DACT1 is Silenced by CpG Methylation in Gastric Cancer and Contributes to the Pathogenesis of Gastric Cancer

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Abstract of thesis entitled: *DACT1* is silenced by CpG methylation in gastric cancer and contributes to the pathogenesis of gastric cancer

Submitted by WANG, Shiyan

for the degree of Doctor of Philosophy in Medical Sciences

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Abstract

Background & Aims: DACT1 is a Dishevelled (DVL) antagonist in planar cell polarity (PCP) signaling. Using methylation sensitive arbitrary primed PCR (MSP) and RT-PCR, DACT1 was preferentially methylated and downregulated in gastric cancer. We aim to clarify its epigenetic inactivation, biological function and clinical significance in gastric cancer.

Materials and Methods: DACT1 expression was tested by immunostaining in 19 pairs of gastric cancer cases. Methylation status was evaluated by MSP and direct bisulfite sequencing (BGS) in 20 gastric cancer cell lines and 205 primary gastric cancers. The effects of DACT1α re-expression were determined in cell growth, apoptosis, proliferation, spreading, migration and invasion assays, along with *in vivo* tumorigenicity assay. Luciferase reporter assays and NF-κB transcription factor binding activity assay were performed to evaluate DACT1α downstream cellular

signaling pathways. DACT1α target genes were identified by Cancer Pathway complementary DNA microarray analysis.

Results: DACT1 was silenced or downregulated in 70% (7/10) of gastric cancer cell lines, but was expressed in normal gastric tissues. Compared with the paired adjacent nontumor tissues, down-regulation of DACT1 was detected in 9 of 19 (47.4%) gastric cancers (P=0.001). MSP and direct BGS results indicated that promoter methylation was a major mechanism for the transcriptional silencing of DACT1 in gastric cancer cells. Ectopic expression of DACT1α in silenced gastric cancer cell lines (AGS, BGC823 and MGC803) by stable transfection of DACT1α suppressed colony formation (P<0.001 for all three cell lines). Overexpression of DACT1α induced apoptosis in BGC823 (P=0.031) and in MGC803 (P=0.004). Induction of apoptosis was mediated by activating caspase 3, caspase 7, caspase 9 and PARP. In addition, DACT1α inhibited cell migration both in AGS (P<0.01) and in MGC803 (P<0.05), suppressed cell invasion in AGS (P<0.05) and in MGC803 (P<0.01) and dampened cell adhesion spreading ability along with reduced actin microfilament (stress fiber) formation. Restoring DACT1α expression in BGC823 cells inhibited tumor growth *in vivo* (P<0.001). Moreover, DACT1α inhibited AP-1 and NF-κB signalings and decreased NF-κB downstream factors including anti-apoptotic BCL2 and BCL-XL and oncogenic IL8 and TNFα. DACT1α also led to downregulation of angiogenic PDGFB, VEGFA as well as multiple cell migration and invasion molecules. DACT1 methylation was detected in 29.3% (60/205) of primary gastric

tumors, but not in 20 normal gastric tissues. DACT1 methylation was significantly associated with advanced tumor size grade (P=0.013), lymph node metastasis (P=0.015), higher TNM stage (P<0.0005) and distant metastasis (P=0.05) respectively. Kaplan-Meier survival curves showed that the overall survival of patients with DACT1 methylation was significantly shorter than that of patients without methylation (P=0.007).

Conclusion: DACT1 acts as a functional tumor suppressor involved in gastric carcinogenesis.

摘要

背景和目的：DACT1 通过拮抗 DVL 分子在平面细胞极性通路中起重要调节作用。 通过 MSP 和 RT-PCR 发现 DACT1 在胃癌中由于甲基化而普遍下调。我们的研究目的是阐明 DACT1 在胃癌中的甲基化作用、功能及临床意义。

材料和方法：通过免疫组化对 DACT1 在 19 对胃癌标本和癌旁组织进行检测。使用 MSP 和直接 BGS 方法对 20 个正常胃组织和 205 个胃癌组织进行甲基化检测。DACT1α 过表达对细胞生长、凋亡、增殖、铺展能力、迁移和侵袭能力的影响作用以及通过裸鼠实验检测体内致瘤性作用。通过荧光素酶报告实验和 NF-κB 转录因子结合实验分析 DACT1α 的下游信号通路。肿瘤信号通路 cDNA 微阵列对 DACT1α 下游靶基因进行分析。

结果：DACT1 在正常胃组织中呈强表达，但在 70% (7/10)的胃癌细胞株中呈现表达沉默。免疫组化发现，与正常癌旁组织相比，DACT1 在 47.4% (9/19)胃癌组织中表达下调 (P=0.001)。MSP 和直接 BGS 表明启动子甲基化导致的表达沉默是 DACT1 下调的主要原因。DACT1α 在三条胃癌细胞株(AGS、BGC823 和 MGC803)中过表达，可显著抑制集落形成能力 (P<0.001)。在 BGC823 和 MGC803 细胞中，DACT1α 过表达显著诱导细胞凋亡 (P<0.05)。细胞凋亡作用是通过激活 caspase 3、caspase 7、caspase 9 和 PARP。DACT1α 可显著抑制 AGS 和 MGC803 细胞的迁移能力和侵袭能力(P<0.05)，并且大大抑制细胞铺展能力以及纤丝状肌动蛋白的聚集。DACT1α 过表达可显著抑制体内肿瘤生长

(*P*<0.001)。DACT1α 可显著抑制 AP-1 和 NF-κB 信号通路，并抑制 NF-κB 下游靶基因，包括抗凋亡蛋白 BCL2 和 BCL-XL 与促瘤分子 IL8 和 TNFα。此外，DACT1α 抑制促血管分子 PDGFB 和 VEGFA 以及多种细胞迁移和侵袭相关分子。在 29.3% (60/205)的胃癌组织中发现 DACT1 甲基化，而在 20 个正常胃组织中没有甲基化。DACT1 甲基化与肿瘤大小、淋巴结转移、远端转移和晚期肿瘤 TNM 分期显著相关 (*P*<0.05)。Kaplan-Meier 生存曲线发现存在 DACT1 甲基化的胃癌病人的生存状况显著比没有 DACT1 甲基化的胃癌病人要差 (*P*=0.007)。

结论：DACT1 在胃癌发生过程中是肿瘤抑制分子。

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Publications

1. Publications related to the thesis

1) **Wang S**, Minnie YY Go, Eagle Chu, Kin F Cheung, Wei Kang, Joanna HM Tong, Qian Tao, Xiaoxing Li, Ka Fai To, Sung JJ, Jun Yu. DACT1 is silenced by CpG methylation in gastric cancer and contributes to the pathogenesis of gastric cancer. **(not published) (The abstract has been accepted for oral presentation in Digestive Disease Week, 2011.)**
2) Yu J, Ma X, Cheung KF, Li X, Tian L, **Wang S**, Wu CW, Wu WK, He M, Wang M, Ng SS, Sung JJ. Epigenetic inactivation of T-box transcription factor 5, a novel tumor suppressor gene, is associated with colon cancer. Oncogene. 2010 Aug 30.
3) **Wang S**, Wan Du, Cheng Y, Lu L, Zhou L, Kin F Cheung, Qian Tao, Wang H, Sung JJ, Jun Yu. ZNF545 is silenced by CpG methylation in gastric cancer and contributes to the pathogenesis of gastric cancer. **(not published)**
4) Wan Du, **Wang S (Co-first author),** Zhou Q, Kin F Cheung, Qian Tao, Sung JJ, Jun Yu. ADAMTS9 is silenced by CpG methylation in gastric cancer and contributes to the pathogenesis of gastric cancer. **(not published)**

2. Publications produced in the course of the study

1) Shen B, Yu J, **Wang S**, Chu ES, Wong VW, Zhou X, Lin G, Sung JJ, Chan HL. Phyllanthus urinaria ameliorates the severity of nutritional steatohepatitis both in vitro and in vivo. Hepatology. 2008 Feb;47(2):473-83.
2) Yu J, Shen B, Chu ES, Teoh N, Cheung KF, Wu CW, **Wang S**, Lam CN, Feng H, Zhao J, Cheng AS, To KF, Chan HL, Sung JJ. Inhibitory role of peroxisome proliferator-activated receptor gamma in hepatocarcinogenesis in mice and in vitro. Hepatology. 2010 Jun;51(6):2008-19.
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Sung JJ. Heme oxygenase-1 protects against steatohepatitis in both cultured hepatocytes and mice. Gastroenterology. 2010 Feb;138(2):694-704, 704.e1. Epub 2009 Oct 7.

4) Zhang LJ, **Wang SY (Co-first author)**, Huo XH, Zhu ZL, Chu JK, Ma JC, Cui DS, Gu P, Zhao ZR, Wang MW, Yu J. Anti-Helicobacter pylori therapy followed by celecoxib on progression of gastric precancerous lesions. World J Gastroenterol. 2009 Jun 14;15(22):2731-8.

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6) **Wang S**, Tian L, Zeng Z, Zhang M, Wu K, Chen M, Fan D, Hu P, Sung JJ, Yu J. IkappaBalpha polymorphism at promoter region (rs2233408) influences the susceptibility of gastric cancer in Chinese. BMC Gastroenterol. 2010 Feb 5;10:15.

8) **Wang S**, Zhang M, Zeng Z, Tian L, Wu K, Chu J, Fan D, Hu P, Sung JJ, Yu J. IκBα polymorphisms were associated with increased risk of gastric cancer in a southern Chinese population: A case-control study. Life Sci. 2011 Mar 2.

Abbreviations

H. pylori, *Helicobacter pylori*; HER2, human epidermal growth factor receptor 2; MSI, microsatellite instability; LOH, loss of heterozygosity; DNMT1, DNA cytosine-5-methyltransferase 1; PML-RAR, promyelocytic leukaemia-retinoic acid receptor-α; HDAC, histone deacetylases; PcG, polycomb group; SUZ12, proteins suppressor of zeste 12; EED, embryonic ectoderm development; PRC2, polycomb repressive complex 2; TSA, Trichostatin A; LMP1, latent membrane protein 1; EBV, Epstein-Barr virus; miRNA, microRNA; ECOP, epidermal growth factor receptor-coamplified and overexpressed protein; MGMT, O6-methylguanine DNA methyltransferase; USF, upstream stimulatory factors; STAT3, signal transducer and activator of transcription 3; EGCG, epigallocatechin-3-galltae; RLGS, restriction landmark genome scanning; HELP, HpaII tiny fragment Enrichment by Ligation-mediated PCR; MSDK, Methylation-specific digital karyotyping; MSRF, Methylation sensitive restriction fingerprinting; MCA-RDA, methylation CpG island amplification-representational difference analysis; DMH, differential methylation hybridization; MALDI-TOF-MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; MeDIP, methyl-DNA immunoprecipitation; HTS, high-throughput sequencing; 5mC, 5-methylcytidine; MSP, methylation specific PCR; COBRA, combined bilufite restriction analysis; BGS, bisulfite genomic sequencing; 5-Aza, 5-Aza-2'-deoxycytidine; RT-PCR, reverse transcript PCR; PARP, nuclear enzyme poly (ADP-ribose) polymerase; PCP, planar cell polarity; JNK, c-Jun

N-terminal kinase; Dvl, dishevelled; DACT, dapper, antagonist of beta-catenin, homolog; Vangl2, vang-like 2; AP-1, activator protein 1; NFκB, nuclear factor kappa-light-chain-enhancer of activated B cells; BCL2, B-cell CLL/lymphoma 2; BCL-XL, apoptosis regulator Bcl-X; IL8, interleukin 8; TNFα, tumor necrosis factor alpha; PDGFB, platelet-derived growth factor beta polypeptide; VEGFA, vascular endothelial growth factor A; ITGA, integrin alpha; ITGB, integrin, beta; PLAU, plasminogen activator, urokinase; MCAM, melanoma cell adhesion molecule; MMP9, matrix metallopeptidase 9; ECM, extracellular matrix;

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Chapter 1 INTRODUCTION

1. Pathogenesis of Gastric Cancer

Gastric cancer is the fourth most common cancer worldwide with 930,000 newly diagnosed cases in 2002. Metastasis is found in 80-90% gastric cancer patients, making it the second most common cause of cancer related death with a high death rate (~800,000 per year) (Parkin DM et al 2005). Gastric cancer is much more common in in developing countries including East Asia (China, Japan), Eastern Europe, and parts of Central and South America and 42% cases were from China alone. But incidence rates are much lower in developed areas. For example, gastric cancer represents about 2% of all new cancer cases in USA.

Most gastric cancers are adenocarcinoma, which originate from glandular epithelium of the gastric mucosa. Gastric adenocarcinoma is classified under the Lauren system into three histologic types: intestinal, diffuse and mixed type. Intestinal type adenocarcinoma is characterized with irregular tubular structures and multiple lumens surrounded by a reduced stroma. Often it associates intestinal metaplasia in adjacent mucosa. Depending on glandular architecture, it may present 3 degrees of differentiation: well, moderate and poorly differentiated. Intestinal-type gastric adenocarcinoma usually occurs at older age with male predominance (male to female ratio of 2:1) and is associated with chronic inflammation (Takaishi S et al

2008). It undergoes a series of precanerous changes (chronic active nonatrophic gastritis → multifocal atrophy → intestinal metaplasia → dysplasia) before progression into gastric cancer. Diffuse type gastric cancer is a poorly differentiated adenocarcinoma characterized as infiltrating and discohesive tumor cells without glandular structure and secreting mucus. It tends to predominate in younger people with a nearly equal gender ratio (Takaishi S et al 2008). Most hereditary gastric cancer displays a diffuse histopathology and E-cadherin muation. Besides, around 5% of gastric cancers are lymphomas (MALT lymphoma).

Helicobacter pylori (*H. pylori*) is a Gram-negative, microaerophilic bacterium that can localize various areas of the stomach. It is strongly associated with the development of duodenal and gastric ulcers and stomach cancer by inducing a chronic inflammation, increased tissue turnover, excessive rate of proliferation (Takaishi S et al 2008). Uemura N *et al* group a long term follow-up (7.8 years) of Japanese patients with duodenal ulcers, gastric ulcers, gastric hyperplasia or nonulcer dyspepsia and found that gastric cancer of both intestinal and diffuse type developed in 2.9% of *H. pylori* infected patients but none in uninfected patients (Uemura N et al 2001). *H. pylori* is classified as class I carcinogen by the International Agency for Research on Cancer. Despite the conflicting data about the association between development of cardia gastric cancer and *H. pylori* infection (Kamangar F et al 2006), *H. pylori* infection is an important risk factor for noncardia gastric cancer and accounts for at least for two thirds of cases (Takaishi S et al 2008). The magnitude of

this association vary depending on length of follow-up and patient characteristics sush as age, gender, histological type and *H. pylori* strain. Besides, Epstein-Barr virus infection induced stomach malignancy accounts for approximately 10% of gastric cancer cases (Takada K et al 2000). Other risk factors include smoking, a high-salt diet, and a low intake of fruits and vegetables.

2. Molecular Mechanisms of Gastric Cancer

2. 1 Oncogene Activation

Many oncogenes are overexpressed in gastric malignancy. Amplified c-myc was found in 25% gastric cancer patients and was significantly correlated with the presence of distant metastasis (Kozma L et al 2001). c-met gene, which encoded hepatocyte growth factor receptor, was amplified in 23% advanced gastric carcinomas, especially scirrhous gastric cancer (Kuniyasu H et al 1992). There were two forms of the transcript of c-met, sized 7.0 kb and 6.0 kb in gastric carcinomas tissues (Kuniyasu H et al 1993). Overexpression of the 6.0-kb transcript of the c-met gene was significantly associated with tumor stage, lymph-node and distant metastasis. More than 20% of gastric cancers showed frequent human epidermal growth factor receptor 2 (HER2/c-erbB-2) overexpression due to aberrant gene amplification (Yokota J et al 1988). HER2 heterodimerized with other members of HER family, leading to activation of the oncogenic pathways including

Ras-Raf-MAPK, PI3K and STAT. This alteration inhibited apoptosis, increased proliferation and invasion. Monoclonal antibody against HER2, trastuzumab, in combination with chemotherapy was effective in treatment of patients with HER2-positive advanced gastric cancer (Bang YJ et al 2010). Other activated or amplified oncogenes include K-sam, EZH2, Semaphorin 5A, BCL2L12 and BUBR1 kinase.

2. 2 Loss of Functions of Tumor Suppressor Gene

2. 2. 1 Genetic Alteration: Mutation, Deletion

Microsatellites are repeated sequences of DNA in genome. Microsatellite instability (MSI) is a form of genetic instability due to defects in the normal DNA repair process and is observed in 13–44% of gastric cancer. MSI in sporadic gastric cancer causes frameshift mutations in sequences of tumor suppressor gene, contributing to cancer pathogenesis. Genomic DNA repair is maintained by mismatch repair genes such as hMLH1, hPMS1, hPMS2, hMSH2 and hMSH6, of which muational inactivation or epigenetic silencing result in DNA replication errors and microsatellite instability. *Halling et al* group found microsatellite instability at one or more loci in 19 out of 117 (16%) gastric carcinomas (Halling KC et al 1999). Immunohistochemical staining showed that all cases with high level MSI showed loss of expression for either hMLH1 or hMSH2, whereas gastric carcinoma with low

level MSI or without MSI had normal hMSH2 and hMLH1 protein expression. Promoter of the DNA repair gene hMLH1 was methylated in the progression of gastric cancer and correlated with microsatellite instability (Oue N et al 2001, Kang GH et al 2002).

Loss of heterozygosity (LOH) is the loss of normal function of one allele of a gene in which the other allele was already inactivated. In tumorigenesis, LOH occurs in tumor suppressor gene when the remaining functional allele is inactivated by mutation. In gastric cancer, frequent LOH has been identified at chromosomes 2q, 4p, 5q, 6p, 7q, 11q, 14q, 17p, 18q and 21q, in which tumor suppressor genes may locate (Tamura G et al 2006).

p53 gene on chromosome 17p was frequently inactivated by LOH and mutation of the remaining allele in about 40% gastric cancer patients (Fenoglio-Preiser CM et al 2003). Furthermore, p53 alteration occurred even in precancerous lesions, suggesting that the presence of p53 missense mutations in gastric adenomas was a key mediator in malignant transformation (Sakurai S et al 1995). Except methylation mediated silencing, E-cadherin were mutated in 50% of the diffuse type and in 14% of the mixed type gastric cancers (Becker KF et al 1994). A germline homozygous intronic mutation in spindle-assembly checkpoint gene BUB1B was identified in a patient with multiple primary invasive adenocarcinomas of both the colon and the stomach (Rio Frio T et al 2010). Mutations in k-ras mostly occurred in codon 12

(about 91.7%) and identified in 9.8% Chinese gastric cancer patients (Chen HC et al 2011).

2. 2. 2 Epigenetic Alteration

Epigenetics is defined as heritable changes in gene expression that are not accompanied by changes in DNA sequence. Vertebrates not only use covalent modifications of histones and other chromatin components but also take advantage of the heritability of DNA cytosine methylation patterns to control gene expression. Thus, DNA methylation and histone modifications are two important epigenetic mechanisms in the control of gene activity and nuclear architecture. Aberrant epigenetic change is central to human cancer pathogenesis. The cancer epigenome is characterized by global changes in DNA methylation and histone modification patterns as well as altered expression of chromatin-modifying enzymes (Sharma S et al 2010).

2. 2. 2. 1 DNA methylation aberrations

DNA methylation primarily occurs by the covalent modification of cytosine residue in CpG dinucleotides. CpG dinucleotides which could be potentially methylated are not randomly distributed in human genome, but instead concentrated in CpG-rich regions called 'CpG islands' and regions of large repetitive sequences

(Sharma S et al 2010). Most CpG islands, which primarily occupy the 5' end region of about 60% human genes (Wang Y et al 2004), are usually unmethylated in normal cells. This unmethylated status in CpG islands allows the gene to be transcribed in response to upstream transcriptional activators. However, some CpG island promoters of germline- and tissue-specific genes are methylated during development. Tissue-specific methylation were overrepresented at numerous gene loci that are essential for development such as HOX and PAX family members (Illingworth R et al 2008). Genomic imprinting is an epigenetic form of gene regulation that entails differential sex-specific hypermethylation of the alleles of a gene to achieve monoallelic expression.

In cancer cells, both genome wide hypomethylation and site-specific CpG island promoter hypermethylation occured (Figure 2.2.2.1) (Esteller M et al 2007). Many tumor suppressor genes are transcriptionally silenced by CpG island promoter hypermethylation, which contribute to the development of tumorigenesis. On the other hand, DNA hypomethylation occurs at repetitive sequences, tissue-specific and imprinted genes during carcinogenesis, which cause loss of cell phenotype, loss of imprinting and genomic instability. The profiles of DNA methylation have been shown to vary with tumor type (Costello JF et al 2000). The patterns of CpG-island methylation that were shared within each tumour type almost define the particular malignancy (Costello JF et al 2000). In addition, some microRNAs that act as tumor suppressors are also subject to epigenetic silencing in cancer cells (Lujambio A et al

2007). During the past few years, genes with key roles in cancer biology have been identified to be silenced by promoter hypermethylation during cancer development (Esteller M et al 2007).

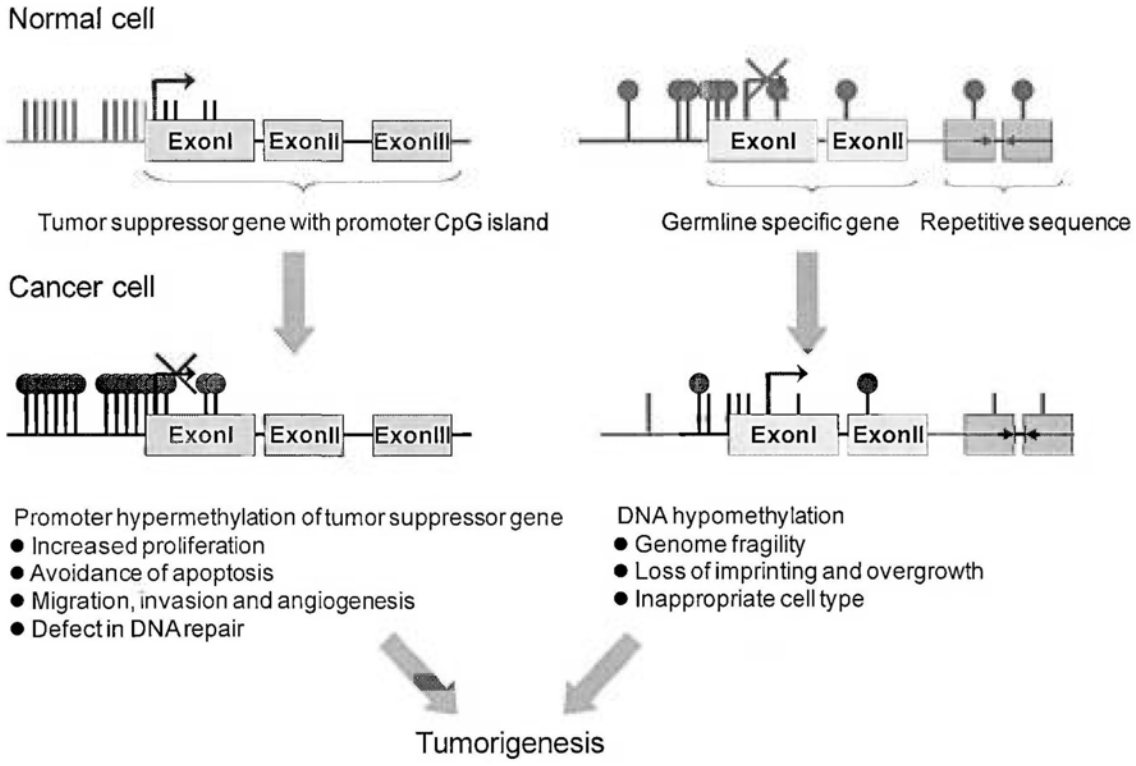

Figure 2. 2. 2. 1. 1 Aberrant DNA methylation in tumorigenesis.

