0	10	0	0	0	5.0
P073	F	40	0	0	0	0	0	0	0	0	0	0	0.0
P079	F	63	0	0	0	0	0	0	0	0	0	0	0.0
P081	F	41	0	0	0	0	0	0	0	0	0	0	0.0
P086	F	46	40	50	40	30	30	30	50	30	40	50	39.0
P088	F	56	0	0	0	0	0	0	0	0	0	0	0.0
P105	M	82	0	0	0	0	0	0	0	0	0	0	0.0
P106	F	46	20	10	10	10	10	20	30	20	10	10	15.0
P121	F	76	20	20	40	20	20	30	40	20	20	40	27.0
P131	F	77	0	0	0	0	0	0	0	0	0	0	0.0
P133	M	22	0	0	0	0	0	0	0	0	0	0	0.0

Table 3.4 Methylation percentage of *PAX5* in GC biopsies.

					Methylation percentage at each CpG site (%)										
Case No.	G	Age	S	Day	1	2	3	4	5	6	7	8	9	10	Average
Z004	M	47	D	337	40	60	60	70	60	60	60	70	80	80	64.0
Z007	M	53	A	351	50	60	60	80	60	60	60	80	80	60	65.0
Z010	M	63	A	356	30	30	10	40	10	10	40	30	30	30	26.0
Z012	M	64	A	363	0	0	0	0	0	0	0	0	0	0	0.0
Z022	M	56	A	357	10	50	50	50	10	10	40	40	50	40	35.0
Z024	M	43	A	362	50	70	90	90	80	80	100	90	100	100	85.0
Z025	F	47	A	369	40	70	50	70	50	50	80	80	70	70	63.0
Z027	M	62	A	354	20	20	20	20	10	10	10	20	10	10	15.0
Z028	F	26	A	385	0	0	0	0	0	0	0	0	0	0	0.0
Z030	M	78	D	123	30	30	30	30	10	30	60	60	40	90	41.0
Z031	M	69	D	105	50	40	50	50	50	60	60	60	60	60	54.0
Z037	M	69	A	402	10	10	0	20	10	30	20	20	20	20	16.0
Z041	M	50	A	408	50	70	60	60	60	60	70	60	70	80	64.0
Z046	F	53	A	425	60	70	60	70	50	20	20	60	70	50	53.0
Z051	M	36	A	432	10	0	10	10	0	0	10	0	10	0	5.0
Z052	M	60	A	433	30	30	30	30	40	50	50	50	50	50	41.0
Z060	M	53	A	491	60	80	70	80	80	80	90	70	60	90	76.0
Z061	M	56	A	494	0	0	0	0	0	0	0	0	0	0	0.0
Z062	F	67	A	498	0	0	0	0	0	0	0	0	0	0	0.0
Z064	F	62	A	456	0	0	0	0	0	0	0	0	0	0	0.0
Z070	F	58	A	496	90	90	80	100	80	90	100	100	90	100	92.0
Z071	M	68	A	498	20	10	50	90	10	10	10	10	20	40	27.0
Z072	M	44	A	498	10	20	10	10	10	10	10	10	10	10	11.0
Z076	M	46	A	531	40	30	10	40	0	0	0	40	20	20	20.0
Z083	M	50	D	148	60	60	50	50	70	80	80	80	90	90	71.0
Z088	F	50	D	237	40	60	60	60	60	80	70	80	60	80	65.0
Z090	F	49	A	574	50	30	40	50	20	10	70	20	30	10	33.0
Z093	M	63	A	582	50	40	50	60	0	0	80	70	50	50	45.0
Z099	M	32	D	320	10	0	10	10	0	20	0	0	20	0	7.0
Z105	M	62	A	620	10	0	10	10	10	10	0	0	10	0	6.0
Z109	M	50	D	600	50	70	60	60	60	70	70	50	70	70	63.0
Z111	F	38	A	570	30	50	50	50	50	60	80	60	70	60	56.0