Genetic alteration is another key cause of malignancy. Genome-wide analysis of 13,023 genes in breast and colorectal cancers revealed that individual tumors accumulate an average of approximately 90 mutant genes but that only a subset of these mutated genes (average of 11 per tumor) contribute to the neoplastic process at significant frequency (Sjöblom T et al 2006). Similarly, a range of 100-400 promoter hypermethylated CpG islands occur in an individual tumor (Weber M et al 2005).

However, the relative contribution of genetic and epigenetic alterations to cancer progression and the synergy between them is still unclear.

It is currently still unclear why many indivitual genes undergo de novo methylation in certain types of tumors whereas others with a typical CpG island remain methylation-free. The DNA hypermethylation does not only occur in the promoter CpG island region of tumor suppressor-like genes, but also encompass a large variety of different gene types that are not necessarily involved in tumorigenesis (Zardo G et al 2002). That means cancer-specific DNA hypermethylation does not preferentially enrich in the genes involved in cell cycle control and apoptosis. Genome wide analysis by methyl-DIP approach in colon and prostate cancer cells demonstrated that tumor specific methylated genes might have common sequence motifs in their promoters and are found in clusters on chromosomes (Zardo G et al 2002). In this regard, most *de novo* methylation in cancer may take place in an instructive manner through interaction between cis-acting sequences on the DNA and trans-acting protein complexes capable of recruiting DNA methyltranferases. Restriction Landmark Genome Scanning to analyze the susceptibility of 1,749 unselected CpG islands to de novo methylation driven by overexpression of DNA cytosine-5-methyltransferase 1 (DNMT1) found that the majority of CpG islands (69.9%) were resistant to de novo methylation, but a subset of methylation-prone CpG islands were consistently hypermethylated in multiple DNMT1 overexpressing clones (Feltus FA et al 2003). Notably, there is a

sequence "signature" associated with susceptibility to, or protection from, aberrant methylation. DNA sequences that are targeted by transcriptional repressors such as Polycomb proteins early in development are more prone to be hypermethylated later in cancer cells (Schlesinger Y et al 2007).

In some cases, aberrant DNA methylation might be directly induced by preceding genetic or molecular alterations. Leukemia promoting promyelocytic leukaemia-retinoic acid receptor-α (PML-RAR) fusion protein induced gene hypermethylation and silencing by recruiting DNA methyltransferases and histone deacetylases (HDACs) to specific target promoters, contributing to its leukemogenic potential (Di Croce L et al 2002). Another example is the oncogene *MYC* transcription factor which is an essential mediator of cell growth and proliferation. Myc repressed p21Cip1 gene expression and caused its promoter methylation by recruiting DNA methyltransferase Dnmt3a (Brenner C et al 2004). It seems likely that targeting of DNA methyltransferases by transcription factors is a wide and general mechanism for the generation of specific DNA methylation patterns during tumor development.

It has been proposed recently that in a stem cell origin of cancer, reversible gene repression is replaced by permanent silencing including DNA methylation induced repression, locking the cell into a perpetual state of self renewal and thereby predisposing to subsequent malignant transformation (Esteller M et al 2007). Stems

cells rely on Polycomb group (PcG) proteins to reversibly repress developmentally important genes through many rounds of cell division (Ringrose L et al 2004). The transcriptional repression in human embryonic stem cells is mediated by the PcG proteins suppressor of zeste 12 (SUZ12) and embryonic ectoderm development (EED), which form the Polycomb repressive complex 2 (PRC2) and which are associated with nucleosomes that are trimethylated at Lys27 of histone H3 (H3K27). Ringrose and his colleague found that some genes with cancer associated methylation contained at least one of these embryonic stem cell repressive marks. Stem cell Polycomb group targets are up to 12-fold more likely to have cancer-specific promoter DNA hypermethylation than non-targets (Ringrose L et al 2004). It suggested that PRC2 distributions and crosstalk between PRC2 and *de novo* DNA methyltransferases in an early precursor cell are similar to that of embryonic stem cells. This finding is contrary with the previous hypothesis that particular genes are selectively silenced during the transformation. Aberrant PRC2–DNA methyltransferase crosstalk occurs at low frequency in stem cells and may not disrupt normal differentiation if the silencing affects a small number of PRC2 targets that are not crucial to differentiation. However, if sufficient numbers of a particular subset are affected, then the resulting DNA methylation seeds could prevent proper differentiation and could predispose to further malignant development (Figure 2. 2. 2. 1. 2).

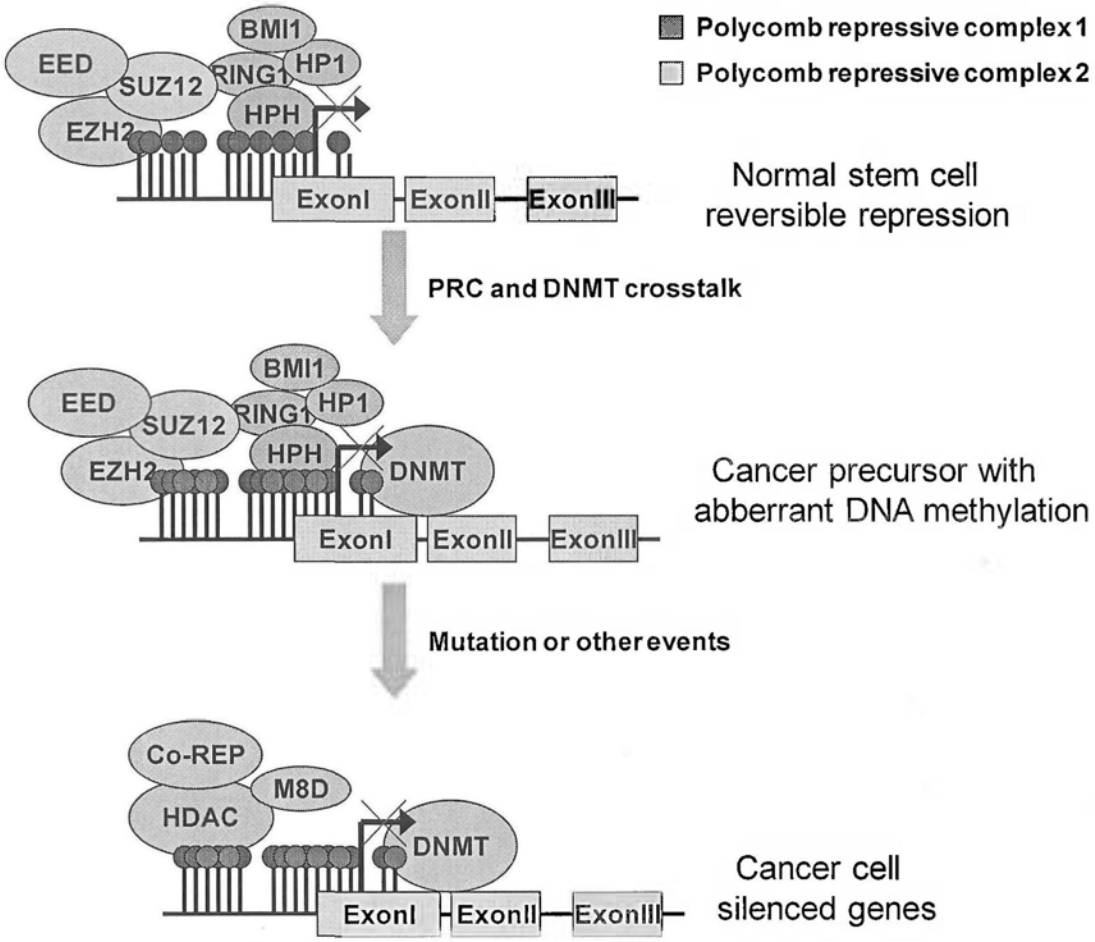

Figure 2. 2. 2. 1. 2. A model for the progression of epigenetic marks from reversible repression in embryonic stem cells to aberrant DNA methylation in cancer precursor cells and persistent gene silencing in cancer cells.

Based on the hypothesis that alterations in DNA methylation play a key role in tumor initiation , methylation markers are ideally suited for detecting cancer in the early stages and prognosis for cancer patients. Belinsky and colleagues evaluated aberrant methylation in a six-gene panel (p16, MGMT, DAPK, RASSF1A, PAX5 β and GATA5) to identify people at high risk for lung cancer incidence in sputum samples taken months to years prior to the clinical onset of cancer (Belinsky SA et al 2006). The concomitant methylation of three or more of these six genes was associated with a 6.5-fold increased risk and a sensitivity and specificity of 64%.

Other applications for detection of noninvasive cancer use bodily fluids such as urine for kidney, bladder, or prostate cancer, and serum or nipple aspirates for breast cancer (Cairns P et al 2001, Krassenstein R et al 2004). Notably, although each tumor type can be assigned a specific DNA hypermethylome, the panels of aberrantly methylated genes in these different tumor types overlap significantly, indicating that assays for cancer methylation markers could be shared for testing.

2. 2. 2. 2 Histone-modification change

There are four core histones H2A, H2B, H3 and H4. A nucleosome consists of two H2.A-H2.B dimers and one H3-H4 tetramer. The post-transcriptional histone modification include acetylation, methylation, phosphorylation, ubiquitination, SUMOylation and ADP-ribosylation. Histone modifications play critical roles in transcriptional regulation, DNA repair and replication and chromosome condensation. Abnormal histone modifications contribute to many disease development, especially in cancer progression. Histone deacetylases inhibitor led to accumulation of acetylated histones H3 and H4 at the promoter region of $p21^{WAF1}$ gene and its re-expression with suppressed tumor cell proliferation (Richon VM et al 2000). Genetic knockdown of histone deacetylases RBP2 triggered senescence of gastric cancer cells by enhancing H3K4 trimethylation in the promoters of p21, p27 and p16 and their expression (Zeng J et al 2009). Suv39h-deficient mice had impaired H3K9 methylation at pericentric region with relaxed chromatin conformation, severe

chromosomal instabilities and increased tumor risk (Peters AH et al 2001).

There are two types of histone modification patterns in chromosomes depending on its chromosomal location : actively transcribed gene-rich region and transcriptionally inactive subtelomeric and DNA repeat region (Figure 2. 2. 2. 2) (Esteller M et al 2007). In normal cells, the promoters in the gene-rich region are enriched with active histone modification marks such as acetylation of H3 and H4 lysine residues (H3K9, H4K5, H4K8, H4K12 and H4K16) and trimethylation of H3K4. In the same cells, the subtelomeric and DNA repeat region is associated with repressive histone marks including trimethylated H3K27, dimethylated H3K9 and trimethylated H4K20. However, cancer cells display a disrupted histone modification pattern which is characterized by loss of the active histone marks on the promoters of gene-rich region and loss of repressive histone marks such as trimethylated H3K27 and trimethylated H4K20) at subtelomeric and DNA repeat region.

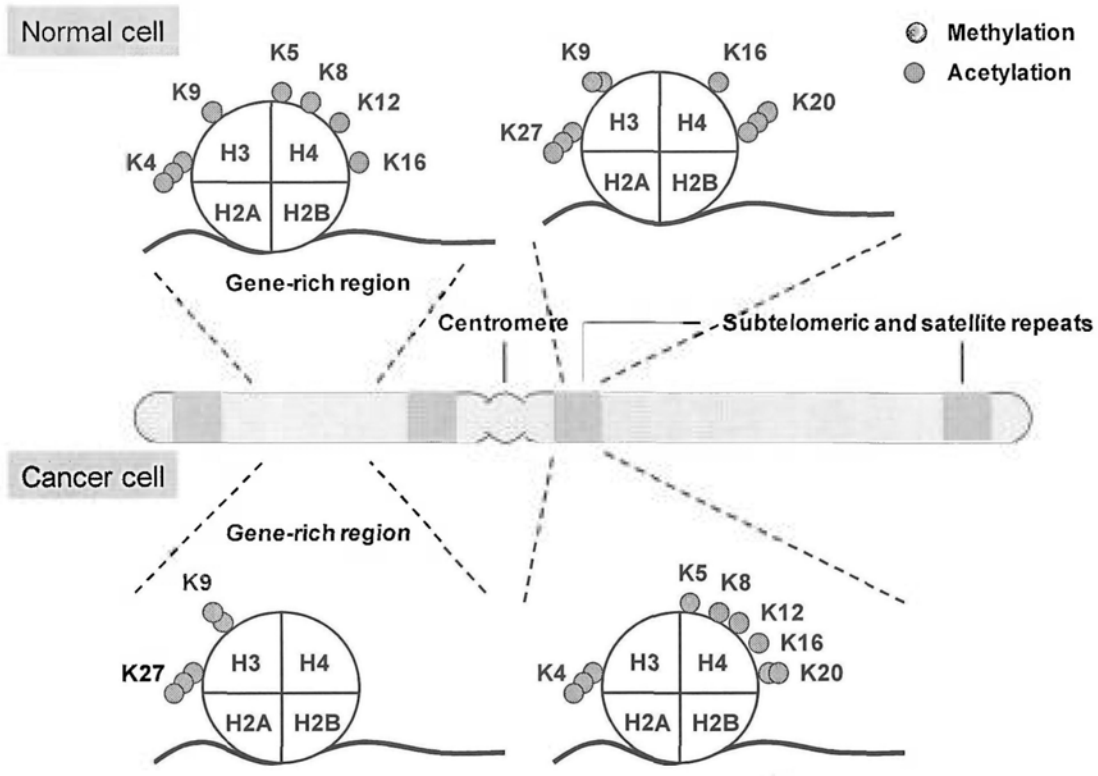

Figure 2. 2. 2. 2. Histone modification patterns in cancer cells are different from those in normal cells.

2. 2. 2. 3 Interplay between histone modification and DNA methylation

Heritable gene silencing may involve some or all of three process : DNA methylation, histone modification and nucleosomal remodeling (Figure 2. 2. 2. 3) (Jones PA et al 2007). In cancer cells, promoter CpG island hypermethylation is associated with changes of key histone code components: gain of repression marks methylated H3-lysine 9 (methyl-H3-K9) and H3K27 trimethylation and loss of activation mark H3K4 trimethylation, and deacetylation of histone H3 and H4 (Nan X et al 1998, Fahrner JA et al 2002). That means DNA methylation mediated transcription inhibition need cooperation with the histone code elements (active or

repressive) and histone modification related proteins at a hypermethylated gene promoter in cancer. Methyl-CpG binding protein MeCP2 mediated transcriptional repression by association and cooperation with Brahma, a catalytic component of the SWI/SNF-related chromatin-remodeling complex (Harikrishnan KN et al 2005). Trichostatin A (TSA), an inhibitor of histone deacetylation, induced chromatin decondensation and gene re-expression. In colon cancer cells, the effects of 5-AZA, an DMNT inhibitor, closely resembled those of TSA, although with subtle differences (Gius D et al 2004). However, how the cascade of events start is still unknow : DNA methylation triggers histone modification or histone alteration triggers silencing with promoting DNA methylation.

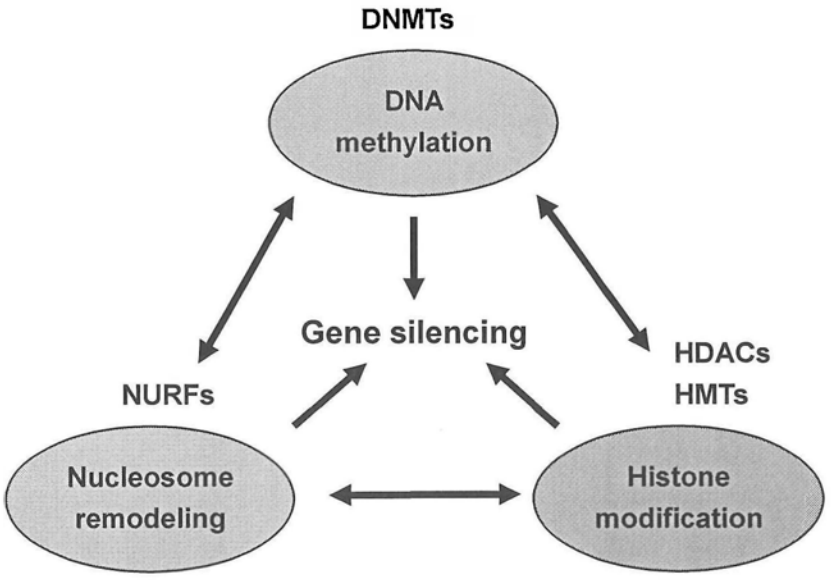

Figure 2. 2. 2. 3. Interplay between histone modification, DNA methylation and nucleo some remodeling.

2. 2. 2. 4 Disruption of the epigenetic machinery in cancer

Abberant changes in DNA methylation or histone modification machineries have also been identified in cancer development (Esteller M et al 2007). A list of disregulated genes of epigenetic modification machineries is provided in Table 2. 2. 2. 4.

Overexpression of DNMT1 and DNMT3b have been found in multiple cancers and associated with poor prognosis of cancer patients (Esteller M et al 2007). It is widely known that Epstein–Barr virus is oncogenic inducer in gastric cancer initiation and progression. The latent membrane protein (LMP1) of Epstein-Barr virus (EBV) suppressed E-cadherin expression by inducing DNMT1, DNMT3b and DNMT 3a expression, leading to reduced cell migration ability in culture cancer cells (Tsai CN et al 2002). DNMT1-targeted inhibition showed an inhibitory effect of cell proliferation in the cancer cells with restoration of many tumor suppressor genes such as CDKN2A, RASSF1A, and RUNX3 (Jung Y et al 2007). Increased DNMT1 expression in gastric cancer significantly correlated with EBV infection and CpG island methylator phenotype and might be responsible for promoter hypermethylation of hMLH1, THBS-1 and E-cadherin genes, indicating that DNMT1 was an important regulator in the development of gastric cancers by inducing frequent DNA hypermethylation of multiple CpG islands (Etoh T et al

2004). However, How DNMTs and Methyl-CpG-binding proteins are involved in the process in which specific areas of hypermethylation in the context of global DNA hypomethylation is still unclear. Genome-wide pigenomic analysis using CHIP with antibodies against DNMTs in normal versus cancer cells might provide one way to explore the mechanisms further.

The expressions of genes of histone modification machineries (histone deacetylases, histone acetyltransferases and histone methyltransferases) are also disrupted in human cancers (Ozdağ H et al 2006). Elevated levels of HDAC1 and HDAC2 are commonly observed in many cancers, including gastric cancer, and were correlated with poor survival of cancer patients (Mutze K et al 2010). Fraga et al found that the reduction of monoacetylated H4K16 and trimethylated H4K20 is a common cancer-specific histone modification alteration with a loss of recruitment of these H4K16 histone acetyltransferases (MOZ, MOF and MORF) to repeat sequences (Fraga MF et al 2005). Among known histone acetyltransferases, p300 and CBP, which are considered as tumor suppressors, were inactivated in several tumors including gastric cancer (Muraoka M et al 1996). EZH2, H3K27 histone methyltransferase of pollycomb-repressive complex 2, plays an important role and shows disregulated expression in cancer development (Tsang DP et al 2011). EZH2 mediated the suppresion of p16INK4alpha tumor suppressor gene by H3K27 trimethylation (Kotake Y et al 2007). EZH2 knockdown reduced growth of estrogen receptor-negative invasive breast cancer cell with increased expression of BRCA1

(Gonzalez ME et al 2009). Histone methyltransferases Suv39h is responsible for H3K9 methylation at pericentric heterochromatin. Suv39h-deficient mice display a different pattern of H3K9 methylation and severely chromosomal instabilities, leading to an increased tumor risk (Peters AH et al 2001).

Table 2. 2. 2. 4. Disregulated genes of DNA-methylation and histone-modification machineries in gastric cancer.

Gene	Alteration	Reference
DNA methyltransferases		
DNMT1	overexpression	Ding WJ et al 2008
DNMT3b	overexpression	Ding WJ et al 2008
Methyl-CpG-binding proteins		
MBD4	Inactivating mutations in gastric cancer patients with microsatellite instability	Yamada T et al 2002
Histone deacetylases		
HDAC1	overexpression	Mutze K et al 2010
HDAC2	overexpression	Song J et al 2005
RBP2	overexpression	Zeng J et al 2009
Histone acetyltransferases		
p300	mutations in gastric cancer patients with microsatellite instability	Muraoka M et al 1996
Histone methyltransferases		
EZH2	Gene amplification, overexpression	Cai GH et al 2010
RIZ1	Inactivation by CpG island hypermethylation	Oshimo Y et al 2004

2. 3 Other molecular alteration

MicroRNA (miRNA) alterations are involved in the development of human cancer. Pri- miRNAs are usually transcribed by RNA polymerase II, processed by RNase III enzyme Drosha and its cofactor Pasha, and transported to cytoplasm and cleaved into mature miRNAs by RNase III enzyme Dicer (Calin GA et al 2006). Through imperfect pairing with mRNA of target genes, miRNA post-translationally represses specific gene products. Disregulated miRNAs in cancer cells consist of both overexpressed and downregulated microRNAs or onco-miRNAs and tumour-suppressor-miRNAs. miR-218 was significantly down-regulated in gastric cancer tissues and in *H. pylori*-infected gastric mucosa (Gao C et al 2010). miR-218 inhibited cell proliferation and increased apoptosis by targeting epidermal growth factor receptor-coamplified and overexpressed protein (ECOP) and thus suppressing nuclear factor kappa B (NF-kappaB) transcriptional activity in gastric cancer cells. Kim YK et al group found that two clusters miR-106b~93 ~ 25 and miR-222 ~ 221 were up-regulated in gastric cancer tissues (Kim YK et al 2009). These cluster of miRNAs suppressed the Cip/Kip family members of Cdk inhibitors : miR-106b and miR-93 regulated p21 while miR-222 and miR-221 targeted both p27 and p57, leading to activation of Cdk2, G1/S phase transition and tumor growth. So far, at least three mechanisms how miRNAs are dis-regulated in cancer progression have been described (Calin GA et al 2006). First, more than half of the known human

miRNAs locate in particular chromosomal regions that frequently harbour abnormalities such as LOH, amplification and breakpoints in cancer. Second, several miRNAs such as miR-127 are controlled by epigenetic regulations (DNA methylation and histone modification) which are aberrant in malignant transformation. Third, abnormalities of miRNA-processing machinery proteins exert great influence on miRNA expression profile. Thomson JM *et al* group revealed that failure at the Drosha processing step contributed to a wide-spread downregulation of miRNAs in cancer (Thomson JM et al 2006).

3. Role of epigenetic alteration in multisteps development of gastric cancer

3. 1 Aberrant CpG island hypermethylation through multistep gastric carcinogenesis

Aberrant CpG island hypermethylaiton occur early in premalignant lesions of gastric mucosa and accumulate along the whole process of gastric carcinogenesis (Kang GH et al 2001, Zou XP et al 2009, Nardone G et al 2007). The methylaiton level and the number of methylated genes greatly increased from atrophic gastritis, intestinal metaplasia, dysplasia to gastric cancer (Figure 3. 1). Many methylated genes, which lost expression due to promoter hypermethylation, play important roles in cell proliferation, cell cycle, apoptosis, angiogenesis, metastasis, thus contributing to the development of gastric cancer (Table 3. 1). On the other hand, elevated

repetitive DNA hypomethylation, another phenomenon occurred in cancer progression, was found at several repetitive sites through the multistep gastric carcinogenesis (Park SY et al 2009). *Park SY et al* group found that DNA hypomethylation at LINE-1 repetitive sites increased with the progression of premalignant lesions from chronic gastritis, intestinal metaplasia to gastric adenoma (Park SY et al 2009). Besides, even in adjasent normal gastric mucosa of gastric cancer patients, methylation levels of many tumor suppressor genes were still significantly higher than healthy controls (Kaise M et al 2008, Tahara T et al 2010).

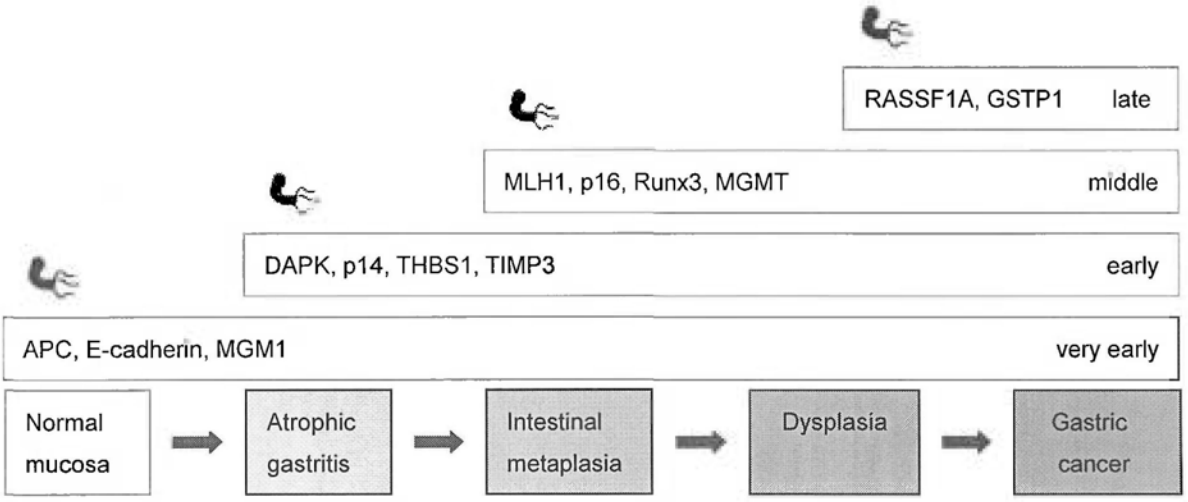

Figure 3. 1. CpG island hypermethylation changes through multistep process of gastric carcinogenesis.

Table 3. 1. A catalogue of some genes silenced by CpG island promoter hypermethylation in human gastric cancer

Gene	Function/pathway
MLH1	DNA mismatch repair
COX2	Cycooxygenase-2
GATA4	Transcription factor
GATA5	Transcription factor
ID4	Transcription factor
WRN	DNA repair
CHFR	Mitotic checkpoint
RUNX3	TGFβ signaling
PAX5	Transcription factor
p14	Cell cycle regulator
p15	Cell cycle regulator
p16	Cell cycle regulator
DAPK1	Regulator of programmed cell death
SLC19A3	Thiamine receptor
DLEC1	Unclear
CMTM3	Chemokine-like factor
ZIC1	Transcription factor
PCDH10	Cadherin-related neuronal receptor, cell-cell connections
DKK3	Regulator of Wnt signaling pathway
Fibulin 1	Secreted glycoprotein that becomes incorporated into a fibrillar extracellular matrix
OPCML	Opioid-binding protein, cell adhesion molecule
UCHL1	Thiol protease that hydrolyzes a peptide bond at the C-terminal glycine of ubiquitin
SFRP2	Modulators of Wnt signaling
E-cadherin	Cell-cell adhesion glycoprotein

Cyclin D2	Cell cycle regulator
HLTF	Regulate transcription of certain genes by altering the chromatin structure
GSTP1	Glutathione S-transferases
RASSF1A	Cell cycle regulator

3. 2 The roles of *H. pylori* infection in aberrant DNA hypermethylation during gastric cancer progression

H. pylori is a Gram-negative, microaerophilic bacterium that can causes chronic stomach inflammation and lead to the development of duodenal and gastric ulcers and stomach cancer. Patients infected with *H. pylori,* had increased risk by 2-fold to 5-fold for development of gastric cancer of both intestinal and diffuse type (Yu J et al 2010). Epigentic aberration was a critical mechanism of carcinogenesis. The correlation between *H. pylori* infection and epigentic change has been noted during past few years. Many studies showed that *H. pylori* infection led to disregulated promoter hypermethylation of multiple tumor suppressor genes through multistep gastric cancer progression from premalignant lesion to adenocarcinoma such as E-cadherin, p16, trefoil Factor 2, runx 3, O6-methylguanine DNA methyltransferase (MGMT) and upstream stimulatory factors 1 and 2 (USF1 and USF2), thereby contributing to gastric cancer initiation and progression (Chan AO et al 2003, Dong CX et al 2009, Peterson AJ et al 2010, Kitajima Y et al 2008, Sepulveda AR et al 2010, Bussière FI et al 2010). DNA hypermethylation of these genes was

significantly higher in patients with *H. pylori* infection than those without infection. *In vitro* study, coculture of gastric cancer cell line AGS cells with *H. pylori* organisms, without the presence of inflammatory cells, contributed to promoter hypermethylation and down-regulation of MGMT (Sepulveda AR et al 2010). In mice chronically infected with H. pylori SS1, active gastritis with metaplasia was developed with increased USF1 and USF2 hypermethylation and decreased expression and binding activity of USF1 and USF2 (Bussière FI et al 2010).

IL-1B-511 TT genotype was associated with increased risk of gastric cancer as IL-1B-511 TT genotype contributed to increased promoter activity and IL-1B production (Figueiredo C et al 2002). The synergistic effect with *H. pylori* infection was also observed. Patients with IL-1B-511 TT genotype and *H. pylori* infection had further increased risk of gastric cancer. Interestingly, patients with *H. pylori* infection and IL-1B-511 TT genotype were predisposed to CpG island methylation in gastric cancer development (Chan AO et al 2007).

The mechanism underlying how *H. pylori* infection induces aberrant DNA methylation and other abnormal epigenetic changes is largely unknown. Current studies suggest that *H. pylori* induces disregulated DNA methylation by its downstream inflammatory signalings (Katayama Y et al 2009, Niwa T et al 2010). *Katayama Y et al* found that *H. pylori* caused Runx3 promoter methylation and mRNA suppression in gastric cancer cell line MKN45 when they were co-cultured

with macrophages (Katayama Y et al 2009). This effect was abolished by an iNOS-specific inhibitor, indicating that *H. pylori* induced nitric oxide production in macrophages contributed to runx3 methylation in gastric epithelial cells. Gerbil model is widely used for studying *H. pylori* induced gastric cancer. In gerbil model of gastric cancer, the temporal methylation level changes of genes which were specificially methylated only in gastric mucosae with *H. pylori* infection, paralleled the expression of several inflammatory genes (CXCL2, IL-1β, NOS2, and TNF-α) (Niwa T et al 2010). Treatment with cyclosporin A canceled *H. pylor*i-specific DNA methylation by inhibiting inflammatory signalings induced by *H. pylori* infection without affecting *H. pylor*i colonization.

Several studies indicated that *H. pylori* infection caused persistent DNA methylation even after *H. pylori* eradication during gastric cancer progression, although the methylation level was greatly alleviated after eradication (Nakajima T et al 2010, Niwa T et al 2010). Successful *H. pylori* eradication in well-differentiated early gastric adenocarcinoma led to a significant decrease in DNA methylation, but methylation level was still higher than those of healthy control without *H. pylori* infection (Nakajima T et al 2010). This phenomenon in DNA methylaion after *H. pylori* eradication was also observed in gerbil model of gastric cancer, suggesting that *H. pylori* induced DNA methylation consisted of temporary and permanent components (Niwa T et al 2010). One hypothesis was proprosed by *Niwa T et al* group that temporary methylation probably occurred in progenitor or differentiated

cells, whereas permanent methylation might be induced in stem cells and keep persistent during progression. However, *H. pylori* eradication was quite effective to lower or abolish aberrant DNA methylation in early stage of the progression of premalignant lesion (Leung WK et al 2006, Chan AO et al 2006). *H. pylori* eradication successfully reversed E-cadherin methylation in patients with chronic gastritis (Leung WK et al 2006, Chan AO et al 2006). Another *H. pylori* eradication study showed that after *H. pylori* eradication, promoter methylation of E-cadherin, p16, and APC was substantially reversed and COX2 methylation completely disappeared in patients with dyspepsia or intestinal metaplasia (Perri F et al 2007).

3. 3 The role of EBV in aberrant DNA hypermethylation during gastric cancer progression

Epstein-Barr virus (EBV) has been identified as gastric cancer-causing infective agent (Fukayama M et al 2008, Fukayama M et al 2010). EBV associated gastric cancer acounts for about 10% of all cases of gastric cancer and is characterized with distinct clinical features including male predominance, yourger age, special location, less involvement of lymph node, alcerated like tumor mucosa and thickening of gastric wall. The oncogenic progression by EBV in gastric cancer is partially mediated by methylation associated silencing of many tumor suppressor genes such as p14, p15, p16, p73, TIMP3, DAPK and MGMT. Several studies suggested that DNMT1 overexpression induced by EBV infection played critical role in

EBV-associated aberrant epigenomic changes and gastric cancer development (Etoh T et al 2004, Tsai CL et al 2006, Hino R et al 2009). Elevated DNMT1 expression was found in EBV infected gastric cancer patients (Etoh T et al 2004). One of EBV genes, latent membrane protein 1 (LMP1) directly activated DNMT1 promoter activity and increased DNMT1 expression through JNK pathway activation, thus contributing to E-cadherin promoter hypermethylation and down-regulation in nasopharyngeal carcinoma cells (Tsai CL et al 2006). EBV latent membrane protein 2A mediated upregulated DNMT1 led to increased methylation of tumor suppressor gene PTEN and expression suppression by activating phosphorylated signal transducer and activator of transcription (STAT3) in gastric cancer (Hino R et al 2009). Elevated DNMT1 expression by EBV was considered as a cellular defense to silence viral protein expression through the methylation of viral DNA. However, DNMT1 mediated hypermethylation of multiple tumor suppressor genes in host cell resulted in tumor progression.

3. 4 Methylation and lifestyle and environmental exposures

Increasing evidences showed that lifestyle, diet and environmental exporsure played important roles in the development of epigenetic changes in normal and cancer tissues (Rashid A et al 2004, Johnson IT et al 2007). Marked geographic variation in DNA methylation pattern was observed in non-small cell lung cancer (Toyooka S et al 2003). Another evidence was seen in epigenetic differences during

the lifetime of monozygotic twins (Fraga MF et al 2005). Monozygous twins, who shared a identical genotype, were epigenetically similar during the early years of life, but exhibited remarkable differences in DNA methylation and histone acetylation pattens when they grew older. Several diet supplements such as folate, S-adenosyl-methionine, and S-adenosyl-homocysteine directly influenced DNMT enzyme activity and methylation reaction *in vivo*. (-)-epigallocatechin-3-galltae (EGCG), an active ingredient in green tea, potently inhibited DNMT activity (Johnson IT et al 2007). Treatment with EGCG reversed promoter hypermethylation of tumor suppressor genes (p16, MGMT and RARbeta) in a dose- and time-dependent manner by inhibiting DNMT in human esophageal cancer (Fang MZ et al 2003). In the study on the relationship between dietary factors and methylation, CDX2 methylation was correlated with the decreased intake of green tea in gastric cancer patients (Yuasa Y et al 2005). Thus the role of lifestyle and environmental exposure is critical for CpG island methylation changes, but further research is needed to explore the mechanisms.

4. How to identify a novel tumor suppressor gene

The principle of DNA methylation detection is to distinguish cytosine from 5-methylcytosine in the DNA sequence. There are four main strategies for identifying genome-wide DNA methylation changes: transcriptional profiling of gene expression reactivated by demethylation agents; the digestion of DNA by a

methylation-sensitive or –insensitive restriction endonuclease; the chemical conversion of cytosine by sodium bisulfite; and immunoprecipitation of 5-methylcytosine. Each strategies offers potential advantages and disadvantages, but no direct comparison has been performed across different platforms.

4. 1 Transcriptional profiling of gene expression reactivated by demethylation agents

Several investigators have used DNA demethylation agents to restore the expression of methylation-mediated silenced genes and then microarray transcription profiling to identify potential targets for aberrant methylation in cancers (Sato N et al 2003, Suzuki H et al 2002).

5-AZA is the most commonly used potent DNA demethylation agent (Holliday R et al 2002). As a cytidine analogue, 5-AZA integrates into genomic DNA when cells are replicating. 5-AZA irreversibly binds to DNA methyltransferase (DNMT) and inhibits its methyltransferase activity during cell division. In this manner, the methylated DNA become fully unmethylated and epigenetically silenced genes are reactivated after several rounds of DNA replication eventually. However, 5-AZA is characterized by high cytotoxicity and low stability and may affect several metabolic pathways such as folate metabolism and nucleotide synthesis (Stresemann C et al 2006). This suggests that some gene may be induced in response to 5-AZA treatment not through its promoter DNA demethylation. Thus gene expression changes before

and after 5-AZA treatment need to be interpreted carefully. Recently, some novel DNA methyltransferase inhibitor compounds have been developed, but none is as potent as 5-AZA in the ability of DNA demethylation (Stresemann C et al 2006). One approach is to target DNA methyltransferases more directly—antisense mediated or small interfering RNA–mediated degradation of DNMT mRNA, although there are substantial procedural issues that remain unresolved (Robert MF et al 2003).

Two types of transcription profiling microarray are currently used: one uses a single fluorophoe; the other uses two different fluorescent dyes to label two different samples. For identifying targets for DNA methylation, the single color platform such as Affymetrix and Genechip is preferred because there is no RNA expression signal due to methylation mediated gene silencing before 5-AZA treatment. However, the two color platform requires detection of both colors for subsequent analysis. Thus most studies taken the approach of the single color platform for DNA methylation screening.

Notablely, potential targets for DNA methylation are not expressed in untreated cells but induced by 5-AZA treatment. Some genes are expressed in untreated cells and induced higher under 5-AZA treatment, but the induction of these genes are not due to their promoter methylation directly. Thus it is not proper that the widely-used statistical algorithms to identify differentially expressed genes such as Significance Analysis algorithms is rotely applied for DNA methylation analysis.

4. 2 Genomic scanning based on the digestion of DNA by methylation-sensitive or –insensitive restriction endonuclease

One application of restriction endonuclease-based approach to genome-wide screening is restriction landmark genome scanning (RLGS), which is followed by two-dimensional gel electrophoresis or CpG island microarray (Akama TO et al 1997, Yoshikawa H et al 1996, Costello JF et al 2000, Costello JF et al 2002, Smith LT et al 2006). Genomic DNA samples are first digested with the rare-cutting methylation-sensitive restriction enzyme *Not*I, which recognizes CG-rich regions and can not cleave DNA sequences when cytosine is methylated.. After digestion, DNA at cleaved *Not*I sites are radiolabeled. Following further endonuclease digestion, two-dimensional electrophoretic separation and autoradiography, the intensity of a DNA fragment refects the methylation status of the digested DNA fragments (Figure 4. 2). For cancer-spesific methylation, RLGS profiles are compared to identify spots that are present in normal samples but not in cancers. Early application of RLGS is time-consuming with low resolution. Besides, this method requires at least a few micrograms of high quality DNA, a limitation for some investigators. The detection is limited to the diversity of the clone library coverage. This approach identifies only CpG islands with *Not*I sites, which represent about 75% CpG islands in human genome. Recently this method is improved by coupling with high-throughput microarray such as CpG island microarray and BAC clone arrays (Ching TT et al

2005, Heisler LE et al 2005).

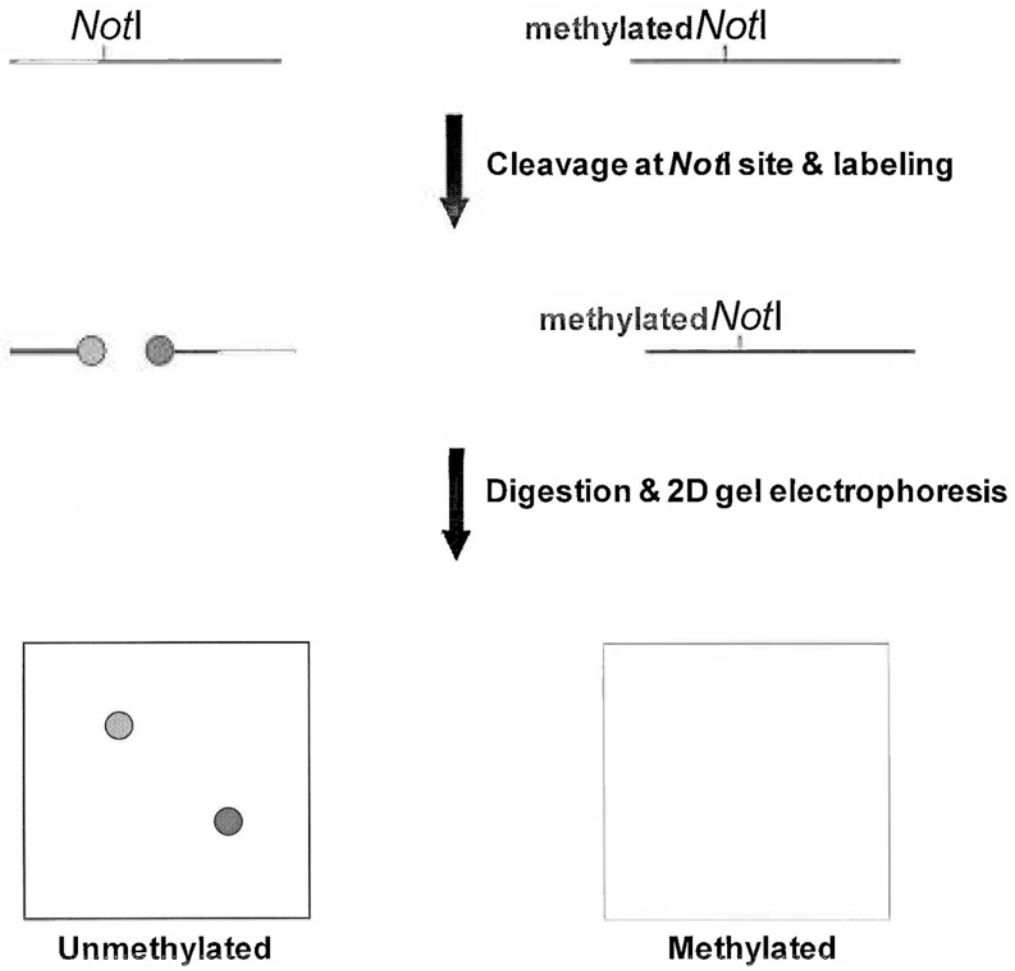

Figure 4. 2. The procedures of restriction landmark genomic scanning (RLGS).

Another technique combining methylation-based restriction enzyme digestion and microarray technology is HpaII tiny fragment Enrichment by Ligation-mediated PCR (HELP) (Khulan B et al 2006). The technique relies upon the properties of two restriction enzymes: HpaII and MspI. Since HpaII digests 5'-CCGG-3' sites when the cytosine in the central CG dinucleotide is unmethylated, only unmethylated DNA is enriched. MspI is a methylation-insensitive isoschizomer. The HELP assay compares representations generated by HpaII and by MspI digestion of the genome followed

by ligation-mediated PCR.

Methylation-specific digital karyotyping (MSDK), like the methods described earlier, used a combination of a methylation-sensitive mapping enzyme (for example, AscI) and a fragmenting enzyme (for example, NlaIII) and mapped the fragments to genome location by SAGE. The number of tags in an MSDK library reflects the methylation status of the mapping enzyme sites (Hu M et al 2006). Although this technology has a relatively low resolution, the tagged DNA is identified through direct sequencing. Thus it avoids some problems from microarray method such as statistical interpretation. *Bloushtain-Qimron N et al* analyzed DNA methylation and gene expression profiles of distinct subpopulations of mammary epithelial cells by MSDK and identified discrete cell-type and differentiation state-specific DNA methylation and expression patterns of genes especially including some transcription factors (Bloushtain-Qimron N et al 2008).

Methylation sensitive restriction fingerprinting (MSRF) is another restriction endonuclease-based approach. The DNA is first fragmented through MseI digestion at TTAA site which is rarely found in CG-rich regions. Then MseI-digested DNA is divided into two aliquots: one is left unmodified and the other is digested with BstUI, a methylation-sensitive restriction enzyme. BstUI cuts at the site CGCG, a sequence that exists in most CpG islands unless the site is methylated. Thus short DNA sequences with methylated BstUI sites are left uncut and can be amplified by PCRs

in the presence of radiolabeled dNTPs. Since this method uses several sets of artitrary PCR primers and gel seperation, only a limited number of candidates can be abtained. *Ho SM et al* applied MSRF to identify epigenetic changes of prostate under developmental exposure to estradiol and bisphenol A, a high risk factor for prostate carcinogenesis (Ho SM et al 2006). They found phosphodiesterase type 4 variant 4, an enzyme responsible for cyclic AMP breakdown, gradually lost its expression with aging due to methylation in normal prostates, but was hypomethylated under estradiol or bisphenol A exposure (Ho SM et al 2006). Other restriction endonuclease-based approaches includes methylation CpG island amplification-representational difference analysis (MCA-RDA) and differential methylation hybridization (DMH) (Table 4. 2).

Table 4. 2. Restriction endonuclease-based approaches.

Conventional RLGS	RLGS uses methylcytosine-sensitive restriction endonucleases to discriminate methylated from unmethylated DNA and maps their position in the genome using two dimentional electrophoretic separation.
RLGS coupled with BAC microarrays	Biotin-labeled DNA is generated from end-filling of genomic DNA samples digested with the rare-cutting methylation-sensitive restriction endonuclease *Not*I. The samples are then hybridized onto a microarray of BAC clones.
HELP	The HELP assay co-hybridizes HpaII digestion products (unmethylated DNA enrichment) with digestion fragments from a methylation-insensitive isoschizomer (MspI) onto a customized array.
MSDK	MSDK uses methylcytosine-sensitive restriction endonucleases to discriminate methylated from unmethylated DNA and maps their position in the genome using SAGE.
MSRF	DNA is digested by MseI and then by BstUI and amplified by several sets of arbitrary PCR
MCA-RDA	DNA is first subjected to SmaI to remove unmethylated SmaI sites and generate blunt end DNA fragments; then digested with SmaI isoschizomer XmaI, which cuts at methylated SmaI sites and sticky end DNA fragments. The sticky end DNA can be amplified by PCR.
DMH	DNA is digested by MseI and then by BstUI and amplified to generate probes for hybridization to a CpG island microarray.

4. 3 High-throughput methods based on the chemical conversion of cytosine by sodium bisulfite

This method based on the fact that cytosine was converted to uracil after bisulfite treatment (Figure 4. 3). After PCR amplication, the sequence is converted to thymine if it is not protected by methylation. Because DNA complexity is reduced after sodium bisulfite conversion, this will creat more problems on PCR than

conventional sequencing. Alternative detections include involvement of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS), microarray and MethyLight.

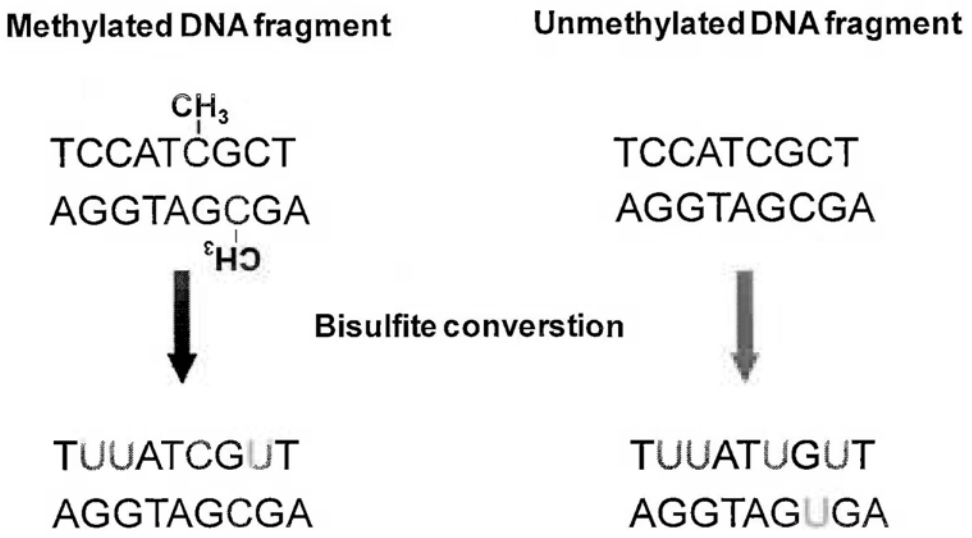

Figure 4. 3. Outline of bisulfite conversion of sample sequence of genomic DNA.