To be continued

					Methylation percentage at each CpG site (%)										
Case No.	G	Age	S	Day	1	2	3	4	5	6	7	8	9	10	Average
Z115	F	40	D	109	50	70	70	70	70	70	70	70	70	70	68.0
Z120	M	76	D	670	30	70	40	40	60	60	90	50	50	90	58.0
Z123	M	68	A	690	40	50	40	50	70	50	60	50	50	80	54.0
Z127	M	47	A	698	50	0	40	30	30	20	40	20	20	10	26.0
Z128	F	37	D	290	30	30	20	40	50	60	60	20	30	60	40.0
Z129	M	65	A	732	30	70	70	80	60	70	80	50	50	80	64.0
Z133	M	67	D	462	60	70	50	80	0	80	80	80	80	90	67.0
Z134	F	72	D	412	60	70	70	80	80	80	80	80	60	80	74.0
Z136	M	47	A	756	10	0	10	10	0	0	10	0	10	0	5.0
Z139	M	46	A	761	10	0	0	0	20	20	20	10	20	20	12.0
Z140	M	35	D	237	60	60	60	60	60	70	70	60	70	70	64.0
Z141	F	35	A	762	50	60	60	70	70	70	70	70	70	70	66.0
Z147	F	75	D	403	30	50	50	70	50	60	60	30	40	40	48.0
Z152	M	63	A	797	50	70	80	80	50	70	70	50	70	70	66.0
Z155	F	65	A	804	10	0	10	10	0	0	0	0	10	0	4.0
Z156	F	54	D	437	20	0	10	10	0	0	0	0	0	0	4.0
Z161	F	58	D	706	0	0	0	0	10	10	10	10	10	10	6.0
Z162	M	80	D	175	80	80	90	90	90	90	90	90	90	90	88.0
Z169	F	57	D	481	10	70	10	0	10	10	0	0	20	0	13.0
Z172	F	58	D	139	80	50	50	70	70	80	80	80	90	90	74.0
Z179	M	33	D	83	0	0	0	50	0	0	50	0	60	70	23.0
Z204	F	45	A	1072	40	80	70	80	60	70	90	70	70	90	72.0
Z208	F	49	A	1086	30	70	50	50	50	70	80	30	50	70	55.0
Z210	M	65	D	7	70	70	70	70	80	80	80	40	50	80	69.0
Z212	F	68	A	1090	10	30	10	40	30	40	40	10	20	40	27.0
Z216	M	70	A	1021	40	80	90	90	90	90	100	100	100	90	87.0
Z218	F	28	D	349	40	70	50	70	70	70	80	50	60	70	63.0
Z222	M	70	D	237	50	90	60	60	80	90	90	90	90	90	79.0
Z238	M	54	A	1164	50	60	50	60	60	70	70	60	70	70	62.0
Z239	F	51	D	373	10	10	10	10	10	10	0	10	20	20	11.0
Z253	M	52	D	501	50	60	50	70	70	70	90	70	70	80	68.0
Z254	M	69	D	76	60	70	40	70	60	80	90	90	80	80	72.0
Z258	M	70	A	1244	0	0	0	0	0	0	0	0	10	0	1.0
Z271	M	61	D	580	20	10	10	20	80	90	90	70	20	80	49.0

To be continued

					Methylation percentage at each CpG site (%)										
Case No.	G	Age	S	Day	1	2	3	4	5	6	7	8	9	10	Average
Z273	M	62	D	1250	40	20	20	50	0	0	70	80	80	30	39.0
Z280	M	66	D	208	60	40	60	60	30	30	30	50	50	50	46.0
Z281	M	60	A	1340	40	60	70	40	70	90	90	80	80	80	70.0
Z282	M	52	D	216	40	70	50	80	70	70	80	70	80	80	69.0
Z286	M	69	D	332	70	80	80	80	80	90	100	90	90	90	85.0
Z287	M	50	A	1354	40	20	30	20	70	80	80	0	10	10	36.0
Z289	F	57	A	1445	30	60	40	60	70	80	80	80	80	80	66.0
Z295	F	40	D	94	60	80	80	80	80	80	90	90	90	90	82.0
Z296	M	63	A	1486	0	0	0	0	10	20	20	10	10	30	10.0
Z311	M	56	A	1583	50	70	60	60	50	50	60	50	60	60	57.0
Z321	M	42	D	446	10	20	10	10	0	20	30	0	20	10	13.0
Z326	F	73	A	1663	30	50	50	80	10	10	20	30	60	40	38.0
Z328	M	67	D	197	70	90	60	60	70	80	90	80	50	100	75.0
Z329	M	59	D	288	30	30	20	30	10	10	0	20	60	40	25.0
Z334	M	75	A	785	60	60	50	80	100	90	100	100	100	80	82.0
Z344	F	53	A	1751	20	20	30	20	10	70	70	70	50	50	41.0
Z346	M	42	D	1203	80	80	80	80	60	60	70	60	80	70	72.0
Z348	M	65	D	347	60	80	90	80	60	70	70	70	80	70	73.0
Z350	F	30	D	154	10	0	20	30	10	0	10	10	20	0	11.0
Z352	M	69	D	854	50	70	50	70	70	80	90	80	80	80	72.0
Z357	F	45	A	1786	80	50	60	60	70	70	70	70	80	80	69.0
Z358	M	59	D	283	40	40	50	50	40	40	20	40	40	50	41.0
Z360	F	54	D	210	0	0	0	0	0	0	50	20	30	20	12.0
Z361	F	85	D	599	20	20	20	20	20	20	20	20	20	20	20.0
Z363	M	72	A	1830	20	50	40	30	40	70	80	50	60	60	50.0
Z367	M	66	A	1869	80	70	80	40	80	80	90	20	20	90	65.0
Z369	M	45	D	351	30	50	50	60	50	80	60	30	20	70	50.0
Z373	F	67	D	1173	30	60	50	50	50	70	80	50	60	80	58.0
Z376	M	64	D	778	20	30	10	50	70	90	80	60	50	80	54.0
Z377	M	74	D	265	50	70	70	80	60	60	70	80	80	80	70.0
Z384	M	53	A	1968	40	60	50	50	50	50	50	70	50	70	54.0
Z386	F	36	D	903	10	50	40	40	30	30	80	30	30	90	43.0
Z390	F	43	A	1996	0	0	0	0	0	0	0	0	0	0	0.0
Z393	F	41	D	240	40	80	50	60	60	60	60	70	60	70	61.0

To be continued

					Methylation percentage at each CpG site (%)										
Case No.	G	Age	S	Day	1	2	3	4	5	6	7	8	9	10	Average
Z395	F	56	D	1316	20	20	10	20	10	10	10	10	10	10	13.0
Z399	F	57	D	1104	30	50	40	40	70	70	70	30	30	80	51.0
Z400	M	74	D	242	40	50	30	30	20	30	30	30	30	30	32.0
Z414	M	27	D	145	40	60	50	70	70	70	70	70	70	70	64.0
Z415	F	39	D	501	50	80	70	80	90	90	90	90	90	90	82.0
Z416	M	53	D	145	40	50	50	50	60	60	60	70	70	70	58.0
Z417	M	54	A	2218	40	70	70	70	70	80	80	80	80	80	72.0
Z419	M	55	D	339	30	50	30	40	50	50	50	50	50	50	45.0
Z420	M	67	D	149	70	50	40	50	70	70	80	70	70	80	65.0
Z421	M	49	A	2215	70	50	30	30	80	70	90	70	70	80	64.0
Z422	F	75	A	2222	20	80	30	70	70	80	100	20	20	60	55.0
Z427	M	35	A	2210	10	0	0	0	0	0	0	0	0	0	1.0
Z434	M	69	D	185	30	30	60	30	30	30	40	30	20	30	33.0
Z436	M	69	A	2342	20	40	40	40	30	30	30	30	50	30	34.0
Z439	M	59	D	2205	20	20	10	20	10	10	20	10	10	20	15.0
Z444	F	45	A	2417	50	70	50	50	70	80	80	70	80	80	68.0
Z447	M	38	D	394	50	50	50	50	50	50	50	30	50	50	48.0
Z448	M	68	D	2301	30	30	30	30	40	50	40	40	50	50	39.0
Z449	F	50	D	1719	20	30	30	30	50	50	50	50	50	50	41.0
Z451	F	50	D	793	20	20	20	10	10	10	20	10	10	10	14.0
Z455	M	58	D	650	10	20	20	20	10	10	20	20	20	20	17.0
Z457	M	49	A	2530	0	10	10	10	10	0	0	10	10	10	7.0
Z458	M	51	D	470	20	50	60	50	60	60	70	50	30	50	50.0
Z462	M	67	D	992	20	20	20	20	20	20	30	20	20	30	22.0
Z463	M	63	D	359	40	50	50	50	80	80	80	90	90	90	70.0
Z481	M	43	A	2742	40	70	40	50	50	60	60	50	60	70	55.0
Z483	M	68	D	482	40	70	50	60	60	50	70	60	60	70	59.0
Z484	M	71	A	2709	20	40	40	30	30	30	40	30	30	30	32.0
Z486	M	66	D	333	10	10	10	10	50	40	40	40	50	50	31.0
Z487	M	70	D	1899	10	10	10	20	20	10	10	20	30	20	16.0
Z502	M	54	A	2928	30	40	60	50	60	70	70	50	50	70	55.0
Z505	M	52	D	255	30	50	50	50	50	50	70	50	70	70	54.0
Z506	F	56	A	223	50	50	50	60	60	70	70	60	60	70	60.0
Z508	M	62	A	196	10	40	30	30	30	40	40	40	40	40	34.0

To be continued

					Methylation percentage at each CpG site (%)										
Case No.	G	Age	S	Day	1	2	3	4	5	6	7	8	9	10	Average
Z509	M	63	D	129	40	50	30	50	60	70	60	70	70	70	57.0
Z513	M	53	A	209	20	50	30	50	50	50	60	50	50	50	46.0
Z516	M	38	A	180	30	50	50	50	60	70	70	60	60	70	57.0
Z519	F	69	D	228	70	90	90	80	90	90	90	100	100	100	90.0
Z541	F	26	A	279	20	20	10	10	20	20	20	20	10	30	18.0
Z551	F	35	A	266	10	10	10	10	20	20	40	30	20	30	20.0
Z559	M	71	A	324	20	30	30	30	30	30	40	30	30	30	30.0
Z561	M	64	A	294	60	70	70	80	80	80	90	80	90	90	79.0
Z563	M	63	A	294	30	40	30	40	30	30	40	30	30	30	33.0
Z564	M	68	A	328	30	50	30	40	50	50	50	70	90	90	55.0
Z567	M	69	A	334	20	20	30	30	30	40	90	40	20	80	40.0
Z569	M	66	A	306	50	30	40	30	40	40	50	50	40	50	42.0
Z576	M	76	D	139	40	50	50	60	70	80	80	80	80	90	68.0
Z577	M	56	A	1065	20	30	20	40	50	50	60	30	30	50	38.0
Z578	M	63	D	363	40	50	50	60	60	50	50	50	60	70	54.0
Z580	F	43	D	500	40	50	50	60	70	70	80	80	80	70	65.0
Z581	M	61	D	364	20	50	30	40	70	80	80	50	70	70	56.0
Z582	M	67	D	145	20	50	40	30	60	50	50	50	60	50	46.0
Z596	M	54	A	1230	20	40	40	30	40	50	50	50	50	50	42.0
Z604	F	74	D	446	40	50	50	50	50	50	50	60	50	50	50.0
Z605	F	65	D	453	30	30	30	40	50	50	50	60	60	50	45.0
Z610	F	68	A	1507	60	50	30	40	40	40	50	60	50	60	48.0
Z613	F	66	A	1553	40	50	50	50	60	50	60	50	50	70	53.0
Z622	M	69	D	127	30	50	50	50	50	50	50	60	50	70	51.0
Z623	M	61	A	1798	50	70	70	70	60	60	60	70	70	60	64.0
Z624	F	44	A	1800	10	20	20	20	30	20	40	30	20	20	23.0
Z625	F	69	D	344	30	50	40	50	30	30	70	20	30	50	40.0

G: Gender, S: Survival; Day: Number of survival day

Methylation ratios in normal samples were significant lower than in GC biopsies ($P < 0.0001$, Figure 3.22). The difference within the normal group or the GC group was not significant.