4. 4 Immunoprecipitation of 5-methylcytosine coupled with DNA microarray

The restriction endonuclease-based methods need to determine the sequence identity of differential spots or bands. Besides, only particular sequence motifs can be analyzed because specific restriction sites are required to be present. Transcriptional profiling of gene expression reactivated by demethylation agents is not a direct method to analyze methylation. Recently, a novel approach, methyl-DNA immunoprecipitation (MeDIP) assays, in combination with hybridization on high-resolution microarrays or high-throughput sequencing (HTS) techniques, are

developed to overcome these problems for identifying methylated CpG-rich sequences (Figure 4. 4) (Weber M et al 2005). In this assay, an antibody specific for 5-methylcytidine (5mC) is used to immunocapture methylated genomic DNA fragments. In brief, genomic DNA purified by standard procedures is sheared through sonication to produce random fragments. Sonication conditions must be optimized to yield fragments ranging in size between 300 and 600 bp. After fragmentation, DNA must be denatured in order to obtain single-DNA fragments, as the anti-5mC has a higher affinity for this form of the 5mC-containing DNA. Immunoprecipitated DNA can then be used for individual analysis of the methylation status of a particular gene by employing PCR with specific primers targeting the specified gene. Alternatively, the MeDIP enrichment in the immunoprecipitated fraction is labeled with Cy3, while the input DNA is labeled with Cy5. The labeled DNAs are co-hybridized to a high-resolution genomic microarrays. Relative signal intensity at each locus indicates the methylation status. Usually several cancer cell lines and normal cells are selected and analyzed by MeDIP for mapping the cancer-specific DNA hypermethylome.

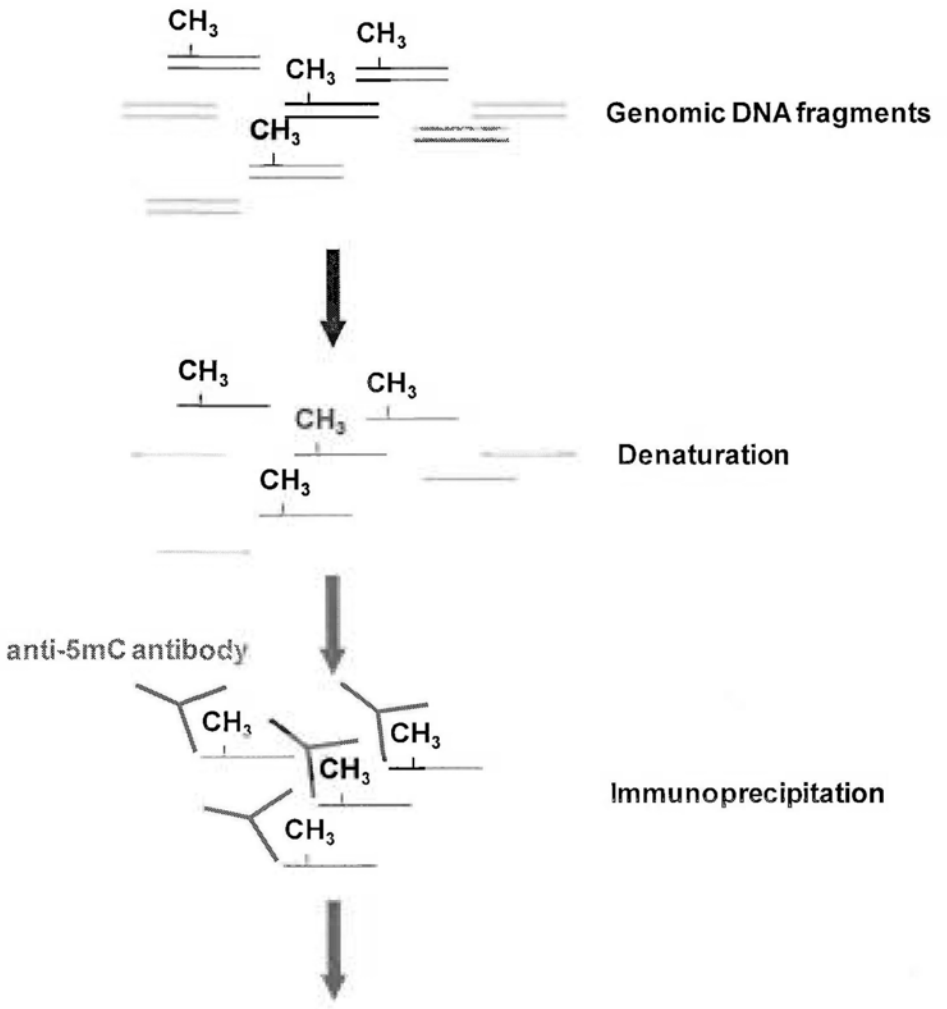

Figure 4. 4. Methylation analysis by methyl-DNA immunoprecipitation (MeDIP) coupled with DNA microarray.

Weber M et al first used MeDIP to analyze the methylation pattern in normal and transformed human cells (Weber M et al 2005). They found that the inactive X chromosome was globally hypomethylated but hypermethylated only at a subset of gene-rich regions. They also found that aberrant methylation occurred in malignant cancer cells, although it is less frequent than previously thought. *Papageorgiou EA et al* employed MeDIP methodology in combination with real-time quantitative PCR to assess fetal-specific differentially methylated regions in maternal peripheral blood,

which provided a noninvasive prenatal diagnosis (Papageorgiou EA et al 2001). *Morris MR et al* determined methylation status of DNA samples from nine renal cell carcinoma tumours and from three non-malignant kidneys. They also analysed genome-wide expression changes in 11 renal cell carcinoma-derived cell lines following treatment with the demethylating agent 5-Aza-20-deoxycytidine using cDNA microarrays. Based on the two types of data, they prioritised those genes from MeDIP methylation array that also showed a significant reexpression in at least two cell lines. A total of 78 genes were identified and 9 genes were confirmed to have methylation in primary tumour samples such as KLHL35, QPCT, SCUBE3 and CORO6 (Morris MR et al 2010).

4. 5 Validation assays of potential target for aberrant methylation

Once a potential CpG island region has been identified in the 5' region of a gene, the methylation status of the gene is validated and analyzed. Many methods for determining the methylation status of individual genes are developed such as methylation-specific PCR (MSP), combined bilufite restriction analysis (COBRA), MSSSCP, MethyLight and bisulfite DNA sequencing (BGS), pyro-sequencing. Each method has advantages and disadvantages. Researchers should consider the aim of methylation analysis and choose proper method.

MSP is a simple rapid and inexpensive method to determine the methylation

status of CpG islands. This approach allows the determination of methylation patterns from very small samples of DNA, with a sensitivity of detecting 0.1% methylated alleles and utilizes the sequence differences between methylated alleles and unmethylated alleles which occur after sodium bisulfite treatment. Results are obtained immediately following PCR amplification and gel electrophoresis. However, MSP is not highly quantitative and may produce false positive results in some circumstances. Since there is no inbuilt measure of adequacy of bisulphite treatment, the possibility of false positives due to inadequate conversion of non-methylated cytosine to uracil exists. Another potential source of false positives is mis-priming, and this may be a greater problem when high numbers of PCR cycles or nested primers are used.

COBRA provides more quantitative and reliable informationon the methylation status of the target sequence. The primers used in the PCR reaction do not contain CpG dinucleotides so that the amplification step retain the mixed sequence according to their original methylation status. Then PCR product is digested by restriction enzyme BstUI (CGCG) and separated by gel electrophoresis. BstUI site CGCG would be retained and cutted if this site is methylated.

BGS and pyro-sequencing allows precise analysis of methylation in a certain region after bisulfite convertion. However, their cost may be expensitive compared with other methods and need sequencing equipment.

In summary, potential target genes for cancer specific methylation is first identified by genome-wide methylation screening. Then the methylation status of the target gene should be validated in detail in cancer cell lines and primary samples by MSP, COBRA, BGS and etc. Moreover, RT-PCR should be performed to find whether the mRNA expression is restored under 5-AZA treatment.

5. DACT1 gene

DACT1, a member of DACT family, was identified by MeDIP coupled with DNA microarray in our lab. DACT1 was an important regulator in planar cell polarity (PCP) pathway (Zhang L et al 2006, Wen J et al 2010, Suriben R et al 2009). PCP is one critical branch of noncanonical WNT signalings involved in developmental processes in vertebrates such as convergent extension movements of mesenchymal cells during gastrulation (Wang Y et al 2009). Tissue-specific downstream PCP effectors convert the upstream PCP signals into specific morphogenetic programs to generate the distinct planar polarity phenotypes. Daam1, Rho GTPase, Rho kinase, JNK, and Profilin are primary downstream PCP effectors engaging in cytoskeleton rearrangements to direct cell polarity and cell movements. Increasing evidences have demonstrated that PCP pathway played important roles in cancer development especially in tumor metastasis and angiogenesis (Wang Y et al 2009). Dishevelled (Dvl) 2, a core PCP component was over-expressed in non-small

cell lung cancer and colorectal cancer and correlated to poor tumor differentiation and advanced TNM stages (Wei Q et al 2008, Metcalfe C et al 2010). Genetic deletion of Dvl2 reduced the intestinal tumor numbers in a dose-dependent way in the Apc(Min) model for colorectal cancer (Metcalfe C et al 2010).

It has been reported that DACT1 played an important role in regulating PCP pathway by post-translationally regulating central PCP component Dvl2 and vang-like 2 (Vangl2) and modulating PCP downstream Rac1/JNK cascade (Zhang L et al 2006, Wen J et al 2010, Suriben R et al 2009). In DACT1 knockout mouse embryo, Dvl2 and Vangl2 were acummulated at the primitive streak where cells ordinarily undergo an epithelial-mesenchymal transition (Wen J et al 2010, Suriben R et al 2009). Since DACT1 expression was down-regulated in hepatocellular carcinoma and in nonsmall cell lung cancer and correlated with aggressiveness and poor prognosis (Yau TO et al 2005, Yang ZQ et al 2010), aberrant inactivation of this developmental regulator may contribute to tumor development. However, the effect of DACT1 on tumorigenicity has never been studied in details to date.

Chapter 6 MATERIALS AND METHODS

6. 1 Gastric cancer cell lines

Gastric cancer cell lines (AGS, Kato III, MKN28, MKN45, N87, SNU1, SNU16, SNU719 YCC1, YCC2, YCC3, YCC6, YCC7, YCC9, YCC10, YCC11, YCC16, BGC823 and MGC803) and normal gastric epithelial GES1 cell were obtained from American Type Culture Collection (Manassas, VA) or Dr Qian Tao (Chinese University of Hong Kong, HK) or Beijing Oncology Hospital (China). Cell lines were maintained in RPMI-1640 or DMEM medium (Gibco BRL, Rockville, MD) with 10% fetal bovine serum (Gibco BRL). Human normal adult tissue RNA samples were purchased commercially (Stratagene, La Jolla, CA or Millipore Chemicon, Billerica, MA).

6. 2 Human tissue samples

Paraffin-embedded tumor tissues samples were obtained from 205 Chinese gastric cancer patients, newly diagnosed in the First Affiliated Hospital of Sun Yat-sen University, Guangzhou from January 1999 to December 2006. Diagnosis was confirmed histologically as gastric cancer. All patients diagnosed to have gastric cancer received surgical resection of the tumor. Clinical data of patients were collected from medical record and structured interview of patients. Clinical

information included age, gender, survival time, TNM stage (I-IV), tumor size (I-IV), lymph node status (postive or negative), distant metastasis (postive or negative), differentiation (poor, moderately or well differentiated), *H. pylori* infection, tumor location, Lauren classification and chemotherapy. 20 age-matched subjects (average age 51.9±17.2) with normal upper gastroscopy were recruited as control. The study protocol was approved by the Clinical Research Ethics Committee of the Sun Yat-sen University of Medical Sciences.

6. 3 Plasmid constructs

6. 3. 1 pCDNA3.1 expression plasmid

The full-length DACT1α cDNA was amplified and cloned into the pCDNA3.1+ expression vector (Invitrogen) (Figure 6. 3. 1).

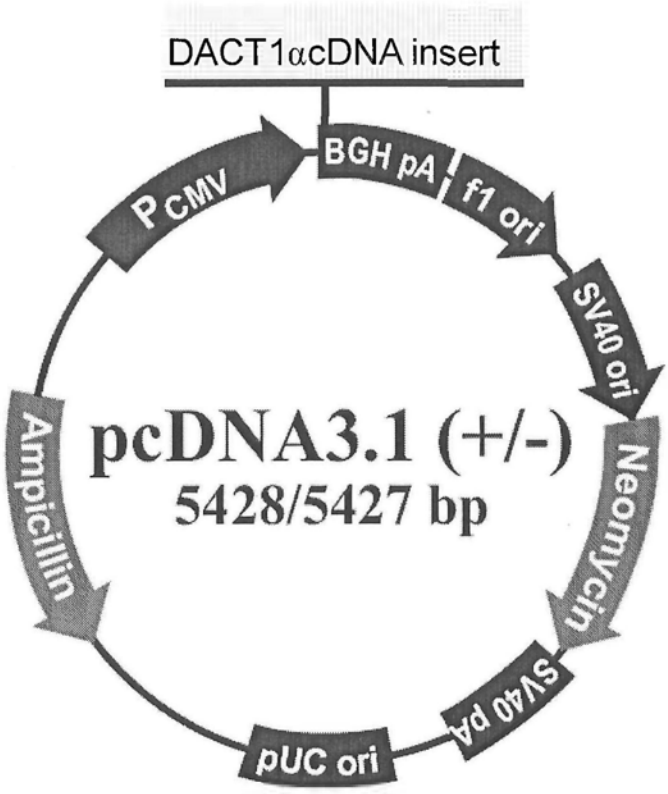

Figure 6. 3. 1. Map of pCDNA3.1- DACT1α.

6. 3. 2 pIRES2-ZsGreen1 expression plasmid

pIRES2-ZsGreen1-DACT1α was generated by inserting the cloned full-length DACT1α into pIRES2-ZsGreen1 empty vector (Clontech) (Figure 6. 3. 2). The internal ribosome entry site (IRES) in pIRES2-ZsGreen1 located between an multiple cloning site (MCS) and the Zoanthus sp. green fluorescent protein (ZsGreen1) coding region. This design permitted the translation of both DACT1α (cloned into the MCS) and the ZsGreen1 gene.

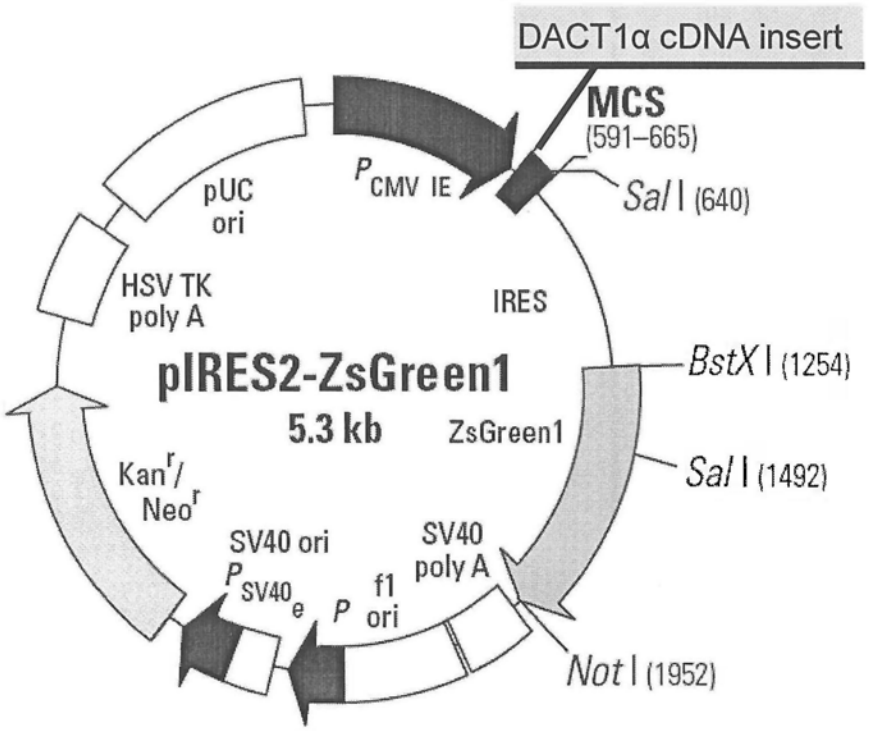

Figure 6. 3. 2. Map of pIRES2-ZsGreen1-DACT1α.

6. 3. 3 Retrovirus expression system

pBABE-puro-DACT1α was cloned by inserting the full-length DACT1α cDNA into pBABE-puro empty vector (Addgene) (Figure 6. 3. 3). Two retrovirus packaging plasmid pUMVC (encoding MuLV gag and pol proteins) and pCMV-VSV-G (encoding VSVG envelope protein) were purchased from Addgene.

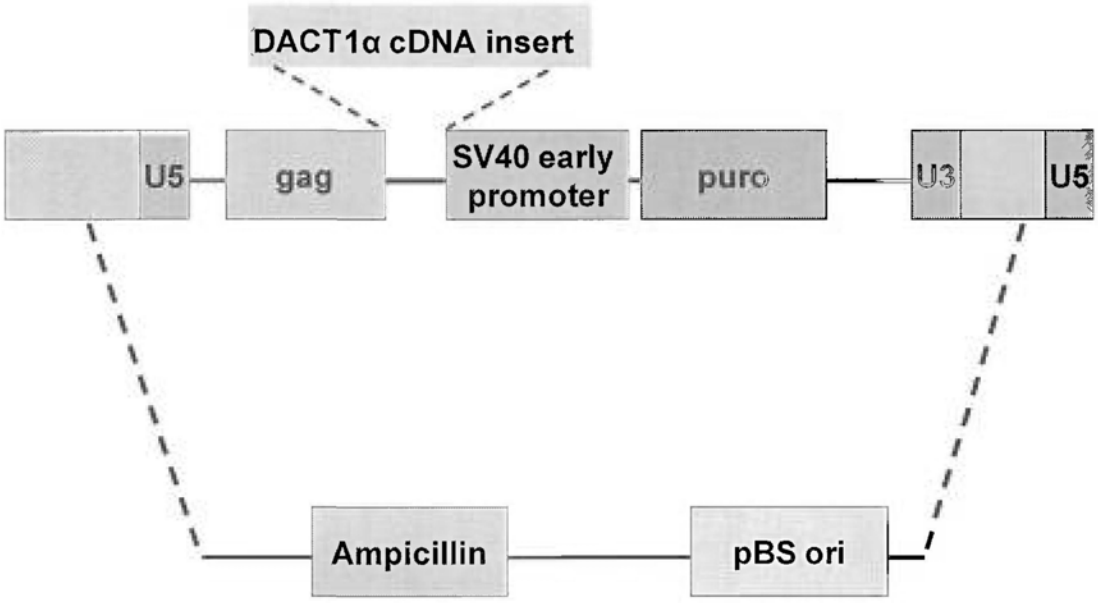

Figure 6. 3. 3. Map of pBABE-puro-DACT1α.

6. 3. 4 ShRNA mediated knockdown system

DACT1 ShRNA contructs and scrambled control ShRNA in plasmid vector pGFP-V-RS were purchased from Origene (Rockville, MD): ShDACT1-1, 5'-AGAGCACAACCACCAGCGACTCTGAAGAA-3' (GI328121); ShDACT1-2, 5'-GATGGCTACATTCTGAGCCTGGTCCAGAA-3' (GI328123); scrambled control ShRNA: 5'-GCACTACCAGAGCTAACTCAGATAGTACT-3' (TR30013).

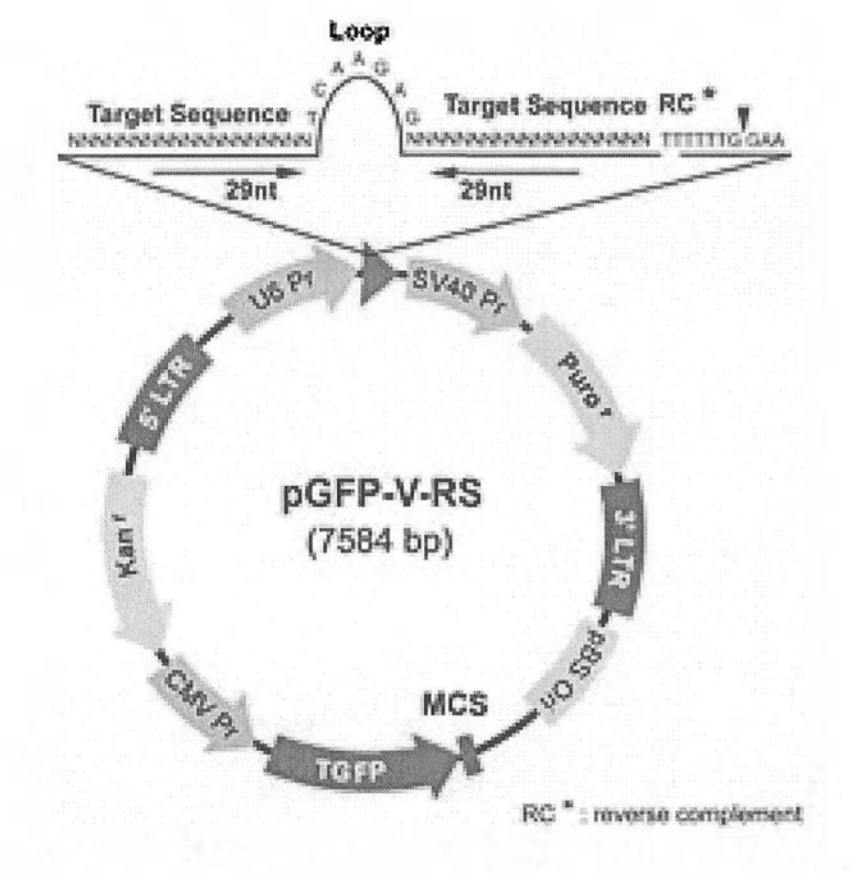

Figure 6. 3. 4. Map of pGFP-V-RS vector.

6. 4 DNA Methylation analysis

6. 4. 1 Bisulfite modification of DNA

Genomic DNA was extracted from fresh gastric tissues of healthy subjects, paraffin-embedded tissues of gastric cancer patients and gastric cancer cell lines using DNA mini kit (Qiagen, Hilden, Germany). To remove the paraffin before tissue digestion, paraffin-embedded tissue was incubated with xylene, collected by centrifuge, then washed by ethanol (96–100%) twice and resuspended in digestion buffer (Buffer ATL containing proteinase K). Fresh gastric tissue was mechanically

disrupted into small pieces before digestion. Tissue or cell pellet in digestion buffer was incubated at 56°C overnight until the tissue was completely lysed. Then Buffer AL added into the sample, incubated at 70°C for 10 min, and mixed with ethanol (96–100%). The mixture was carefully applied to the QIAamp Mini spin column, centrifuged, washed by Buffer AW1 and AW2 and eluted by H_2O.

Extracted DNA was treated with sodium bisulfite using a EZ DNA Methylation-Gold™ Kit (Zymo Research, Orange, CA). Bisulfite induces deamination of unmethylated cytosines, converting unmethylated CpG sites to UpG without modifying methylated sites. This allows their differentiation by methylation-specific polymerase chain reaction (MSP) and direct bisulfite genomic sequencing. 2 µg DNA in 20 µl volume was mixed with 130 µl of the CT Conversion Reagent and incubated under the conditions as follows: 98°C for 10 min, followed by 64°C for 2.5 h and stored at 4°C up to 20 h. The sample was loaded to Zymo-Spin™ IC Column containing 600 µl of M-Binding Buffer, centrifuged, washed by M-Wash Buffer and desulphonated by adding M-Desulphonation Buffer to the column and incubating at room temperature (20°C – 30°C) for 15 - 20 minutes. After washing, bisulfite modulated DNA was eluted and stored for subsequent MSP and direct bisulfite genomic sequencing.