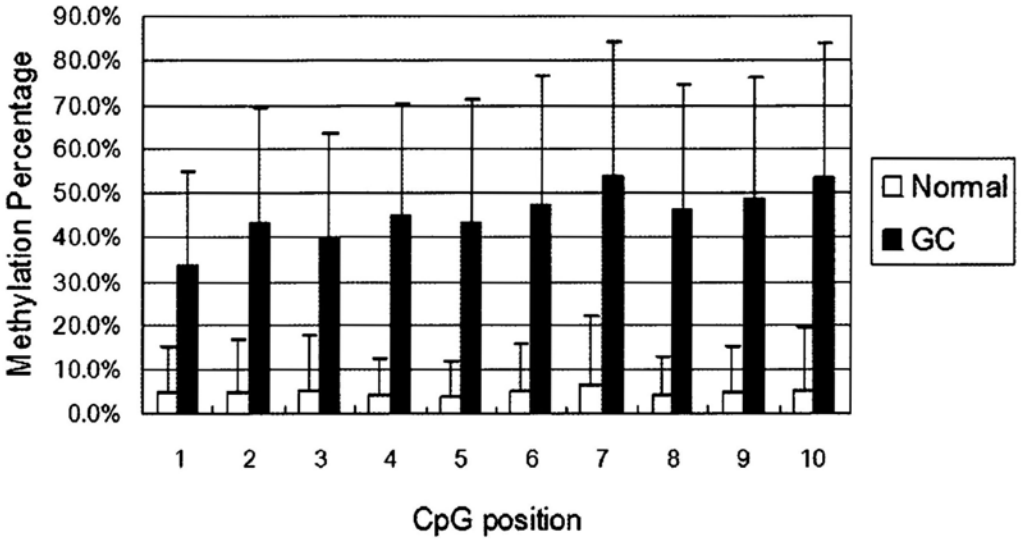


Figure 3.22 Summary of methylation status in primary GC and normal gastric tissues by BGS.

3.4.2 Cut off value for distinguishing methylation and unmethylation

As a biomarker, it was important to determine the methylation status using a cutoff value. ROC curve is a useful tool for organizing classifiers and visualizing their performance. In this study, ROC was used to determine the cutoff value for *PAX5* methylation status in patient. The Area under the ROC Curve (AUC) was 0.937 (Figure 3.23). Considering the balance of sensitivity (90.1%) and specificity (84.2%), 8.5% was chosen as the cutoff value (Methylated sample: methylation% > 8.5%, Unmethylated sample: methylation% ≤ 8.5%). Using this cutoff value, 145 of 161 (90.1%) GC samples and 3 of 19 (15.8%) normal samples were methylated.

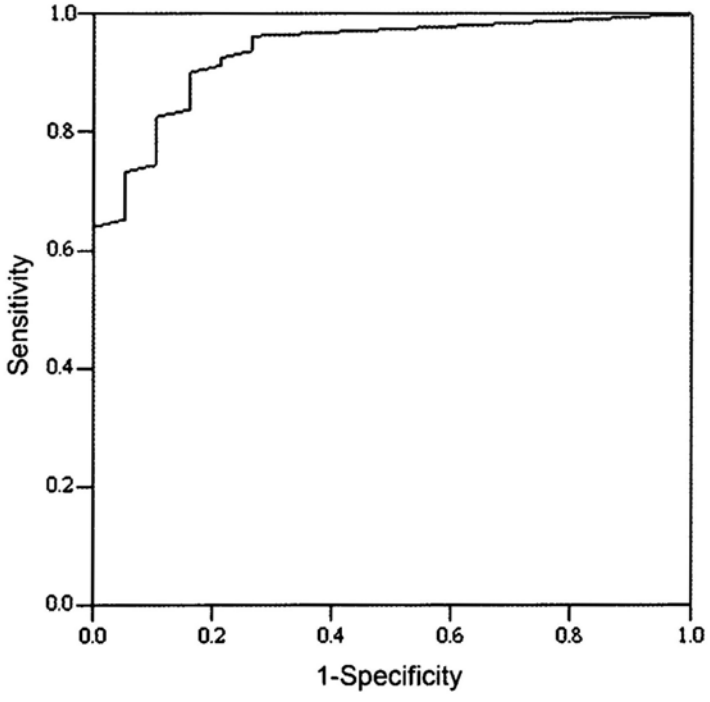


Figure 3.23 ROC curve for the methylation status classifier.

3.4.3 Association between *PAX5* methylation and clinical characteristics

To evaluate the clinical application of *PAX5* in gastric tumors, we analyzed the correlation between *PAX5* methylation and clinical features including patient age, gender, tumor stage, *H. pylori* infection status, Lauren type, tumor differentiation, and survival data. No statistically significance difference was found in the features shown in Table 3.5. However, patients with *PAX5* methylation in tumor tissues (methylation, median survival of 670 days) had poorer survival than others (unmethylation, median survival of 2,205 days) (Figure 3.24). This difference is statistically significant based on the log-rank test (P = 0.0201, Hazard Ratio = 2.212). We could not observe significant difference of survival after stratified by TNM staging. This might due to the limited sample size.

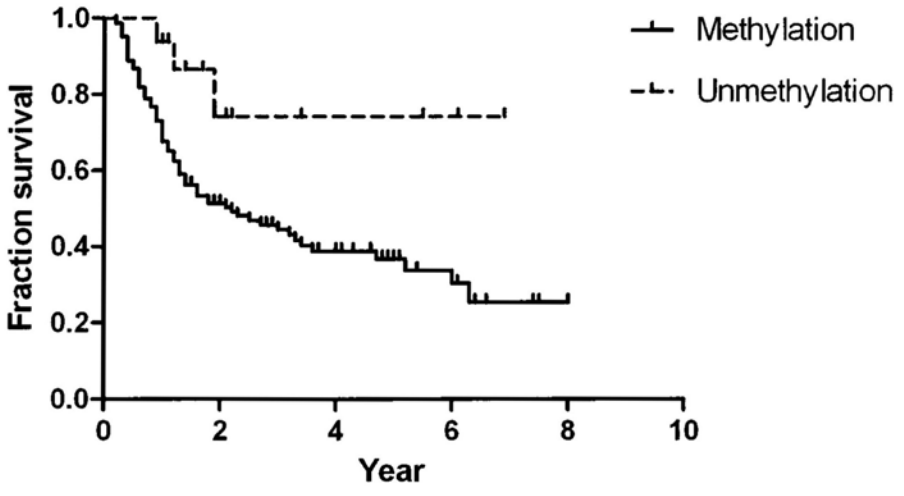


Figure 3.24 Kaplan–Meier survival curves for GC patients.

Table 3.5 Clinicopathologic features of *PAX5* methylation in GC patients.

Variable	Methylated (n=145)	%	Nonmethylated (n=16)	%	P value
Mean age	57.4±12.3		51.6±13.8		0.082
Gender					0.362
M	98	91.6%	9	8.4%	
F	47	87.0%	7	13.0%	
***H. pylori* infection**					0.597
Positive	26	89.7%	3	10.3%	
Negative	60	85.7%	10	14.3%	
TNM					0.268
I	18	90.0%	2	10.0%	
II	19	82.6%	4	17.4%	
III	43	87.8%	6	12.2%	
IV	50	96.2%	2	3.8%	
Lauren					0.708
intestinal	122	90.4%	13	9.6%	
diffuse	12	92.3%	1	7.7%	
mix	10	83.3%	2	16.7%	
Differentiation					0.497
Low	84	89.4%	10	10.6%	
Moderate or High	40	93.0%	3	7.0%	

CHAPTER 4 Discussion & Conclusion

4.1 Methylation and Expression of *PAX5* Gene

As a member of the *PAX* gene family, *PAX5* (*BSAP*) plays an important role in the development of B cell (Nutt et al., 1997). Several reports demonstrated the effect of PAX5 in cancer. Up-regulation of *PAX5* was reported to be related to the genesis of medulloblastoma (Kozmik et al., 1995). It was also reported that *PAX5* gene was involved in the chromosomal translocation of t(9;14)(p13;q32), which contributed to down-regulation of *PAX5* in the diffuse large-cell lymphoma (Busslinger et al., 1996; H. Ohno et al., 2000). However, *PAX5* was absent or down-regulated in tumor cell lines including brain, melanoma, breast, colon, lung, leukemia, ovarian, prostate and renal cancer (Figure 1.9) (Muratovska et al., 2003). Correspondingly, *PAX5* expressed broadly in almost all kinds of normal adult and fetal tissues in our study. As inactivation of *PAX5* gene was frequently observed in GC cell lines and down-regulation of *PAX5* was demonstrated in gastric tumors compared with their adjacent non-cancerous tissues. Thus, we hypothesized that inactivation of *PAX5* might play an important role during the development of GC.

Aberrant methylation of CpG islands within the promoter regions of genes was recognized as a major mechanism of inactivating tumor suppressor in cancer (H. Takada, Imoto et al., 2005; Jung, Park et al., 2008; Yu, Tao et al., 2008). It has been reported that some *PAX* genes, such as *PAX4* and *PAX6*, were involved in epigenetic aberrant expression in cancer cells (Salem et al., 2000; Li et al., 2006). Additionally, some researchers noticed the down-regulated expression was induced by the aberrant promoter methylation of *PAX5* gene in human breast and

lung cancer (Palmisano, Crume et al., 2003). To investigate the relationship between the epigenetic modification and expression regulation in *PAX5*, the promoter region and CGI were identified, and the methylation status of CGI was evaluated by MSP and BGS. The reduced expression of *PAX5* was linked closely with promoter methylation. Moreover, treatment of *PAX5* methylated GC cell lines with demethylating agent (5-Aza), the inhibitor of DNA methyltransferases (Juttermann, Li et al., 1994), restored its mRNA expression, indicating that promoter methylation is the principal regulatory mechanism of *PAX5* inactivation in GC. One unmethylation cell line (SNU16) showed no *PAX5* expression, suggesting that other transcription regulating mechanisms such as histone modification (P. L. Jones, Veenstra et al., 1998) or up-steam transcriptional regulation (B. L. Lee, Kim et al., 2008) may also contribute to the gene silencing. Collectively, our data revealed the epigenetic regulation mechanism on the *PAX5* gene expression in GCs.

By using microarray based methylation analysis in mice, *H. pylori* infected mice had a significant higher *PAX5* methylation level compared with the *H. pylori* negative mice (Alfred SL Cheng, unpublished data). This suggested that the methylation of *PAX5* promoter region might be induced by *H. pylori* infection.