6. 4. 2 Methylation-specific PCR and direct bisulfite genomic sequencing

The bisulfite-modified DNA was amplified by using primer pairs that specifically amplify either methylated or unmethylated sequences of the DACT1 gene (Table 6. 4. 2). The primer pairs were within the CpG island of DACT1 promoter. The PCR was performed in a 20 μl reaction volume containing 1× AmpliTaq Gold PCR buffer II (Applied Biosystems, Foster City, CA), 0.1ul AmpliTaq Gold® DNA Polymerase, 1.6 μl 25mM $MgCl_2$, 1.2 μl 2.5mM dNTP, 0.3 μl sense primer (10μM), 0.3 μl anti-sense primer (10μM) and 2 μl of bisulfite-treated DNA template. The PCR was performed under the conditions as follows: denaturation at 95°C for 10 min, followed by 94°C (30 sec), annealing temperature (60°C for M-MSP, 58°C for U-MSP) (30 sec), and 72°C (30 sec) for 40 cycles, and followed by a final extension of 72°C for 7 min. The PCR product was separated by electrophoresis on 1% agarose with ethidium bromide staining.

Table 6. 4. 2. 1. List of MSP and BGS primers.

Primer Name	Sequence (5'-3')	Annealing Temperature
Methylation specific PCR (MSP)		
DACT1-MSP-MF	CGGGATAGTAGTAGTCGGC	60
DACT1-MSP-MR	CGCTAAAACTACGACCGCG	
DACT1-MSP-UF	GTTGGGATAGTAGTAGTTGGT	58
DACT1-MSP-UR	AAACACTAAAACTACAACCACA	
Bisulfite genomic sequencing (BGS)		
DACT1-BGS-F	GTTTGGGAAGTGAAAGAAATTTAATT	55
DACT1-BGS-R	CTAAAACCCCAACATCCTATTACAAT	

For direct bisulfite genomic sequencing, 2 μl of bisulfite-treated DNA was amplified using primers listed in Table 6. 4. 2. 1. The PCR was performed under the conditions as described above (annealing temperature 55°C). The amplicon were purified by using the ExoSAP-IT PCR Clean-up Kit for the removal of unwanted primers and dNTPs (GE Healthcare, Pittsburgh, PA). PCR product was mixed with ExoSAP-IT at the ratio of 5:2 and incubated for 15 min at 37 °C followed by enzyme inactivation at 80 °C for a further 15 min. The purified DNA was examined by direct sequencing PCR with the Big Dye Terminator Cycle Sequencing kit (Applied Biosystems) under the condition as follows: denaturation at 96°C for 1 min, followed by 96°C (10 sec), 50°C (5 sec), and 60°C (4 min) for 32 cycles (Table 6. 4. 2. 2).

After column purification using ~7.5% (g/ml) Sephadex-G50 (Amersham Biosciences, UK), sequencing PCR product was detected by ABI PRISM 7000 Sequence Detection System (Applied Biosystems).

Table 6. 4. 2. 2. Sequencing PCR condition using Big Dye Terminator Cycle Sequencing kit.

Reagent	Volume (μl)
Big Dye v3.1	2
BigDye® Terminator 3.1 Sequencing Buffer (5X)	2
DACT1-BGS-F primer（1μM）	3.2
H2O	0.8
Purified DNA template	1
Total volume	10

6. 4. 3 Demethylation with the DNA demethylating agent 5-Aza-2'-Deoxycytidine

Gastric cancer cells were seeded at a density of $1*10^6$ cells/mL. After 24 hours, cells were treated with 2 μmol/L of the DNA demethylating agent 5-Aza-2'-deoxycytidine 5-Aza (Sigma–Aldrich, St. Louis, MO) for 96 hours. Cells then were harvested for DNA and RNA extractions.

6. 5 Array comparative genomic hybridization (Array CGH)

Array CGH is used to detect genomic copy number variations at a higher resolution level. Genomic copy number change might be caused by gene amplifications, non-reciprocal translocations and interstitial deletions. Breakage of a chromosome or a non-reciprocal translocation event may result in low level copy number changes whereas homozygous or heterozygous deletions would lead to high level copy number changes. DNA from five gastric cancer cell lines (MKN45, MKN28, KatoIII, N87, SUN1) and normal gastric tissue reference sample are labelled differentially, using different fluorophores, and hybridized to Array-CGH. The procedure was performed according to protocol (Agilent Oligonucleotide Array-Based CGH for Genomic DNA Analysis) and the result was analyzed by Agilent G4175AA CGH Analytics 3.4 (Agilent Technologies, USA). The ratio of the fluorescence intensity of the gastric cancer cell line to that of the normal reference DNA is then calculated, to measure the copy number changes for a particular location in the genome. Two probes were employed to detect the copy number change of DACT1 gene: locus I was at chr14, from 58177255 to 58177309, locus II was located at chr14, from 58179290 to 58179349.

6. 6 Colony formation assay

For overexpression assay, AGS, BGC823 and MGC803 cells were plated in a 12-well plate and transfected with pcDNA3.1- DACT1α or empty pcDNA3.1. For knockdown assay, GES1 cells were seeded in 12-well plate and transfected with

ShControl (TR30013, Origene) or ShDACT1-1 or ShDACT1-2. After 48 h transfection, cells were subsequently split at 1:20 ratio on six-well plates with neomycin (G418) or puromycin (Invitrogen). Colonies were fixed with methanol/ acetone (1:1) and stained with gentian violet. Colonies with cell numbers of more than 50 cells per colony were counted. All the experiments were performed in triplicate wells in 3 independent experiments.

6. 7 Cell growth curve assay

For overexpression assay, $10^{\wedge 4}$ AGS cells stably transfected with pcDNA3.1-DACT1α or empty pcDNA3.1 were seeded into a 12-well plate. The number of viable cells was counted every day for 4 days.

6. 8 RNA extraction

Cell pellet or tumor tissue was homogenized and lysed by 1 mL TRIzol® Reagent (Invitrogen) at room temperature for 10 min. Add 0.2 ml of chloroform per 1 mL of TRIzol® Reagent, shake vigorously by hand for 15 sec and incubate for 2–3 min at room temperature. Then centrifuge the sample at 12,000 × g for 15 min at 4°C and transfer the upper aqueous phase by pipetting into a new tube. Next add 0.5 ml of 100% isopropanol to the aqueous phase, incubate at room temperature for 10 min and centrifuge at 12,000 × g for 10 min at 4°C. Wash the RNA pellet with 1 ml of

75% ethanol twice, air dry the RNA pellet for 5–10 min and resuspend the RNA pellet in RNase-free water.

6. 9 Westernblot

Total cellular proteins were extracted with CytoBuster™ Protein Extraction Reagent (Novagen). The cell pellet was resuspended in the recommended amount of CytoBuster with protease inhibitor cocktail (Roche) and allowed to be lysed at room temperature for 5 min. Centrifuge for 5 min at 16,000 × g at 4°C and collect the supernatant as whole cell extract. The concentration of total celluar protein from the extract was measured by Bio-Rad *DC*™ Protein Assay (Bio-Rad). First, add the appropriate amount of BSA (10μg/μl) and H_2O to each tube to prepare standards (5×, 3×, 1.5×, 1×, 0.5×). Mix Reagent A and S at the ratio of 5:1 and add 25μl mix and 5μl sample to each well. Then add 200μl Reagent B to each well, incubate at room temperature for 5 min and read the absorbances at 750 nm.

30 μg protein per well was mixed with 6 × SDS loading buffer (Tris, pH 6.8, Bromophenol blue, 4% w/v SDS, 10% w/v beta-mercaptoethanal, 20% glycerol) and denatured at 95°C for 10 min and cooled on ice. Then the protein was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). After the electrophoresis, protein was transferred from polyacrylamide gel onto nitrocellulose membrane using semi-dry or wet transfer method. Next the membranes were blocked

by 5% non-fat milk in TBST and probed with primary antibodies against DACT1 (ab42547, Abcam), cleaved-PARP, cleaved caspase 3, 7, 9 (#5625, #9664, #9491, #9501, Cell Signaling), Phospho-SAPK/JNK (Thr183/Tyr185) (#9255, Cell Signaling) and Dvl-2 (10B5) (sc-8026, Santa Cruz Biotechnology) followed by anti-rabbit or anti-mouse secondary antibody and developed with enhanced chemiluminescence (Amersham Corporation, Arlington Heights, IL). GAPDH signal served as a loading control.

6. 10 Celluar localization by immunofluorescent staining

DACT1α-expressing cells used for celluar localization of DACT1α was generated by transfecting BGC823 and MGC803 cells with pIRES2-ZsGreen1-DACT1α. Cells with DACT1α immunofluorescence signal should also show the signal of green fluorescent protein. After 48 hours post-transfection, cells seeded on the cover slips were fixed with 3% paraformaldehyde (in PBS) at room temperature for 25 min. Fixation buffer was discarded and 0.05M ammonium chloride (in PBS) was added to stop the reaction for 10 min. Then cells were washed with PBS three times and permeated using 0.1% Triton X-100 (in PBS) for 3 min. After washing, cells were blocked by 5% Normal Goat Serum (NGS) (in PBS) (Invitrogen) at room temperature for 30 min. For DACT1 immunofluorescence staining, cells were incubated with the primary antibody DACT1 (ab 51260, Abcam) (1:100 dilution, in 1% BSA and 2% NGS) for 2

hours at room temperature. After thorough washing, cells were then incubated with Texas Red–conjugated goat anti-rabbit IgG (1:500 dilution, Santa Cruz Biotechnology). Finally, were washed and mounted with Mounting Medium containing DAPI (Vector Laboratories).

6. 11 Annexin V apoptosis assay

Apoptosis was determined by dual staining with APC Annexin V (Cat. No. 550474, BD Biosciences, Bedford, MA) and 7-amino-actinomycin (7-AAD) (Cat. No. 559925, BD Biosciences). Cell populations were divided as viable (APC Annexin V negative, 7-AAD negative), early apoptotic (APC Annexin V positive, 7-AAD negative), necrotic (APC Annexin V negative, 7-AAD positive), and late apoptotic cells (APC Annexin V positive, 7-AAD positive). BGC823 and MGC803 cells stably transfected with pcDNA3.1 or pcDNA3.1-DACT1α were harvested and resuspended in 1X Binding Buffer at a concentration of 1 x 10^6 cells/ml. For each reaction, 5 μl of APC Annexin V and 5 μl of 7-AAD were added to 100μl sample (about 1 x 10^5 cells) and incubated for 15 min at room temperature in the dark. Add 400μl 1X Binding Buffer before loading sample in flow cytometry. Sample fluorescence of 10,000 cells was analyzed using FACSCalibur System (Becton Dickinson Pharmingen, San Jose, CA). The relative proportion of Annexin V-positive and 7-AAD negative cells was determined using the ModFitLT software (Becton Dickinson, San Diego, CA) and counted as early apoptotic cells. All the experiments

were performed three times independently.

6. 12 Cell invasion

To measure the cell invasion activity, Transwell assays were done using a BD BioCoat™ Growth Factor Reduced MATRIGEL™ Invasion Chamber (Cat. No. 354483, BD Biosciences). AGS and MGC803 cells were seeded into six-well dishes. After 24 h, the cells were transfected with pcDNA3.1-DACT1α or the empty control vector. After 48 h transfection, AGS and MGC803 cells (2.5×10^4 in 500 uL) suspended in serum-free RPMI 1640/DMEM containing 0.1% bovine serum albumin were applied to the upper chamber. RPMI 1640/DMEM containing 20% fetal bovine serum was added to the lower chamber. After the cells were incubated at 37°C for 24 hours, the number of cells that migrated to the lower side of the upper chamber was counted.

6. 13 Cell migration assays

To carry out the wound healing assay, AGS and MGC803 stably transfected with pcDNA3.1-DACT1α or pcDNA3.1 control vector were seeded into 6-well culture plates. After 24 h, the monolayer AGS and MGC803 cells were then scratched manually with a plastic pipette tip, and after being washed with PBS, the wounded monolayers of the cells were allowed to heal for 24 hours in reduced serum

RPMI 1640 and DMEM medium.

6. 14 Cell spreading assay

BGC823 and MGC803 cells stably transfected with pcDNA3.1-DACT1α or pcDNA3.1 were collected by trypsinization, washed twice with DMEM medium and were resuspended on cover slips and culture plate. Cells were allowed to spread for 6 hours in DMEM containing 10% FBS at 37°C and then photographed. The cell spreading rate was calculated as: number of spreading cells / number of total cells (%). The cover slips with spreading cells were fixed with 3% paraformaldehyde for F-actin staining.

6. 15 F-actin staining

Cell spreading assay was performed using BGC823 and MGC803 cells stably transfected with pcDNA3.1-DACT1α or pcDNA3.1. The cover slips with spreading cells were fixed with 3% paraformaldehyde (in PBS) at room temperature for 15 min. Then the cells were washed three times with 1×TBS and permeabilized with 0.1% Triton X-100 at room temperature for 5 min. Then cells were washed three times with 1×TBS and blocked with 1% BSA at room temperature for 30 min. For F-actin staining, cells were stained with Rhodamine phalloidin (1:40 dilution in 0.2% BSA) (Invitrogen) at 37°C for 20 min in dark. Finally, were washed and mounted with

Mounting Medium containing DAPI (Vector Laboratories).

6. 16 Dual-luciferase reporter assay

To elucidate the signaling pathways modulated by DACT1α, serveral signaling pathway luciferase reporters were examined in DACT1α-transfected AGS and BGC823 cells, including NF-κB-luc (5xNFκB binding sites), AP1-luc (7 x AP1 binding sites) and TOPFlash (4 x TCF binding sites). The signaling pathway luciferase reporter activity was analyzed by the dual-luciferase reporter assay system (Promega, Madison, WI). In this assay, the luciferase activities of firefly (*Photinus pyralis*) encoded by the pathway reporter plasmid and Renilla (*Renilla reniformis*) encoded by internal control pRL-CMV vector were measured sequentially from a single sample in a single experiment. Thus activity of pathway reporter was normalized by the activity of the internal control to provide more reliable result.

There were three main steps. First, gastric cancer cells were plated in 24-well at the first day and co-transfected with luciferase reporter plasmid (200ng per 24-well) and pRL-CMV vector (5ng per 24-well) with DACT1α-pcDNA3.1 (600ng per 24-well) or pc-DNA3.1 (600ng per 24-well) using lipofectamine 2000 Transfection Reagent (Invitrogen) within 24 hour after cell seeding. Second, after 48 hours post-transfection, cell lysates containing the luciferases were harvested. Briefly, remove the growth medium and gently apply 500μl PBS to wash. Add 100μl 1X

Passive Lysis Buffer to each well and place the culture plate on a rocking platform at room temperature for 15 min. Transfer the lysate to a tube, centrifuge at 10,000Xg at 4°C for 5 min and transfer cleared lysates to a new tube for subsequent reporter activity assay. In the third step, predispense 100μl Luciferase Assay Buffer II into the 96-well, add 20μl cell lysate and place it in the luminometer and initiate reading the firefly luminescence encoded by the pathway reporter. Then dispense 100μl Stop & Glo® Reagent and place it in the luminometer and initiate reading the Renilla luminescence encoded by the internal control pRL-CMV vector. Reporter activity was normalized to the control *Renilla*. Experiments were repeated in triplicate.

6. 17 NF-κB p50 and p65 Transcription Factor Binding Activity Assay

Nuclear extracts were obtained from BGC823 cells stably transfected with pcDNA3.1-DACT1α or pcDNA3.1 empty vector using a Nuclear Extract kit (Milipore) in the presence of phosphatase inhibitors. Briefly, the cells were collected by trypsinization, washed and allowed to swell on ice in 5 cell pellet volumes of ice cold 1x Cytoplasmic Lysis Buffer containing 0.5mM DTT and protease inhibitor cocktail for 15 min. The cells were centrifuged at 4°C for 5 min, resuspended in 2 cell pellet volumes of ice cold 1x Cytoplasmic Lysis Buffer containing 0.5mM DTT and protease inhibitor cocktail and disrupted using a syringe with a 27-gauge needle. After centrifuge, the remaining nuclear pellet was resuspended in 2/3 of the original cell pellet volume of ice cold Nuclear Extraction Buffer containing 0.5mM DTT and

protease inhibitor cocktail, disrupted using a syringe with a 27-gauge needle, gently rocked at 4°C for 30 min. After centrifuge. the supernatant was the nuclear extract, of which protein concentration was determined by Bio-Rad *DC*™ Protein Assay (Bio-Rad).

NF-κB binding activity assay was performed using NF-κB (human p50/p65) combo transcription factor assay kit (Cayman) according to the manufacturer's protocol. Briefly, the wells of a 96-well plate were pretreated with the NF-κB consensus sequence oligonucleotides. 90 μl Complete Transcription Binding Assay Buffer and 10μl nuclear extract / positive control was added to the wells. Following one-hour incubation at room temperature without agitation, the wells were washed five times with Wash Buffer (TBS-Tween). Then the rabbit primary NF-κB p50 or p65 antibody was diluted 1:100 and added to the wells, incubated for 1 h without agitation, and washed five time with Wash Buffer. The goat-anti-rabbit secondary horseradish peroxidase-conjugated antibody was diluted 1:100 and added to the wells, incubated for 1 h without agitation, and washed five time with Wash Buffer. The 100μl Developing Solution was added and incubated for 15 min with gentle agitation before the reaction was stopped in the Stop Solution (100μl). The absorbance was read on a spectrophotometer at 450 nm. The relative binding activity is given by ($absorbance^{treatment}$-$absorbance^{blank}$)/ μg protein and normalized to the fold change detected in the corresponding control cells, which was defined as 1.0.

6. 18 Cancer PathwayFinder PCR Array and real-time PCR validation

Gene expression profiles of BGC823 cells stably transfected with pcDNA3.1-DACT1α or pcDNA3.1 empty vector were analyzed by a commercial gene expression array system named the Human Cancer PathwayFinder™ RT² Profiler™ PCR Array (96-well) (SABiosciences, Frederick, MD). This array contains 84 functionally well characterized genes representative of the six biological pathways involved in human tumorigenesis listed below (http://www.sabiosciences.com) (Table 6. 18. 1).

Table 6. 18. 1. List of cancer-related genes representative of the six biological pathways in the Human Cancer PathwayFinder™ RT² Profiler™ PCR Array.

Cell Cycle Control & DNA Damage Repair:

ATM, BRCA1, CCNE1 (cyclin E1), CDC25A, CDK2, CDK4, CDKN1A (p21Waf1), CDKN2A (p16Ink4), CHEK2 (chk2 / Rad53), E2F1, MDM2, RB1, S100A4, TP53 (p53)

Apoptosis and Cell Senescence:

APAF1, BAD, BAX, BCL2, BCL2L1 (bcl-X), CASP8, CFLAR (CASPER), GZMA, HTATIP2, TERT (telomerase), TNFRSF1A (TNF-a receptor), TNFRSF10B (DR5), TNFRSF25 (DR3)

Signal Transduction Molecules and Transcription Factors:

AKT1, ERBB2, ETS2, FOS, JUN, MAP2K1 (MEK), MYC, NFKB1 (NFκB), NFKBIA (IκBα), PIK3R1 (PI3K p85α), RAF1, SNCG

Adhesion:

ITGA1 (integrin α1), ITGA2 (integrin α2), ITGA3 (integrin α3), ITGA4 (integrin α4), ITGAV (integrin αV), ITGB1 (integrin β1), ITGB3 (integrin β3), ITGB5 (integrin β5), MCAM, MTSS1, PNN, SYK, UCC1

Angiogenesis:

ANGPT1 (angiopoietin-1), ANGPT2 (angiopoietin-2), COL18A1 (endostatin), FGFR2, IFNA1 (IFNα), IFNB1 (IFNβ), IGF1, IL8, PDGFA, PDGFB, TEK (tie-2), TGFB1, TGFBR1 (ALK-5), THBS1 (thrombospondin-1), TNF, VEGFA

Invasion and Metastasis:

MET, MMP1 (collagenase-1), MMP2 (gelatinase A), MMP9 (gelatinase B), MTA1, MTA2, NME1, NME4, PLAU, PLAUR, S100A4, SERPINB5 (maspin), SERPINE1 (PAI1), TIMP1, TIMP3, TWIST1

RNA from BGC823 cells stably transfected with pcDNA3.1-DACT1α or pcDNA3.1 empty vector was extracted according the procedure mentioned above. Reverse transcription was conducted using RT² First Strand Kit (SABiosciences), which contained an effective genomic DNA elimination step and a built-in external RNA control and test for DNA contamination. Briefly, 1 μg RNA in 10 μl Genomic DNA Elimination mixture was incubated at 42°C for 5 min and immediately placed on ice for at least 1 min. Then add 10μl the RT cocktail (4μl 5X RT Buffer 3, 1μl Primer and External Control Mix, 2μl RT Enzyme Mix 3, 3μl H_2O) and mix well, incubate at 42°C for exactly 15 min and then immediately stop the reaction by heating at 95°C for 5 min. Add 91 μl of H_2O to each 20μl of cDNA synthesis reaction (Total volume 102μl).

To perform real-time PCR array, prepare the cocktail mixture according the manufacturer's instructions showed below (Table 6. 18. 2) and add 25μl the cocktail mixture per well for 96-well custom PCR arrays. Detection was conducted on ABI PRISM 7500 Fast Real-Time PCR System (Applied Biosystems) under the condition: denaturation at 95°C for 10 min, followed by 95°C (15 sec) and 60°C (1 min) for 40 cycles. Data analysis was performed by PCR Array Data Analysis Software from the following address: http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php. Gene expression with fold-changes ≥1.5 or ≤1.5 was considered to be of biological significance and further validated by real-time PCR.

Table 6. 18. 2. The real-time PCR array cocktail mixture.

Reagent	Volume (µl)
2X SABiosciences RT2 qPCR Master Mix	1350
Diluted First Strand cDNA Synthesis Reaction	102
H_2O	1248
Total volume	2700

For real-time PCR validation analysis, reverse transcription was performed using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according the manufacturer's instructions. Briefly, 2 µg RNA in 10µl H_2O was mixed with 10µl 2× RT master mix consisting of 2µl 10× RT Buffer, 0.6µl 25× dNTP Mix (100 mM), 2µl 10× RT Random Primers, 1µl MultiScribe™ Reverse Transcriptase, 1µl RNase Inhibitor and 3.2µl Nuclease-free H_2O. The 20µl mixture was incubated at 25°C for 10 min, then at 37°C for 120 min, 85°C for 5 min and stored at 4°C. Next aliquots of double-stranded cDNA were amplified using a SYBR Green PCR Kit (Applied Biosystems) and ABI PRISM 7500 Fast Real-Time PCR System. The real-time PCR was performed in a 25 µl reaction volume containing 2µl 100× dilution cDNA product, 12.5µl 2 × SYBR Green Master Mix (Applied Biosystems), 0.3µl forward primer and 0.3µl reverse Primer. Ct was measured during the exponential amplification phase, and the amplification plots were analyzed using SDS 1.9.1 software (Applied Biosystems). The relative expression level (defined as fold change) of target gene is given by $2^{-\Delta\Delta Ct}$ ($\Delta Ct = \Delta Ct^{target} - \Delta Ct^{\beta actin}$; $\Delta\Delta Ct =$

$\Delta Ct^{DACT1\alpha\text{-expressing}} - \Delta Ct^{control}$) and normalized to the fold change detected in the corresponding control cells, which was defined as 1.0. All reactions were performed in duplicate. Primer sequences of target genes are listed in Table 6. 18. 3.

6. 18. 3. List of real-time PCR primers for validation.

Primer Name	Sequence (5'-3')	Annealing Temperature
β-actin-F	GTCTTCCCCTCCATCGTG	60
β-actin-R	AGGGTGAGGATGCCTCTCTT	
TNF-F	CCCGAGTGACAAGCCTGTAG	60
TNF-R	GCTGGTTATCTCTCAGCTCCA	
NFKB1-F	TTTCAACCACAGATGGCACT	60
NFKB1-R	TAGAGGCACCAGGTAGTCCAC	
BCL2L1-F	TCCCGACCTGTGATACAAAAG	60
BCL2L1-R	TCCAAAGCCAAGATAAGATTCTG	
ITGA1-F	GTCAGCCCCACATTTCAAGT	60
ITGA1-R	GGAACCATCCAGCACTATGAC	
ITGA2-F	TTGACCTATCCACTGCCACA	60
ITGA2-R	CTCCAGTTCCCATGTTCCTG	
ITGA3-F	TCCATCGGCAGACAGAGC	60
ITGA3-R	GCACAGGTACACAGCACCAG	
ITGB3-F	CTCAAGTCAGTCCCCAGAGG	60
ITGB3-R	TCCACAGGGTAATCCTCCAC	
VEGFA-F	CTACCTCCACCATGCCAAGTG	60
VEGFA-R	TGATTCTGCCCTCCTCCTTCT	
PDGFB-F	ATCCGCTCCTTTGATGATCT	60
PDGFB-R	GGGTCATGTTCAGGTCCAAC	
IL8-F	GAACTGAGAGTGATTGAGAGTGGA	60
IL8-R	CTCTTCAAAAACTTCTCCACAACC	
MMP9-F	CCTGGAGACCTGAGAACCAATC	58
MMP9-R	CCACCCGAGTGTAACCATAGC	
PLAU-F	AACGTACCATGCCCACAGAT	60
PLAU-R	TCTTGGACAAGCGGCTTTAG	

6. 19 pBABE-puro-DACT1α or control retroviruses production

The retrovirus production system is used to deliver and express DACT1α. The major components of the system include: 1) The expression plasmid containing DACT1α (pBABE-puro-DACT1α) or empty plasmid (pBABE-puro vector) under the control of a choice of promoters, and elements that allow packaging of the construct into virions. 2) An optimized mix of the two packaging plasmids that supply the structural and replication proteins including pUMVC (encoding MuLV gag and pol proteins) and pCMV-VSV-G (encoding VSVG envelope protein). 3) The 293FT cell line, which allows production of lentivirus by cotransfection of the expression plasmid and the packaging plasmids.

For retroviruses production, 293FT cells (Invitrogen) were co-transfected with pBABE-puro-DACT1α or pBABE-puro empty vector, two packaging plasmids pUMVC (Addgene) and pCMV-VSV-G (Addgene) at the ratio of 1: 0.9: 0.1. At 24 hours post-transfection, cells were feeded with fresh medium. After 48 hour post-transfection, the supernatant containing retrovirus pBABE-puro-DACT1α or pBABE-puro control was harvested and stored at -80°C. To generate stable DACT1α expressing or control cell line, retrovirus pBABE-puro-DACT1α or pBABE-puro control-containing supernatant was added to BGC823 cells. After 24 hour transduction, the media containing retrovirus was removed and replaced with fresh

medium containing 0.5 ug/ml antibiotic puromycin (Invitrogen) for selection. The diagram below described the general steps mentioned above (Figure 6. 19).

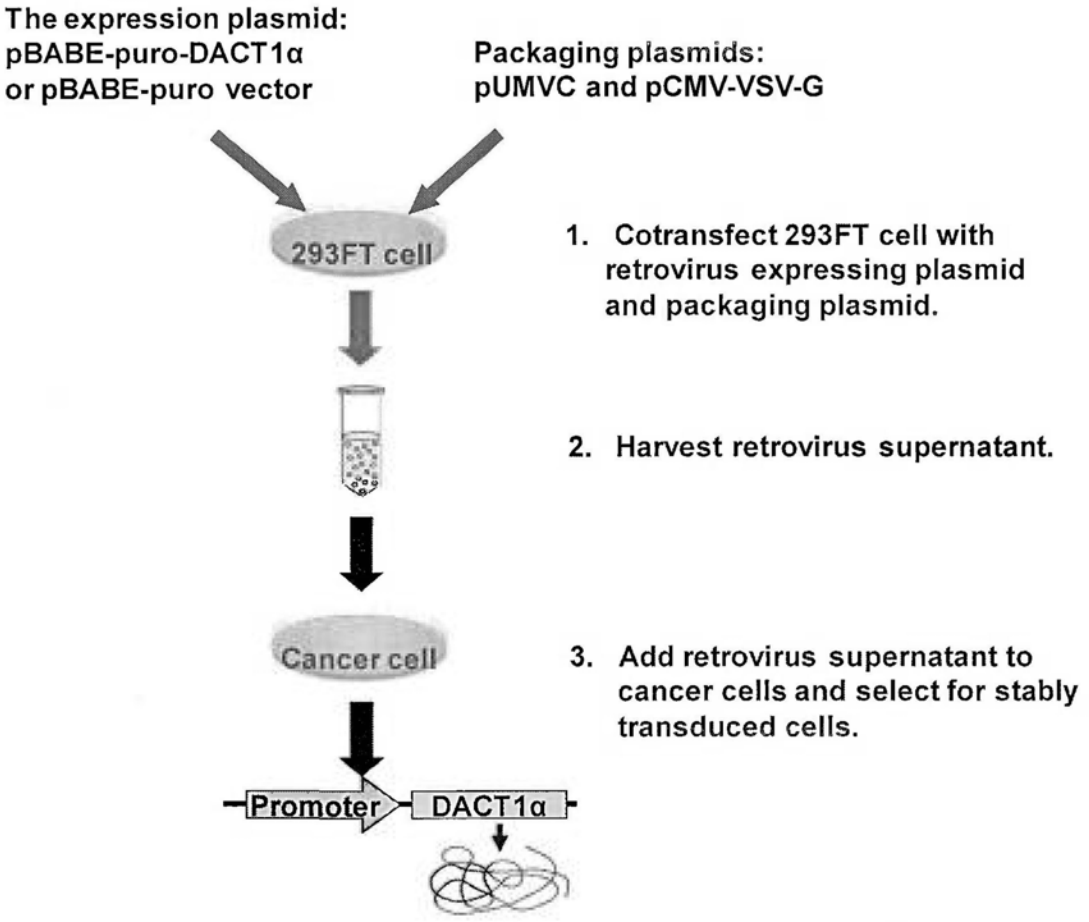

Figure 6. 19. The general steps required to generate stable DACT1α-expressing cells.

6. 20 *In Vivo* Tumorigenicity

Stable DACT1α expressing or control BGC823 cells (5×10^5 cells in 0.2 mL phosphate buffered saline) was injected subcutaneously into the left or the right dorsal flank of four 4-week-old male Balb/c nude mice, separately. Tumor diameter

was measured every 3 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= (shortest diameter)^2 × (longest diameter) × 0.5. On the day of harvest, the mice were euthanized and tumor weight was measured. A portion of the tumor tissue was excised and embedded in paraffin for subsequent histological examination. The remaining tissue was flash-frozen in liquid nitrogen and stored at -80°C for RNA and protein extraction. Care of animals and all experimental procedures were approved by the Animal Ethics Committee of the Chinese University of Hong Kong.

6. 21 Immunohistochemistry

Immunohistochemistry was performed using Histostain®-Plus Bulk Kit, Invitrogen® 2nd Generation, LAB-SA Detection System (Invitrogen). The paraffin-embedded tissue sections were deparaffinized with xylene and rehydrated in a graded series of ethanol (95%, 85%, 70% and 50%). Wash tissue sections in a PBS bath for 10 min before starting staining. Then endogenous Peroxidase activities was blocked by submerging slides in Peroxidase Quenching Solution (30% hydrogen peroxide : absolute methanol = 1: 9) for 10 min and wash with PBS three times for 2 min. Transfer to distilled water for 1 min and rinse briefly with PBS. Microwave 2 min at P-10 followed by 10 min at P-2 in Citrate Buffer (PH 6.0) to make antigen retrieval. Aftering incubating with Serum Blocking Solution (Reagent A) for 10 min, apply the primary antibody (DACT1 (ab42547, Abcam), 1: 100 dilution; Ki-67

(Clone SP6) (Lab Vision), 1: 200 dilution; NF-κB p65 (A) (sc-109, Santa Cruz Biotechnology), 1: 50 dilution) to the slides and incubate in moist chamber for 2 hours at room temperature. Apply Biotinylated Second Antibody (Reagent B) to the sections, incubate for 10 min and wash with PBS 3 times for 2 min. Next apply Enzyme Conjugate (Reagent C) to the sections, incubate for 10 min and wash with PBS 3 times for 2 min. DAB staning (Invitrogen) were employed to visualize antibody and finally counterstained with hematoxylin. The number of NF-κB p65/ Ki-67 nuclear positive cells was counted per 1000 tumor cells in 5 fields. The percentage of NF-κB p65 or Ki-67 positive nuclei were representated as NF-κB activity or cell proliferation rate respectively. Besides, DACT1 antibody is not specific enough to be used to assess DACT1 experession in clinical-pathological analysis.

6. 22 Terminal deoxynucleotidyl transferase–mediated nick end labeling (TUNEL) analysis

The tumor tissues from nude mice were excised and embedded in paraffin for TUNEL analysis. This procedure was performed using DeadEnd™ Colorimetric TUNEL System (Promega). The paraffin-embedded tissue sections were deparaffinized by immersing the slides in fresh xylene for 5 min twice. Then the slides were washed in 100% ethanol for 3 min, and then rehydrated by sequentially washing with graded ethanol (95%, 85%, 70% and 50%) for 3 min each. Next the

slides were washed in 0.85% NaCl for 5 min and then PBS for 5 min and fixed by 4% paraformaldehyde solution. Then add 20μg/ml Proteinase K solution to each slide and incubate for 25 min to permeabilize tissues. After washing and re-fixing, the sections are equilibrated and incubated with Terminal Deoxynucleotidyl Transferase, Recombinant, (rTdT) enzyme reaction mix at 37°C for 60 min inside a humidified chamber to allow the end-labeling reaction to occur. In this step, biotinylated nucleotide is incorporated at the 3′-OH DNA ends by rTdT. The reaction was terminated by immersing the slides in 2X SSC for 15 min at room temperature. After washing, the slides was immersed with 0.3% hydrogen peroxide in PBS for 3–5 min to block endogenous peroxidases. Then add the Streptavidin HRP solution and incubate for 30 min at room temperature to bound to these biotinylated nucleotides. Finally, apoptotic nuclei are stained dark brown by adding DAB solution and developing for approximately 10 min. The apoptotic index was expressed as the percentage of TUNEL-positive tumor cells out of the total number of tumor cells counted.

6. 23 Statistical Analysis

Unless otherwise indicated, data are presented as mean ± SD of 3 independent experiments. The independent Student's t test was used to compare the difference between any 2 preselected groups. Difference in DACT1 protein expression in paired adjacent normal and tumor tissues was analyzed by contingency tables and Pearson's

$\chi 2$ test. The difference in cell growth between the two treatments or the tumor growth rate between the two groups of nude mice was determined by repeated-measures analysis of variance. Relationships between the distributions of DACT1 methylation and clinicopathologic characteristics of gastric cancer patients were compared using contingency tables and Pearson's $\chi 2$ test. Kaplan-Meier survival curves and the log-rank test for trend were used to evaluate the relationship between DACT1 methylation and the prognosis from the date of primary diagnosis to the end of follow-up. The univariate Cox regression analysis was performed to assess the prognostic value of DACT1 methylation and other clinicopathologic characteristics. The multivariate Cox regression analysis was also conducted to estimate the Hazard ratios (HR) for DACT1 methylation with adjustment for age, gender and TNM stage. All analyses were performed using SPSS statistical package for Windows (version 16; SPSS). A P value of less than .05 was taken as statistical significance.

Chapter 7 RESULTS

7. 1 Silence or down-regulation of DACT1 in gastric cancer

7. 1. 1 Silence or down-regulation of DACT1 in gastric cancer cell lines

DACT1 was expressed in all normal adult tissues and fetal tissues including normal stomach tissue as well as normal human gastric epithelial cell GES1 as demonstrated by reverse-transcription PCR, (Figure 7. 1. 1). In contrast, the mRNA expression of DACT1 was silenced or reduced in 7 (7 of 10, (70%)) gastric cancer cell lines.

7. 1. 2 Down-regulation of DACT1 in primary gastric cancer tissues

To further investigate whether DACT1 expression was down-regulated in gastric cancer progression, DACT1 expression was tested by immunostaining in 19 pairs of gastric cancer cases. Compared with the paired adjacent nontumor tissues, down-regulation of DACT1 was detected in 9 of 19 (47.4%) gastric cancers ($\chi 2$ test, P=0.001) (Figure 7. 1. 2).

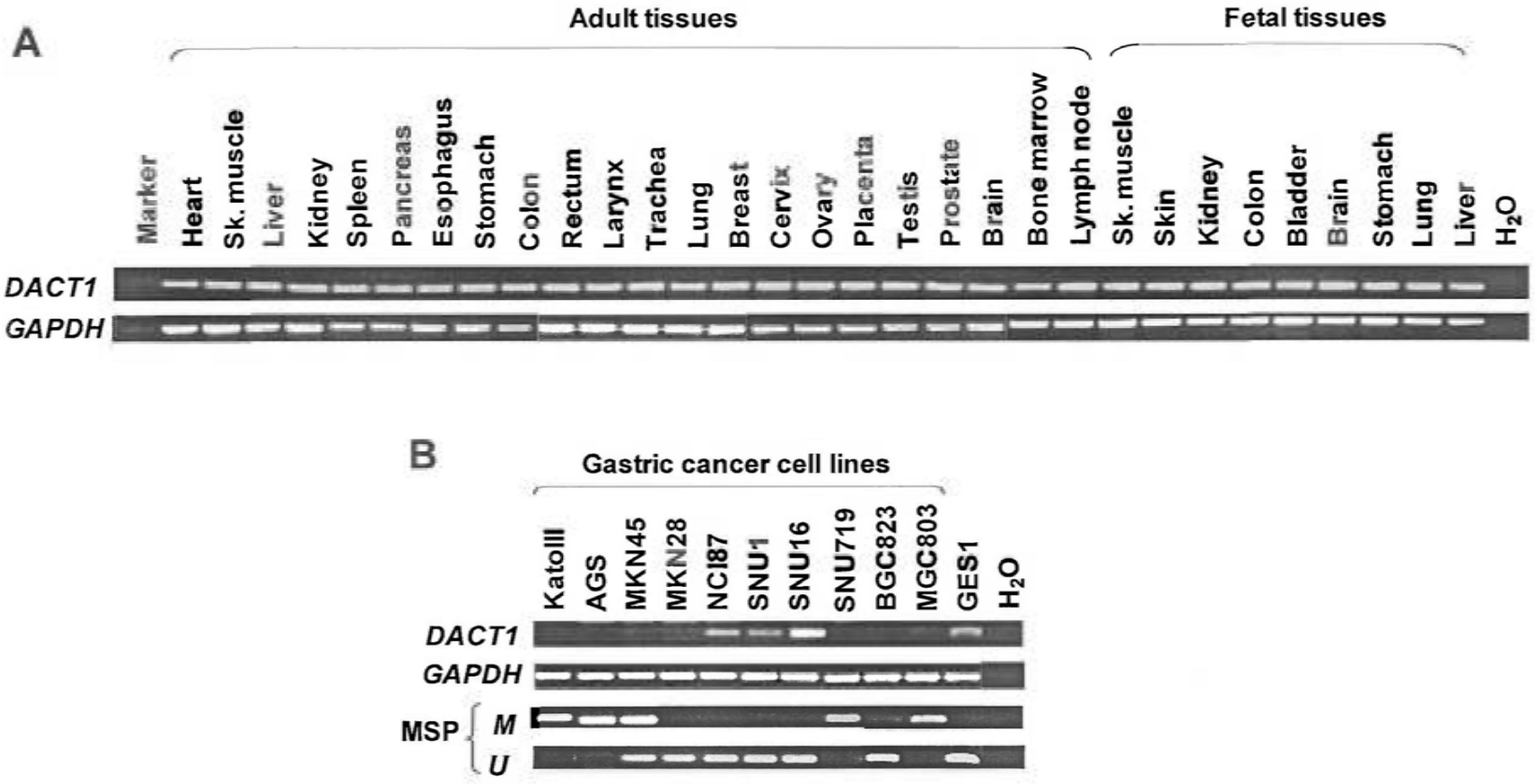

Figure 7. 1. 1. DACT1 was primarily inactivated in gastric cancer cell lines. (A) Robust mRNA expression of DACT1 in normal adult tissues and fetal tissues. (B) DACT1 was frequently silenced or reduced in gastric cancer cell lines by semi-quantitative RT-PCR.

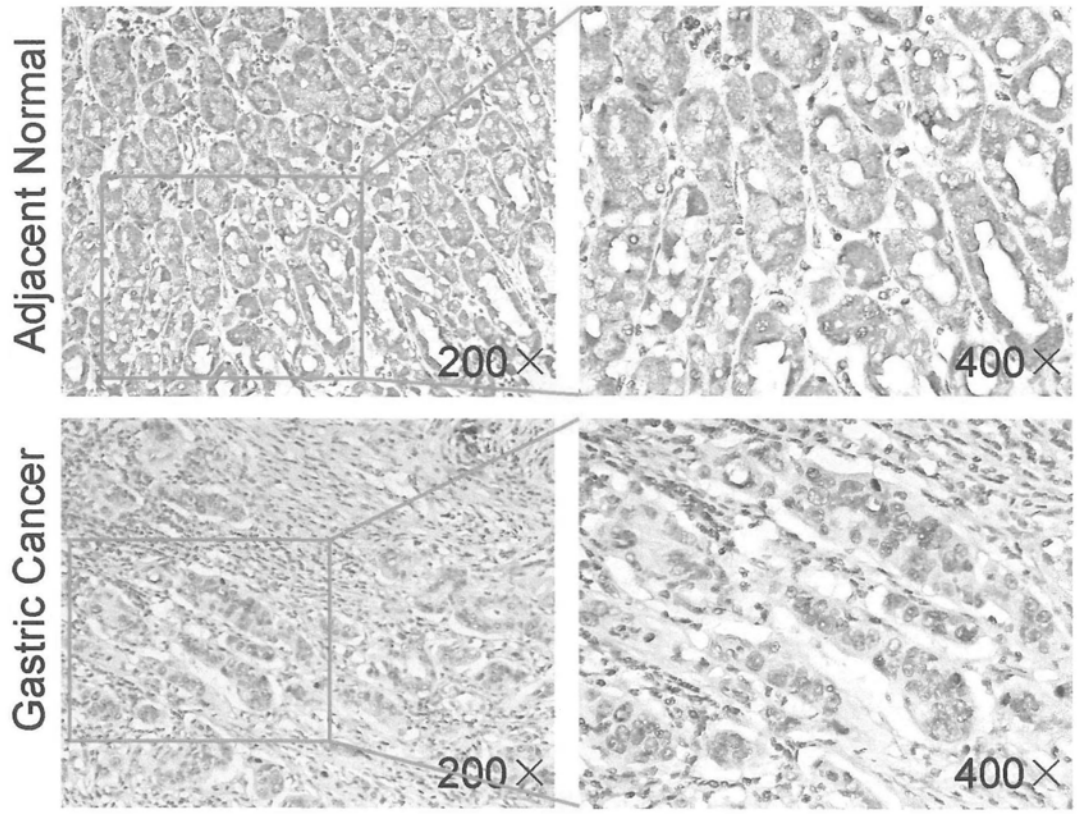

Figure 7. 1. 2. Downregulation of DACT1 in primary gastric cancer specimens. The immunohistochemical staining of DACT1 expression in adjacent normal and cancerous gastric tissues

7. 2 Mechanism(s) of DACT1 transcriptional repression in gastric cancer

7. 2. 1 Frequent methylation of DACT1 in gastric cancer cell lines

To elucidate the role of promoter methylation in the down-regulation of DACT1, DACT1 methylation status was examined by methylation-specific PCR. Full methylation was observed in 4 silenced cell lines (KatoIII, AGS, SNU719, MGC803),

whereas partial methylation was detected in MKN45 cell line (Figure 7. 2. 1, A). Moreover, DACT1 methylation was not detected in normal human gastric epithelial cell GES1 (Figure 7. 2. 1, A).

We further analyzed DACT1 methylation in more detail using direct bisulfite genomic sequencing analysis. The BGS results were consistent with those of MSP in which dense methylation was found in methylated cell lines by MSP (MKN45 and KatoIII), but not in unmethylated MKN28 and normal gastric tissues (Figure 7. 2. 1, B).

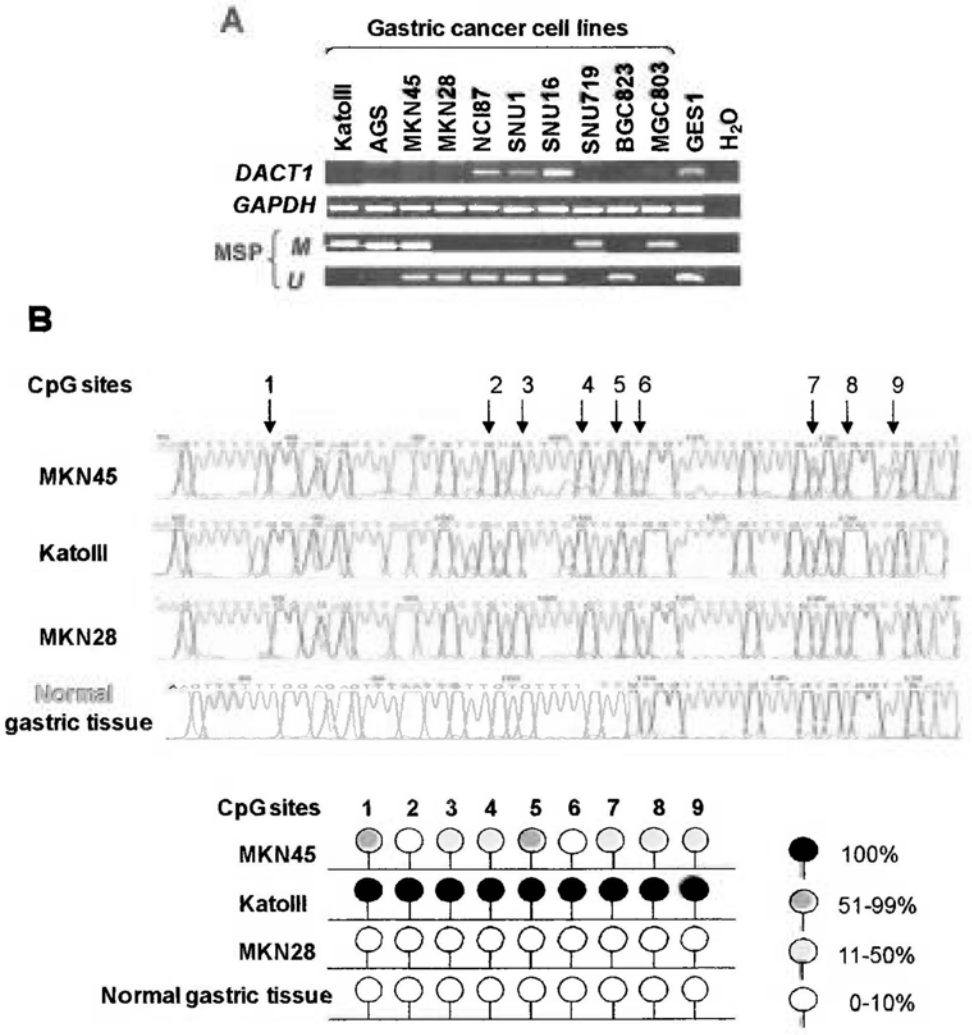

Figure 7. 2. 1. DACT1 was primarily inactivated by promoter methylation in gastric cancer. (A) Methylation of DACT1 was determined by MSP. M, methylated; U, unmethylated. (B) Direct BGS analysis confirmed the methylation status of the DACT1 in gastric cancer cell lines and in normal gastric tissues.

7. 2. 2 Restoration of DACT1 expression by pharmacologic demethylation with 5-Aza

To test whether methylation directly mediates DACT1 silencing, we treated 3 gastric cancer cell lines that showed silencing of DACT1 with 5-Aza. This treatment

resulted in the restoration of DACT1 expression in AGS and KatoIII cell lines with DACT1 full methylation. However, 5-Aza failed to activate DACT1 expression in unmethylated MKN28 cells (Figure 7. 2. 2). These results suggested that promoter methylation was one of the mechanisms for the transcriptional silencing of DACT1 in gastric cancer cells.