4.2 Biological Function of PAX5 Protein as a Tumor Suppressor

PAX gene family is an essential transcription factor family participated in the development of all kinds of tissues, segmented structures (Deutsch, Dressler et al., 1988), muscle (Goulding, Lumsden et al., 1994), brain (Asano & Gruss, 1992), eye (Del Rio-Tsonis, Washabaugh et al., 1995), nasal (Grindley, Davidson et al., 1995), and kidney (Rothenpieler & Dressler, 1993). Most of these members in *PAX* family were considered as oncogene, especially members of subgroups II and III (Shapiro et al., 1993; Khoubehi et al., 2001; Silberstein et al., 2002). Only one of the *PAX* members (*PAX6)* had been reported to function as tumor suppressor gene with convincing evidence of suppressing tumor growth both *in vitro* and *in vivo* (Y. H. Zhou et al., 2005; Shyr et al., 2009).

In this study, the tumor-suppressive property of *PAX5* in GC was investigated by both *in vitro* and *in vivo* assays. Re-expression of *PAX5* significant suppressed the tumor size of nude mice *in vivo*. It also showed marked cell growth suppressing effect in colony formation and in cell viability. Flow cytometry analysis of *PAX5*–re-expressed AGS cells revealed that the cell cycle was arrested in G1 phase. Our finding was opposite to former study for the role of *PAX5* gene in other types of cancer including neuroblastoma (Baumann Kubetzko et al., 2004) and lung cancer (Kanteti et al., 2009), which indicated that over-expression of PAX5 increased cell proliferation in neuroblastoma cell and down-regulation of PAX5 in small cell lung cancer cell line decreased cell growth. It had been reported that some genes can function as both oncogene and tumor suppressor depending on the

tumor type (Loeb & Sukumar, 2002; L. Yang, Han et al., 2007). The *WT1* gene was identified as a tumor suppressor gene and mutations of *WT1* were associated with the development of kidney tumors (Rauscher, 1993). However, wild-type, full-length *WT1* acted as an oncogenic role in tumor formation in human leukemia, breast cancer and majority of Wilms' tumors (Miwa, Beran et al., 1992; Menssen, Renkl et al., 1995; Nakatsuka, Oji et al., 2006).

GC in late stage is associated with poor prognosis mainly because of the malignant invasion and migration. Using wound healing and matrigel invasion assay, we demonstrated that PAX5 could repress the mobility and invasiveness in the GC cells. Moreover, PAX5 induced cell apoptosis as evidenced by Annexin V FACScan and TUNEL assay. Our data confirmed *PAX5* as an important tumor suppressor in GC.

4.3 Molecular Mechanism of Tumor Suppressive Function

As a member of transcription factor family, *PAX5* was reported to regulate several genes in development of B cell. The promoter of CD19, a signal-transducing receptor on the surface of all B-lymphoid cells, was occupied by PAX5 protein both *in vitro* and *in vivo* (Kozmik et al., 1992).

To elucidate the mechanisms of PAX5 in GC tumorigenesis, we conducted a cDNA array to characterize the molecular basis of the regulative effect of PAX5 on the down-stream gene expression. Expression of 5 genes (*BAX*, *P21*, *MTSS1*, *TIMP1* and *P53*) were advanced by PAX5 re-induction, and mRNA level of 3 genes (*BCL2*, *MET* and *MMP1*) was declined. According to the result mentioned above, a suggestive mechanism about the molecular modulation induced by PAX5 protein is shown in Figure 4.1.