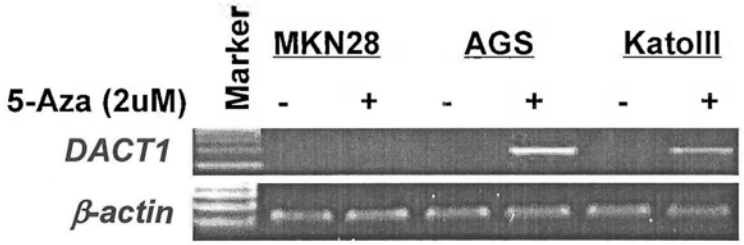

Figure 7. 2. 2. Pharmacologic demethylation with Aza restored DACT1 expression.

7. 2. 3 DACT1 silencing by other genetic alteration mechanism

We next performed Array-CGH to detect genomic copy number variations in five gastric cancer cell lines (MKN45, MKN28, KatoIII, N87, SUN1). DNA from normal gastric tissue served as reference control. The relative copy number changes of DACT1 gene from gastric cancer cell lines were shown in Table 7. 2. 3.. MKN28 without DACT1 methylation appeared as relative copy number loss in both locus of DACT1 gene. A relative low level copy number loss in one locus of DACT1 gene was seen in MKN45 with partial methylation. However, there were no copy number changes in DACT1 expressing cell lines or silenced cell lines with DACT1 full methyaltion. This suggested that other genetic aberration also mediated DACT1

transcriptional repression such as breakage of a chromosome or a non-reciprocal translocation event.

Table 7. 2. 3. The relative copy number changes of DACT1 gene from gastric cancer cell lines (normalized to normal reference DNA).

	The relative copy number change		DACT1	DACT1
Cell line	**Locus I**	**Locus II**	**expression**	**methylation**
MKN45	0.797	0.983	none	parital
MKN28	0.754	0.684	none	none
KatoIII	0.930	0.919	none	full
N87	1.047	0.953	**yes**	none
SUN1	1.052	0.990	**yes**	none

7. 3 Tumor suppressive effect by DACT1α in gastric cancer cell lines *in vitro*

7. 3. 1 Over-expression of DACT1α inhibited gastric cancer cell growth

To elucidate the function of DACT1α in gastric cancer, we examined the effect of DACT1α re-expression and knockdown on growth characteristics of gastric cancer cells by colony formation and growth curve assays. DACT1α–expressing plasmid was stably transfected into AGS, BGC823 and MGC803 cells with no DACT1α

expression. The colonies formed in DACT1α-transfected cells were significantly fewer in number and smaller in size than in control vector-transfected cells (Figure 7. 3. 1, A). Besides, DACT1α over-expression dramatically reduced cell growth in AGS cells (Figure 7. 3. 1, B).

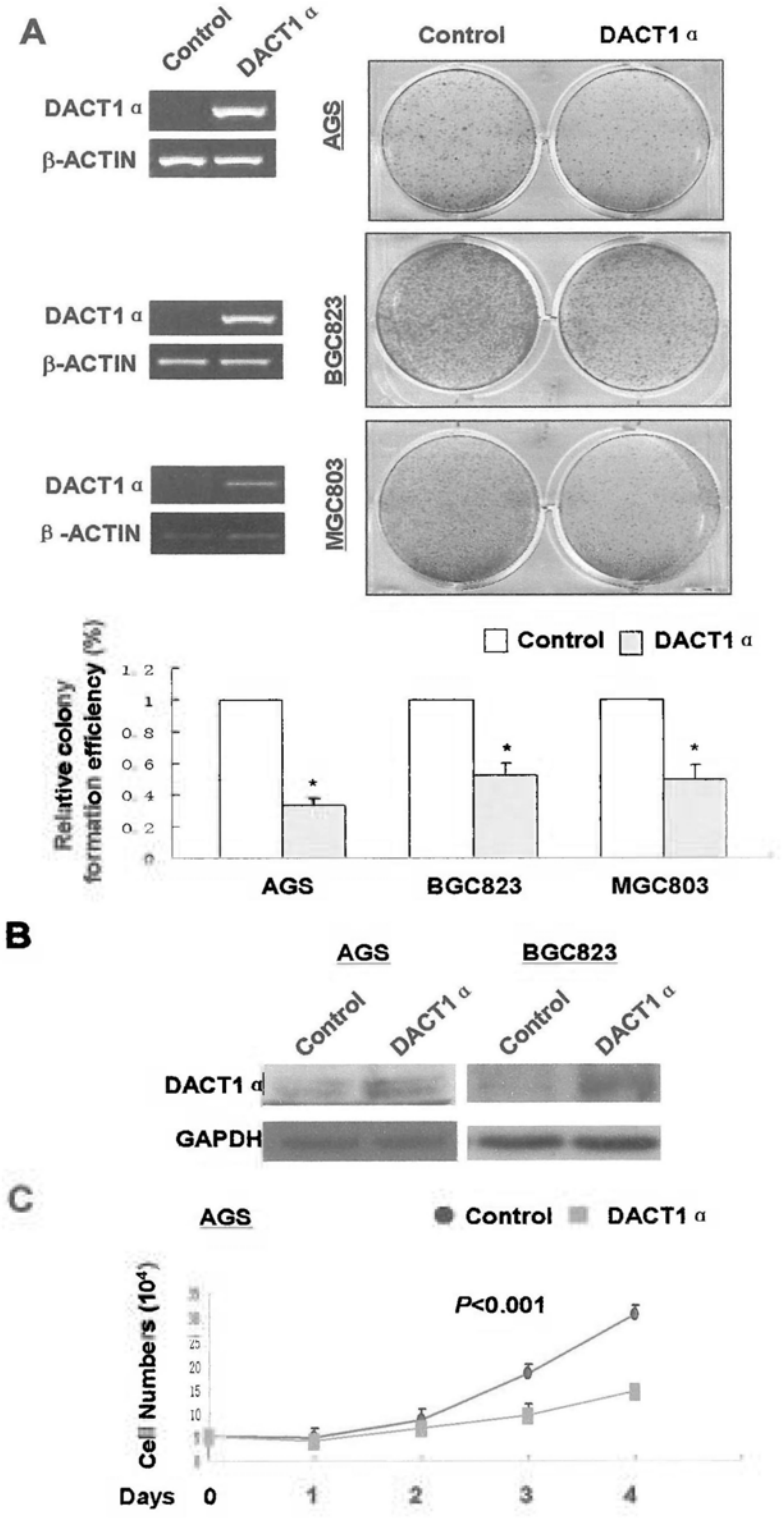

Figure 7. 3. 1. DACT1α over-expression inhibited tumor cell growth and clonogenicity. (A) Effect of DACT1α overexpression on colony formation in AGS, BGC823 and MGC803 cells. Ectopic DACT1α expression in cancer cells was

confirmed by RT-PCR and westernblot (B). (C) Cell growth curve by DACT1α overexpression in AGS cells. * $P<0.01$.

7. 3. 2 Knockdown of DACT1α promoted gastric cancer cell growth

On the other hand, ShRNA mediated knockdown of DACT1 in normal gastric epithelial GES1 cell significantly increased colony formation (Figure 7. 3. 2). Knockdown of DACT1α in the transfected cells was confirmed by RT-PCR (Figure 7. 3. 2).

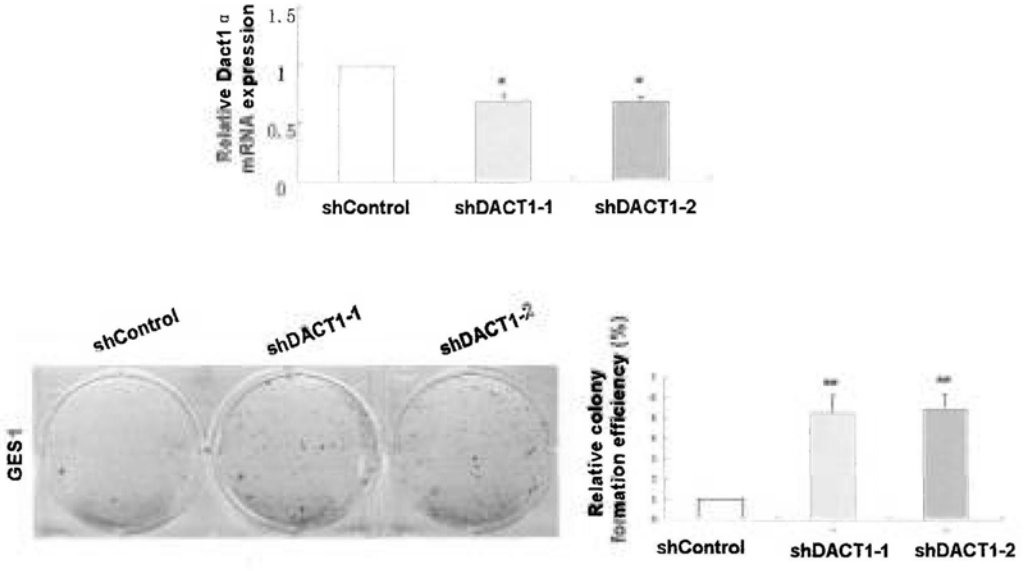

Figure 7. 3. 2. Effect of DACT1α knockdown on colony formation in GES1 cells. DACT1α expression was confirmed by real time PCR. *$P<0.05$, **$P<0.01$.

7. 3. 3 Induction of apoptosis by DACT1α in gastric cancer cells

To examine the contribution of apoptosis to the observed growth suppression by DACT1α, we performed apoptosis assay using flow cytometry with Annexin V-APC and 7-AAD double staining. In BGC823 and MGC803 cells, ectopic DACT1α expression led to a significant increase of early apoptotic cells as compared with control (BGC823: 2.73 ± 0.06% vs. 3.60 ± 0.46%, P=0.031; MGC803: 2.36 ± 0.25% vs. 3.63 ± 0.25%, P=0.004) (Figure 7. 3. 3, A). Induction of apoptosis was further confirmed by analysis the expression of apoptosis-related proteins. Ectopic expression of DACT1α could significantly enhance the cleavage of caspase-3, 7, 9 and poly(ADP-ribose) polymerase (PARP) in stably transfected AGS, BGC823 and MGC803 cells as compared with controls (Figure 7. 3. 3, B).

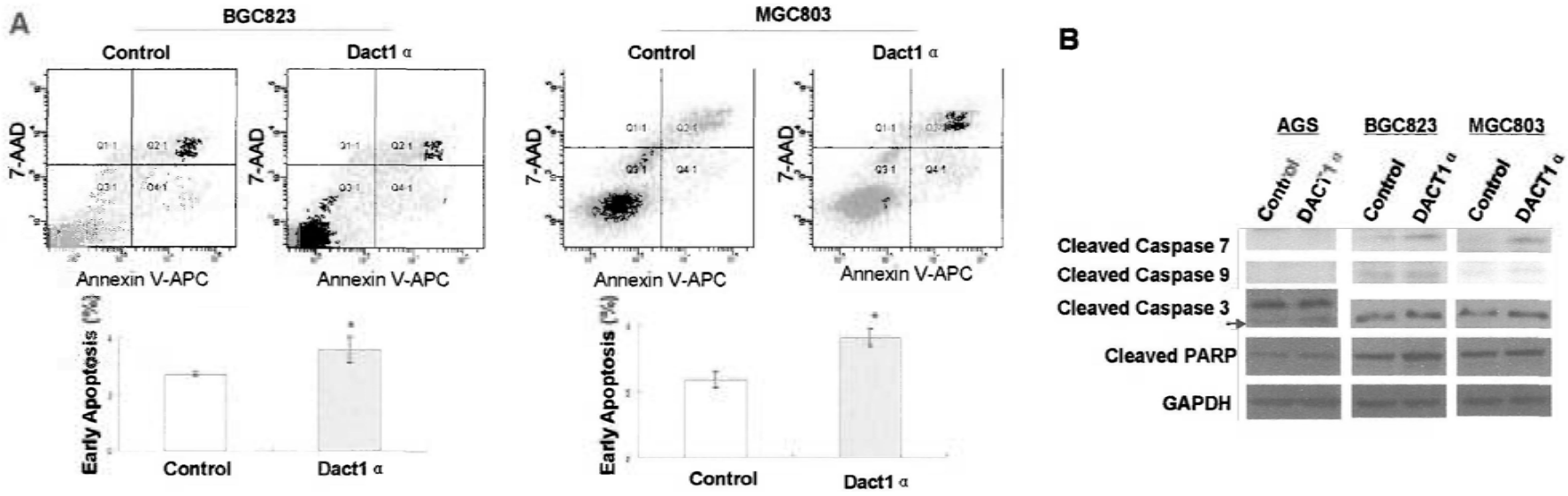

Figure 7. 3. 3. DACT1α induced apoptosis of gastric cancer cells. (A) Average of the early apoptotic cells was compared between DACT1α and control vector transfected BGC823 and MGC803 cells measured by flow cytometry analysis of Annexin V-APC and 7-AAD double-staining. Region Q1 shows the necrotic cells, Q2 shows the late apoptotic cells, Q3 shows the live cells, and Q4 shows the early apoptotic cells. *P<0.05, **P<0.01. (B) Protein expression of cleaved caspase-3, -7, -9 and PARP was evaluated by Western blot. GAPDH was used as loading control.

7. 3. 4 Inhibition of cell spreading, migration and invasion by DACT1α

We investigated whether DACT1α participates in cell spreading, migration, invasion and cytoskeletal regulation through transfection experiments with AGS, BGC823 and MGC803 cells.

7. 3. 4. 1 Inhibition of gastric cancer cell spreading

DACT1α over-expressing cells showed a remarkable reduction in cell spreading. In comparison with control cells, the extent of spreading of individual cells was substantially inhibited in stably transfectants with DACT1α in BGC823 and MGC803 cell lines (BGC823, P=0.003; MGC803, P=0.001. Figure 7. 3. 4. 1, A). DACT1α also down-regulated actin microfilament (stress fiber) formation as determined by staining with rhodamine-labeled phalloidin (Figure 7. 3. 4. 1, B).

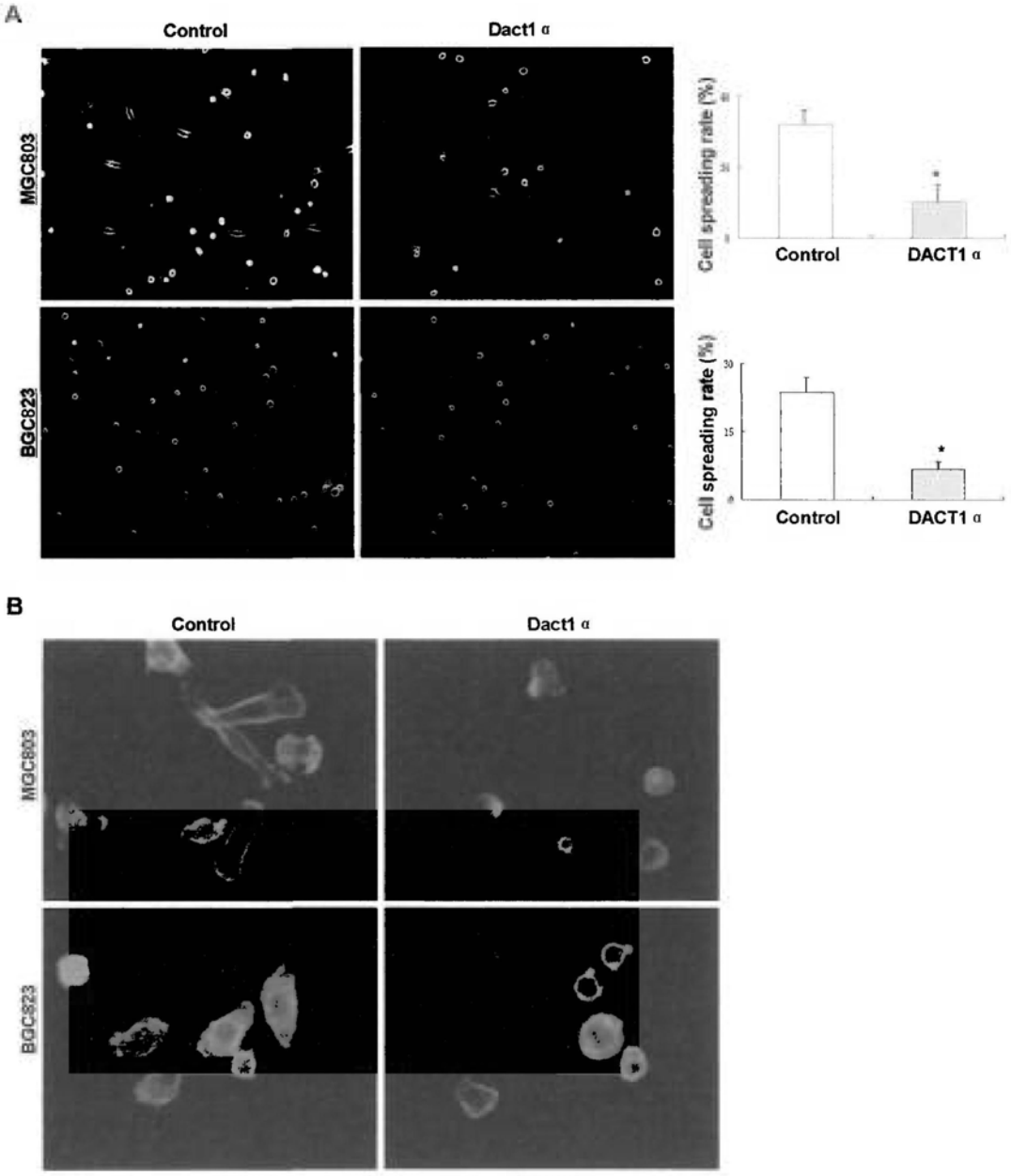

Figure 7. 3. 4. 1. DACT1α suppressed cell spreading and F-actin formation. pcDNA3.1 and pcDNA3.1-DACT1α vector transfected BGC823 and MGC803 cells were allowed to spread on cover slips for 6 hours and then photographed (A) and stained with rhodamine-phalloidin (B). Cell spreading rate of pcDNA3.1 and pcDNA3.1-DACT1α vector transfected BGC823 and MGC803 cells were compared. *P <0.01.

7. 3. 4. 2 Inhibition of gastric cancer cell migration

In vitro wound healing assay shown that a significant decrease of the cell migration ability in wound closure was observed in AGS and MGC803 cells transfected with DACT1α as compared with cells transfected with empty vector (Figure 7. 3. 4. 2).

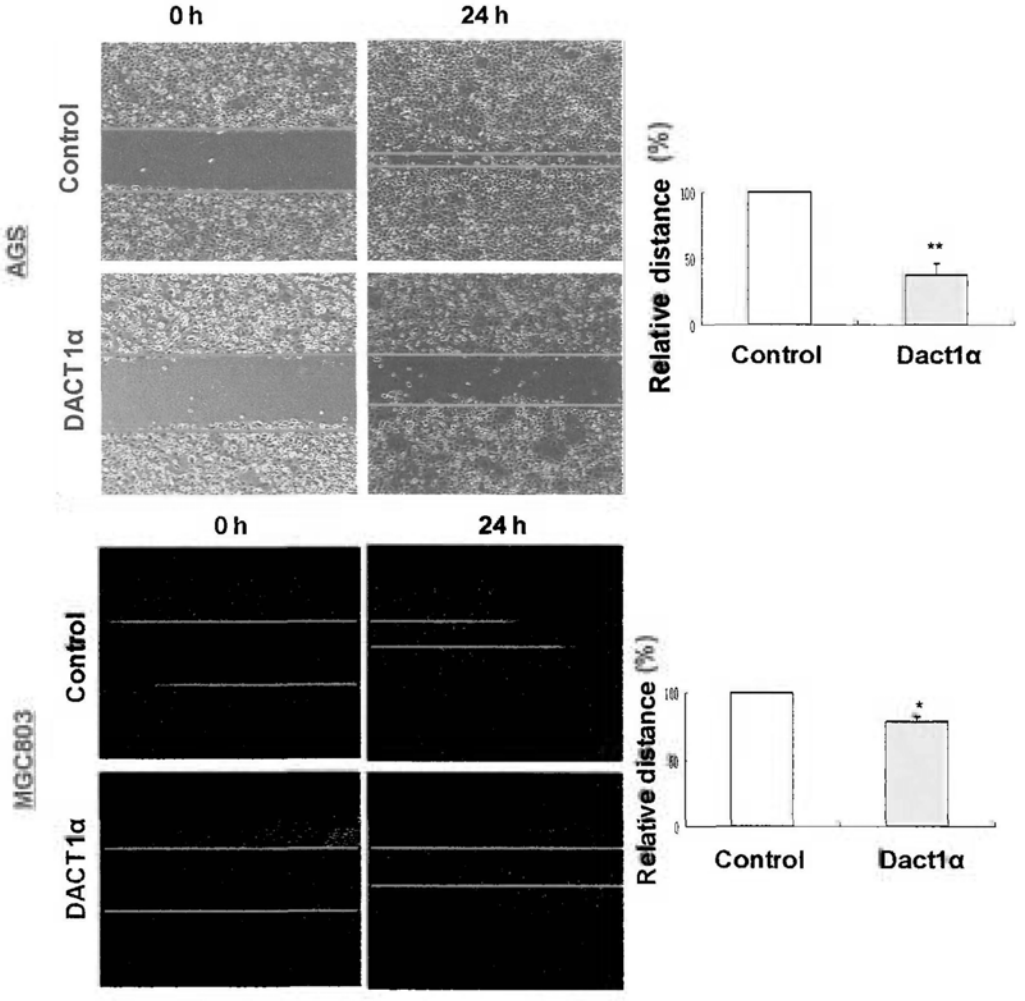

Figure 7. 3. 4. 2. DACT1α inhibited gastric cancer cell migration ability. Cell migration rates of pcDNA3.1 and pcDNA3.1-DACT1α vector transfected AGS and MGC803 cells were compared via wound healing assays. Microscopic observation was recorded at 0 and 24 hours after scratching the surface of a confluent layer of cells. *P<0.05, **P<0.01.

7. 3. 4. 3 Inhibition of gastric cancer cell invasion

DACT1α over-expression significantly dampened cell invasion ability as measured by transwell assays in AGS and MGC803 cells respectively (Figure 7. 3. 4. 3).