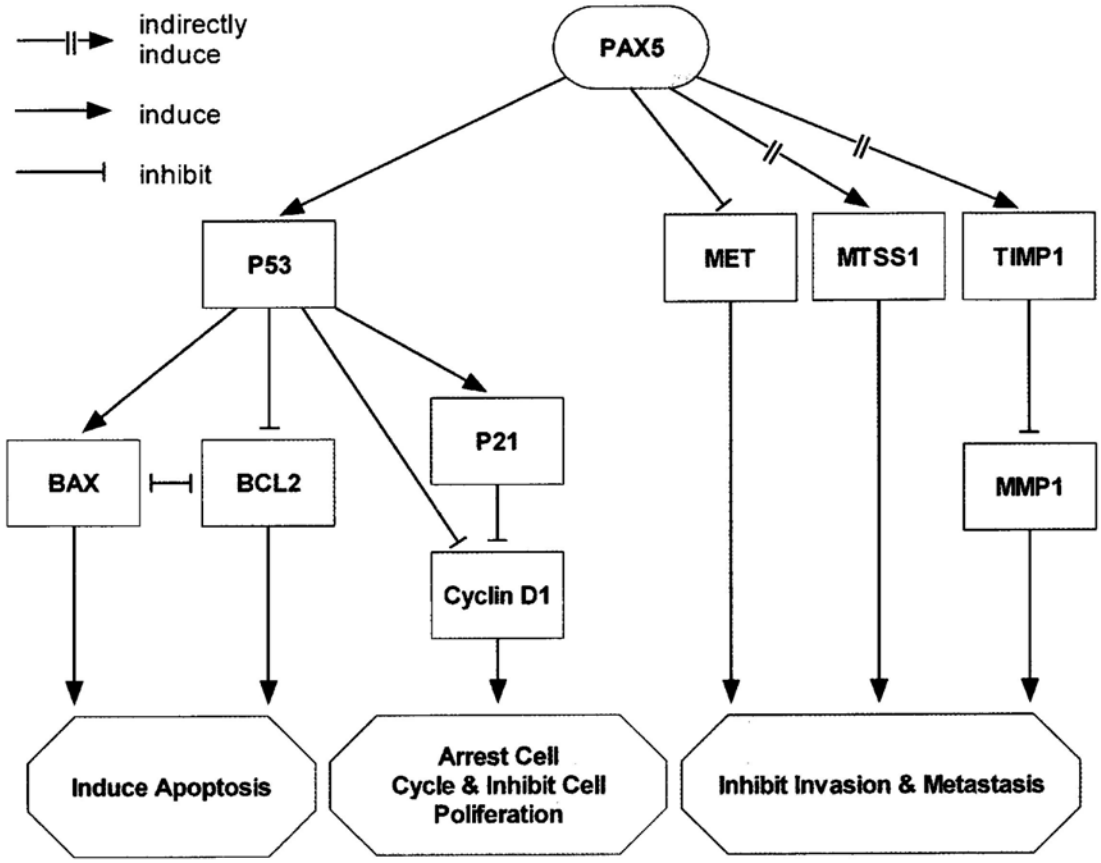


Figure 4.1 Schematic diagram for the mechanisms of anti-tumorigenesis functions of PAX5.

The anticancer effect of PAX5 is mainly included three parts: apoptosis induction; cell growth arrest in G-1 phase and proliferation inhibition; mobility and invasiveness suppression.

P53 gene plays a key role in both apoptosis and cell cycle arrest. P53 (TP53) discovered in 1979 (DeLeo, Jay et al.) is a very important tumor suppressive gene in many human cancers (Levine, 1989; Kishimoto, Murakami et al., 1992; Malkin, Sexsmith et al., 1994). The *P21* (*WAF1/CDKN1A, Cyclin-dependent kinase inhibitor 1A*) gene contains several P53 response elements that mediate the direct binding of the P53 protein resulting in transcriptional activation of P21 (El-Deiry, Tokino et al., 1993). P53 can also suppress *cyclin D1* (*CCND1*) transcription indirectly (Rocha, Martin et al., 2003). The P21 protein binds directly to CDK4 complexes that drive forward the cell cycle (G. He, Siddik et al., 2005). Suppression of cyclin D1, as a regulatory subunit of CDK4, inhibits the cell cycle entry into S phase (Quelle, Ashmun et al., 1993; Musgrove, Lee et al., 1994). Direct interaction between the PAX5 protein and promoter of *P53* has been identified in astrocytomas (Stuart, Haffner et al., 1995). As a result, it is reasonable to speculate that PAX5 may also perform a similar regulation in GC. In our study, we demonstrated *P53* was one of the direct targets of PAX5 by using ChIP-qPCR.

P53 protein can also down-regulate *BCL2* (Haldar, Negrini et al., 1994) and up-regulate *BAX* (Miyashita & Reed, 1995), which are involved in the process of program cell death. BCL2, discovered by Tsujimoto et al. in 1984, is a protein

acting as an apoptotic blocker (Hockenbery, Zutter et al., 1991; Ruvolo, Deng et al., 2001). Over-expressed BAX induces apoptotic cell death by cytokine deprivation and represses activity of BCL2 (Oltvai et al., 1993), whereas BCL2 can promotes cell survival by forming heterodimerizes with BAX (Hanada, Aime-Sempe et al., 1995). Meanwhile, it has been reported that *P21*, which is involved in the cell cycle control, also has something to do with programmed cell death (Ho, Wang et al., 1996). These finding suggest a model in which the increase of P53, BAX, P21 and the decline of BCL2 induced by over-expression of PAX5 may determine survival or death following an apoptotic stimulus.

There are several genes recruited in the biological function of migration and invasion, such as *MET* (*c-MET*), *MTSS1*, *TIMP1* and *MMP1*. MTSS1, also known as missing in metastasis (MIM), is a potential metastasis suppressor involved in cytoskeletal organization (Y. G. Lee, Macoska et al., 2002). TIMP1 indentified by Docherty et al. (1985) acts an inhibitory role against most of the known MMPs except MMP14 (Visse & Nagase, 2003). MMP1 that contains a zinc ion at its active site can degrade collagen, elastins and other components of the extracellular matrix in normal physiological processes, resulting in cell migration and invasion (Chambers & Matrisian, 1997; Sauter et al., 2008). The proto-oncogene *MET* encodes a trans-membrane receptor for tyrosine-kinase activity (Bottaro, Rubin *et al.*, 1991). It is the receptor for a molecule known as scatter factor or hepatocyte growth factor (SF/HGF), which stimulates cell motility and invasiveness thought MET activity (Giordano, Zhen et al., 1993). It

has been reported that *MET* was positively and directly regulated by PAX5 protein in small-cell lung cancer (Kanteti et al., 2009). In contrast, according to our cDNA array and ChIP-PCR result, we found that *MET* expression was directly repressed because of PAX5 re-expression in GC cell line. It is interesting that *MET* could be regulated in dual-direction by PAX5, but this phenomenon was not observed for the first time. The repression domain (octapeptide) and the activation domain allow Pax5 protein to convert from transcriptional activator to repressor through interaction with co-repressors from the Groucho protein family (Eberhard et al., 2000). Several studies considered *PAX5* as an oncogene in tumorigenesis such as in neuroblastoma (Baumann Kubetzko et al., 2004; Souabni et al., 2007), but our findings revealed that *PAX5* could play a tumor suppressive role in GC.

4.4 Potential of *PAX5* for Clinical Application

Many studies show that alterations in DNA methylation play a key role in tumor initiation (Gaudet, Hodgson et al., 2003). Therefore, methylation markers are ideally suited for detecting cancer in the early stages (Laird, 2003; Paluszczak & Baer-Dubowska, 2006). In *PAX* gene family, a panel of methylation markers including *PAX1* was developed to detect cervical cancer (Lai et al., 2008).

In our study, promoter methylation of *PAX5* was detected more frequent in primary GC tissues (90.1%) than in the normal gastric mucosa (15.8%) by using BGS. This finding suggests that *PAX5* promoter methylation is a common tumor-specific event in GC. In other words, *PAX5* methylation may serve as a potential diagnostic biomarker of gastric carcinoma.

We found that *PAX5* hypermethylation in GC tissues was associated significantly with short survival. Therefore, this study is inline with several other studies that tumor suppressor gene methylation is related to poor prognosis (Ye, Xia et al., 2007; Wanajo, Sasaki et al., 2008; Yu, Cheng et al., 2009). This indicates that *PAX5* may also utilize as a prognosis marker for GC.

However, expression of *PAX5* seems not related with CRC (Figure 3.6B). In another study of our team, down-regulation was observed in HCC tumors compared with the paired adjacent normal tissues (Xiaoxing LI, Kin F Cheung, Qian Tao and Jun Yu, unpublished data). The *PAX5* methylation level was also higher in HCC cell lines than in the normal liver cell line (Weili Liu, Qian Tao and

Jun Yu, unpublished data). These results indicated that *PAX5* methylation status had the potential to use as a molecule marker for HCC.

However, either diagnosis marker or prognosis marker, there is further work to do, such as development of non-invasive method using serum samples, and improvement of statistical significant by larger sample size. Further more, *PAX5* should be combined with other biomarkers to achieve higher specificity and efficiency.

4.5 Limitation of this Study

4.5.1 Sampling

For the primary GC biopsies being used in this study, there are various cell types, which also include the non-cancerous cells. Hence, mRNA level in paired GC specimens may not reflect the actual expression of *PAX5* in real GC cells. It may be the reason that some of the paired samples showed the reverse *PAX5* level with the trend in most of the samples. The percentage of *PAX5* methylation in the GC biopsies may also be underestimated in the BGS.

In the methylation detection assay using bisulfite genomic sequencing, all the samples were from the population in Guangzhou. Although this sample size is essential for biomarker selection, it may not be enough for validation. Additionally, although the survival time showed significant difference between methylation group and non-methylation group, no difference was observed while analyzing patients' methylation status in TNM stage I, II, III and IV, respectively. This may due to the limited sample size. Thus, further investigation using enhanced sample size and specimens collected from other population would facilitate the significant value of this potential biomarker.

4.5.2 Methodology

From the technical point of view, methylation specific PCR (MSP) and bisulfite genomic sequencing (BGS) were performed to detect the *PAX5* promoter methylation status in GC tissues. MSP is a qualitative method which provides a simple and rapid detection of methylation status. However, it may omit the methylation change outside the MSP primer. Thus, selection of regions for MSP primer design is very important. More CpG sites including in the primers may lead to more sensitive result. Direct BGS using PCR product is a quantitative approach to estimate the methylation level on each CpG sites of the target sequence. In our study, results of these two methods matched well while scanning methylation degree in the GC cell lines. However, BGS assay is quite labor intensive and costly for clinical use, so using other high-throughput quantification scanning method, such as Mass-CLEAVE assay, may increase the clinical value for acting as the diagnosis or prognosis marker.

Wound healing assay, which is simple and inexpensive, allows us to study cell mobility in a visible way. However, the scrape may also be recovered because of the strong proliferative ability of the cancer cell, but not their mobility. Hence, matrigel invasion assay was employed to validate the information from the scrape healing, making the conclusion more reliant.

4.5.3 Molecular basis

Our functional assays reveal the tumor suppressor role of PAX5 in gastric carcinogenesis. There should be hundreds of genes recruited in its gene regulatory network. These data can only show the tip of the iceberg. Further investigations on the molecule mechanism should be explored, such as finding upstream gene controlling the *PAX5* expression, cooperator interacted with the PAX5 protein, and other downstream targets undiscovered in this study. These may present other mechanisms that have not been clarified in this study. For example, how could PAX5 act as a tumor suppressor in GC while being an oncogene in other cancers such as lymphoma? And what is the functional domain of PAX5 protein during the tumorigenesis procedure?

4.6 Conclusion

In conclusion, our study indicated for the first time the importance of *PAX5* gene in the tumorigenesis of GC.

Firstly, our results demonstrate *PAX5* promoter methylation directly mediates the transcription silencing or down-regulation in GC cell lines. This phenomenon commonly occurs in gastric tissues from GC patients. As the expression of *PAX5* can be restored by demethylation treatment, it suggests that *PAX5* may act as target of epigenetic therapies in GC.

Secondly, the frequency of *PAX5* promoter methylation in GC patients is much higher, compared with the normal group. Additionally, in the GC cancer group, low methylation patients show a better survival rate than high methylation patients. Thus, detection of *PAX5* methylation status may be utilized as a specific biomarker for prognosis of GC patients.

Finally, by performing a series of functional assay, PAX5 protein is demonstrated to act as a functional tumor suppressor in gastric carcinogenesis by playing an important role in depression of cell proliferation, migration, invasion, and induction of cell apoptosis. These findings further support the essential role of *PAX5* gene in the anti-tumorigenesis in GC.

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