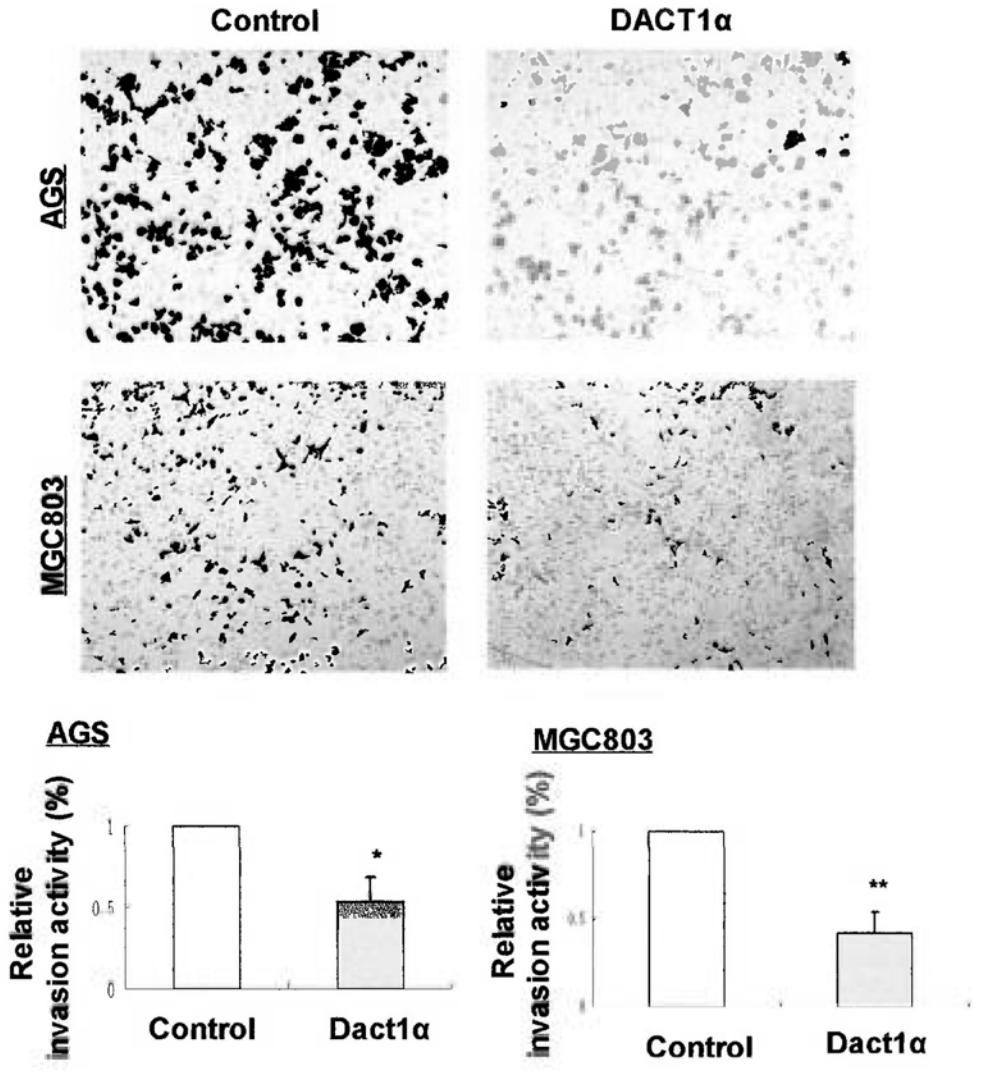

Figure 7. 3. 4. 3. DACT1α inhibited gastric cancer cell invasion ability. Invasion rates of pcDNA3.1 and pcDNA3.1-DACT1α vector transfected AGS and MGC803 cells. Number of cells that invaded through the Matrigel was counted in 10 fields under the ×20 objective lens. *P< 0.05; **P< 0.01.

7. 4 Mechanisms of tumor inhibition effect by DACT1α in gastric cancer

7. 4. 1 Localization of DACT1α in gastric cancer cells

We examined the subcellular localization of DACT1α in pIRES-ZsGreen1-DACT1α transfected gastric cancer cells. Direct DACT1 immunofluorescent staining showed that DACT1α was cytoplasmic protein (Figure 7. 4. 1).

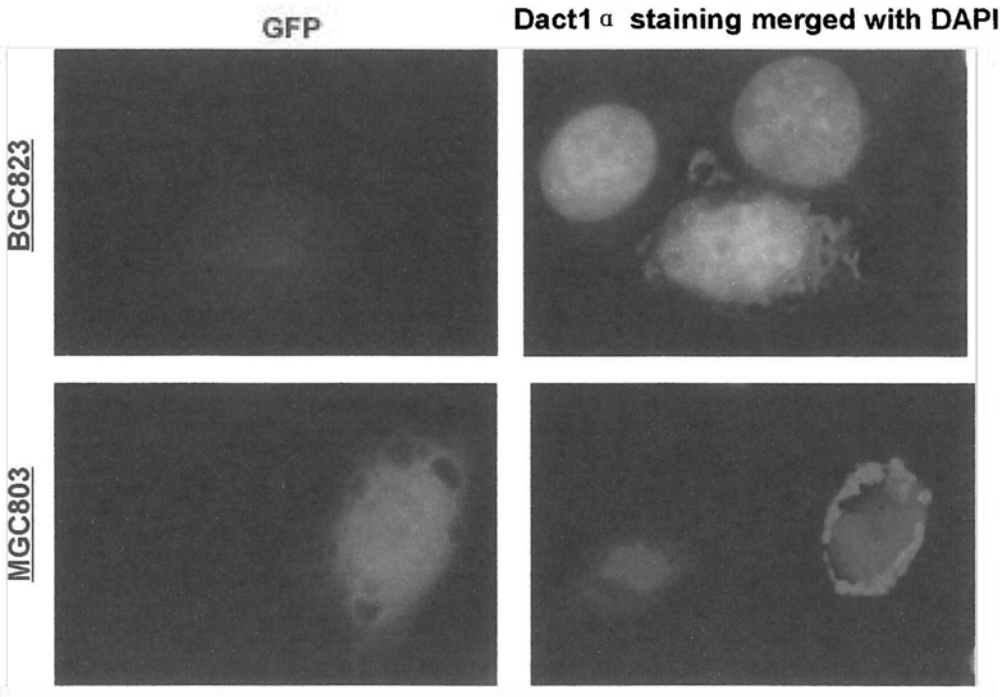

Figure 7. 4. 1. DACT1α was a cytoplasmic protein in gastric cancer cells. (A) Localization of DACT1α by direct immunofluorescent staining in pIRES-ZsGreen1-DACT1α transfected BGC823 and MGC803 cells.

7. 4. 2 Regulation of planar cell polarity (PCP) pathway by DACT1α

To examine whether DACT1α regulated PCP pathway in gastric cancer cells as

previously reported (Cheyette BN et al 2002, Zhang L et al 2006, Suriben R et al 2009, Wen J et al 2010), expression of central PCP component Dvl-2 and activation of PCP downstream JNK pathway were determined by westernblot in pcDNA3.1 and pcDNA3.1-DACT1α stably transfected AGS cells. As shown in Figure 7. 4. 2, DACT1α regulated PCP pathway by promoting Dvl-2 degradation and suppressing the active form of JNK in AGS cells.

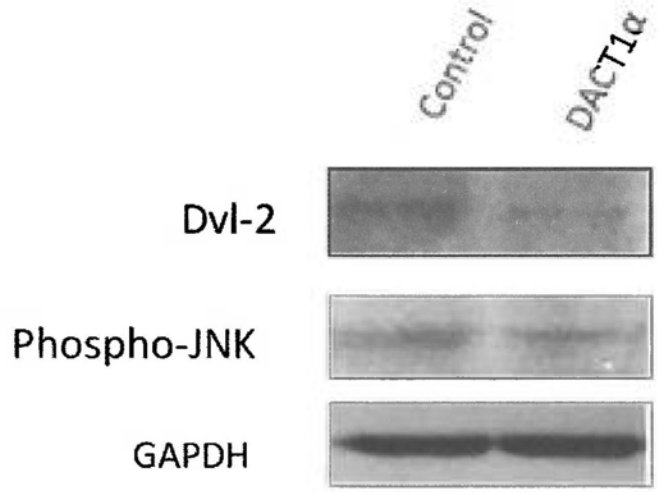

Figure 7. 4. 2. DACT1α regulated PCP pathway by promoting Dvl-2 degradation and suppressing the activation of JNK pathway in AGS cells.

7. 4. 3 Cellular signaling pathways modulated by DACT1α

To elucidate the downstream signaling pathways modulated by DACT1α in tumor inhibition, we performed promoter-luciferase activity assays using several pathway luciferase reporters including NF-κB-Luc, AP-1-Luc and TOPFlash in AGS and BGC823 cells. Ectopic expression of DACT1α significantly suppressed NF-κB reporter activities in both cell lines, while no significant activity changes in TOPFlash pathway reporters were observed (Figure 7. 4. 3, A). Moreover, DACT1α

over-expression led to the inhibition of AP-1 activity in JNK-activated AGS cells. Inhibition of NF-κB activity was further confirmed by NF-κB transcription factor binding activity assay. As shown in Figure 7. 4. 3, B, both NF-κB p50 and p65 binding activity were significantly reduced in stably DACT1α transfected BGC823 cells as compared with control cells.

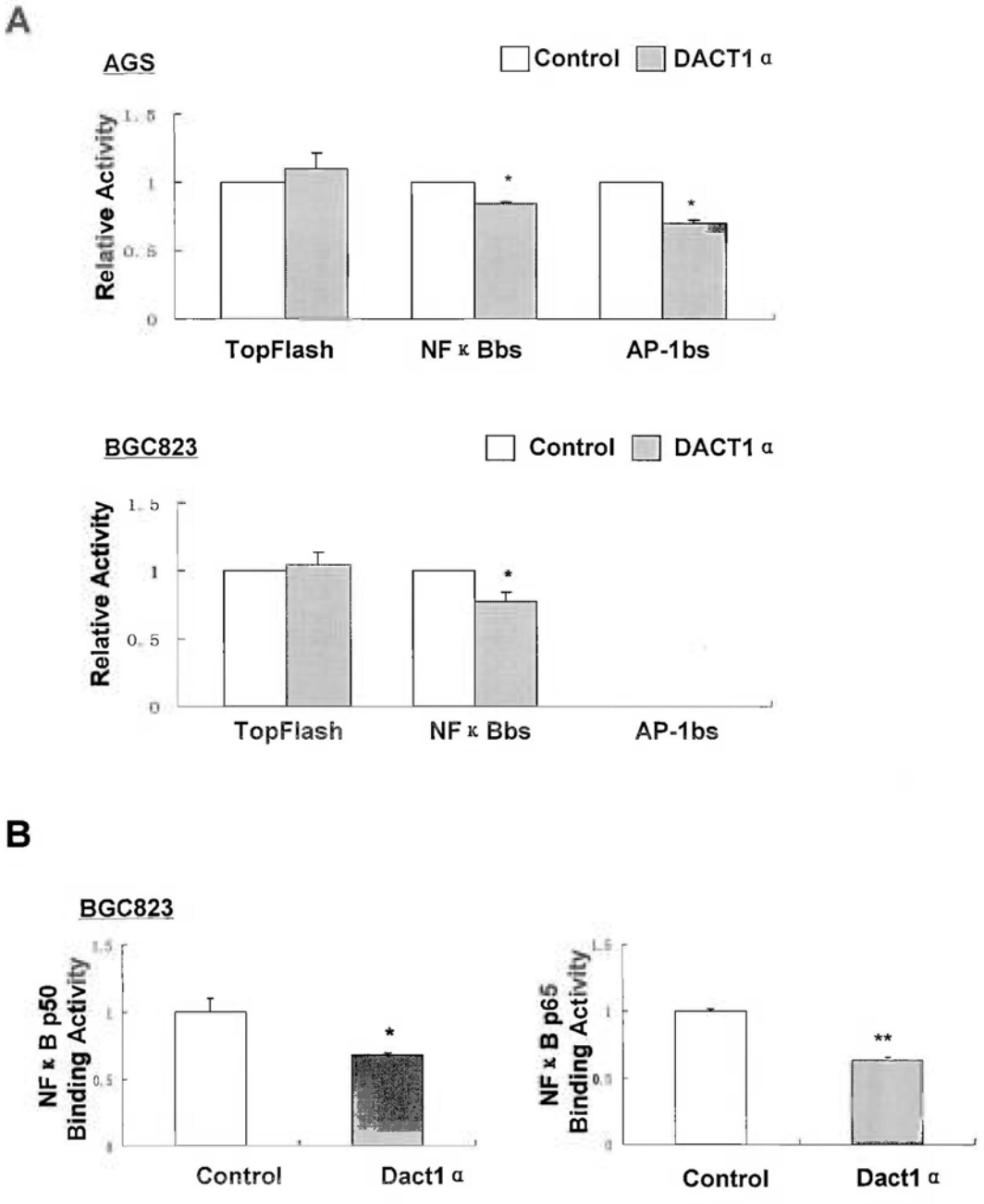

Figure 7. 4. 3. DACT1α inhibited the NF-κB and JNK/AP-1 signaling pathways in gastric cancer cells. (A) The effects of DACT1α on several signaling pathways (NF-κB, JNK/AP-1 and β-catenin/TOPFlash) in AGS and BGC823 cells were assessed by dual-luciferase reporter assays. (B) The effects of DACT1α on NF-κB p50 and p65 transcription factor binding activity in BGC823 cells. $*P< 0.05$; $**P< 0.01$.

7. 4. 4 Downstream target cancer-related genes regulated by DACT1α

To gain insight into the molecular mechanisms underlying the tumor suppression of DACT1α, gene expression profile in DACT1α stably transfected BGC823 were analyzed by Cancer Pathway cDNA microarray and further validated by real-time PCR (Figure 7. 4. 4, A). Compared to control vector transfected cells, the anti-tumorigenesis effect by DACT1α was mediated by regulating important genes in apoptosis, cell proliferation, angiogenesis, adhesion, migration and invasion (Table 7. 4. 4, Figure 6. 4. 4, B). DACT1α decreased the expression of NF-κB signaling mediator NFKB1 (p50) and its downstream factors including anti-apoptotic BCL2 and BCL-XL and oncogenic interleukin 8 (IL8) and tumor necrosis factor (TNFα). DACT1α also led to downregulation of angiogenic platelet-derived growth factor beta polypeptide (PDGFB), vascular endothelial growth factor A (VEGFA) as well as multiple cell migration and invasion molecules integrin, alpha 1 (ITGA1), integrin, alpha 2 (ITGA2), integrin, alpha 3 (ITGA3), integrin, beta 3 (ITGB3), melanoma cell adhesion molecule (MCAM), matrix metallopeptidase 9 (MMP9) and plasminogen activator, urokinase (PLAU).

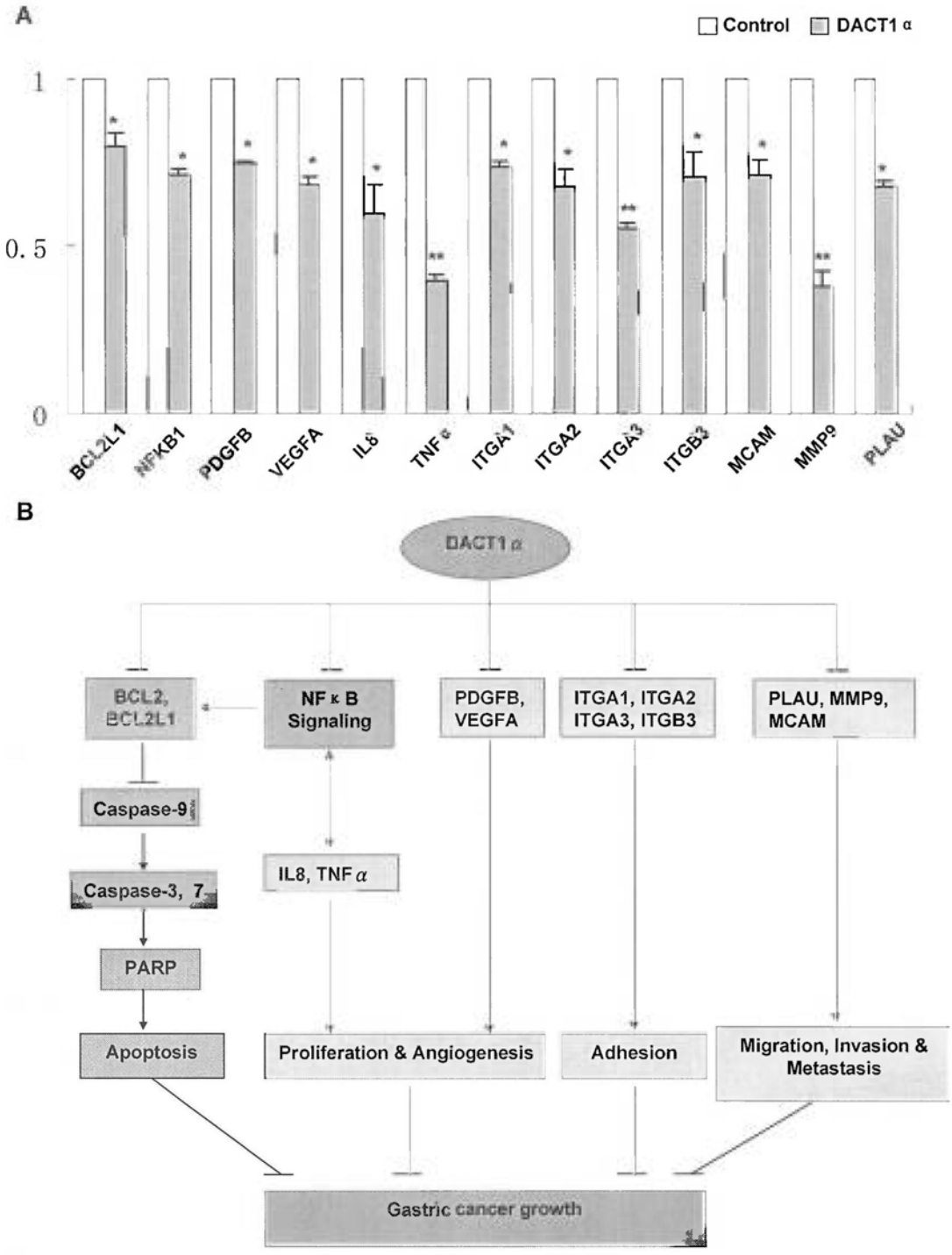

Figure 7. 4. 4. The molecular mechanisms underlying the tumor suppression of DACT1α in gastric cancer. (A) Real-time PCR validation for gene expression profile in DACT1α stably transfected BGC823 cells screened by Cancer Pathway cDNA microarray. (B) Schematic diagram for the molecular basis of DACT1α as a tumor suppressor gene in gastric cancer. Ectopic expression of DACT1α suppressed gastric cancer cell growth was associated with several biological effects: 1) DACT1α induced tumor cell apoptosis through reducing anti-apoptotic BCL2 and BCL-XL

and activating cleavage of downstream apoptosis executors caspase-7, caspase-9, caspase-3 and PARP via inhibiting NF-κB signaling. 2) Down-regulation of NFκB-dependent TNFα and IL-8 and suppression of PDGFB and VEGFA contributed to dampened cell proliferation and angiogenesis. 3) DACT1α mediated cell spreading adhesion, migration and invasion inhibition were associated with the suppression of ITGA1, ITGA2, ITGA3, ITGB3, MCAM, MMP9 and PLAU

Table 7. 4. 4. The effect of DACT1α on the gene expression profiles of cancer pathways

Genebank Accession	Gene Symbol	Gene Name	Gene Function	Fold Change (DACT1α/control)
NM_000633	BCL2	B-cell CLL/lymphoma 2	Anti-apoptosis	-1.9
NM_138578	BCL-XL	BCL2-like 1	Anti-apoptosis	-1.6
NM_003998	NFKB1 (p50)	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1	NFκB signaling	-1.4
NM_000584	IL8	Interleukin 8	Angiogenesis	-1.6
NM_000594	TNFα	Tumor necrosis factor (TNF superfamily, member 2)	Angiogenesis	-1.5
NM_002608	PDGFB	Platelet-derived growth factor beta polypeptide (simian sarcoma viral (v-sis) oncogene homolog)	Proliferation & Angiogenesis	-1.5
NM_003376	VEGFA	Vascular endothelial growth factor A	Proliferation & Angiogenesis	-1.5
NM_000212	ITGB3	Integrin, beta 3 (platelet glycoprotein IIIa, antigen CD61)	Adhesion	-1.6
NM_181501	ITGA1	Integrin, alpha 1	Adhesion	-1.5
NM_002203	ITGA2	Integrin, alpha 2 (CD49B, alpha 2 subunit of VLA-2 receptor)	Adhesion	-1.5
NM_002204	ITGA3	Integrin, alpha 3 (antigen CD49C, alpha 3 subunit of VLA-3 receptor)	Adhesion	-1.5
NM_006500	MCAM	Melanoma cell adhesion molecule	Invasion & Metastasis	-1.5
NM_004994	MMP9	Matrix metallopeptidase 9 (gelatinase B, 92kDa gelatinase, 92kDa type IV collagenase)	Invasion & Metastasis	-1.5
NM_002658	PLAU	Plasminogen activator, urokinase	Invasion & Metastasis	-1.8

7. 5 Tumor growth inhibition by DACT1α *in vivo*

We also tested whether DACT1α could suppress the growth of gastric cancer cells in nude mice *in vivo*. BGC823 cells were transduced with retrovirus pBABE-puro-DACT1α or pBABE-puro empty and selected by puromycin to generate stably DACT1α-expressing BGC823 cells or control cells. The subcutaneous tumor growth curve of stably DACT1α-expressing BGC823 cells or control cells in nude mice was shown in Figure 7. 5. 1. The tumor volume was greatly lower in DACT1α group as compared with the control group ($P<0.001$). At the end of experiments, tumors were isolated and weighted. The mean tumor weight was significantly less in DACT1α transfected nude mice as compared with the control vector mice ($P<0.01$), suggesting that DACT1α did function as a tumor suppressor in gastric carcinogenesis.

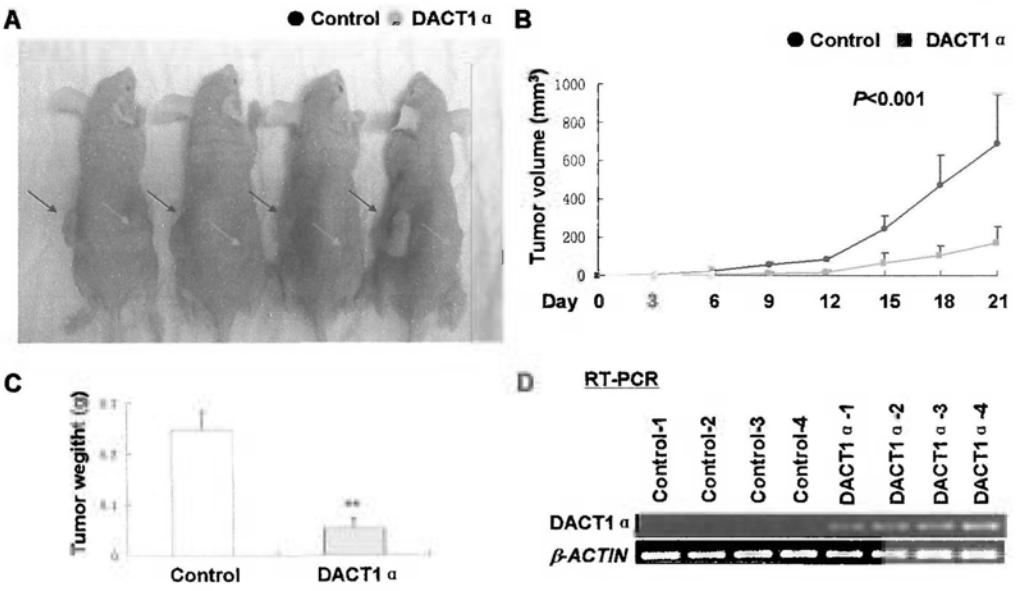

Figure 7. 5. 1. DACT1α inhibited growth of tumors derived from BGC823 cells *in vivo*. (A) A representative picture of tumor growth in nude mice subcutaneous inoculated with DACT1α-expressing cells or control cells. (B) Subcutaneous tumor growth curve of DACT1α-expressing BGC823 cells in nude mice was compared with control cells. The DACT1α group showed a retarded tumor growth compared to the control group ($P< 0.001$). (C) Histogram represents mean of the tumor weight from DACT1α and control groups. (D) Ectopic DACT1α expression from tumor tissues in nude mice was confirmed by RT-PCR. *$P<0.01$.

The cell proliferation and apoptotic index in the xenograft tumor of nude mice was evaluated using Ki-67 immunostaining and TUNEL assay respectively. Gastric cancer tumors from DACT1α group displayed significantly less proliferative cells (88.0% ± 6.4% vs. 64.0% ± 9.8%, P=0.017) and more apoptotic cells (2.04% ± 0.72% vs. 3.41% ± 0.45%, P=0.049) as compared with control group (Figure 7. 5. 2, A and B). Moreover, tumors form DACT1α group exhibited a significant decrease of NF-κB activaty as indicated by NF-κB p65 immunostaining in cell nuclei (7.75‰ ± 1.71‰ vs. 2.50‰ ± 1.50‰, P=0.008) (Figure 7. 5. 2, C), as well as the gene expression of NF-κB downstream effectors TNFα and IL-8 ($P<0.05$, Figure 7. 5. 2, D).

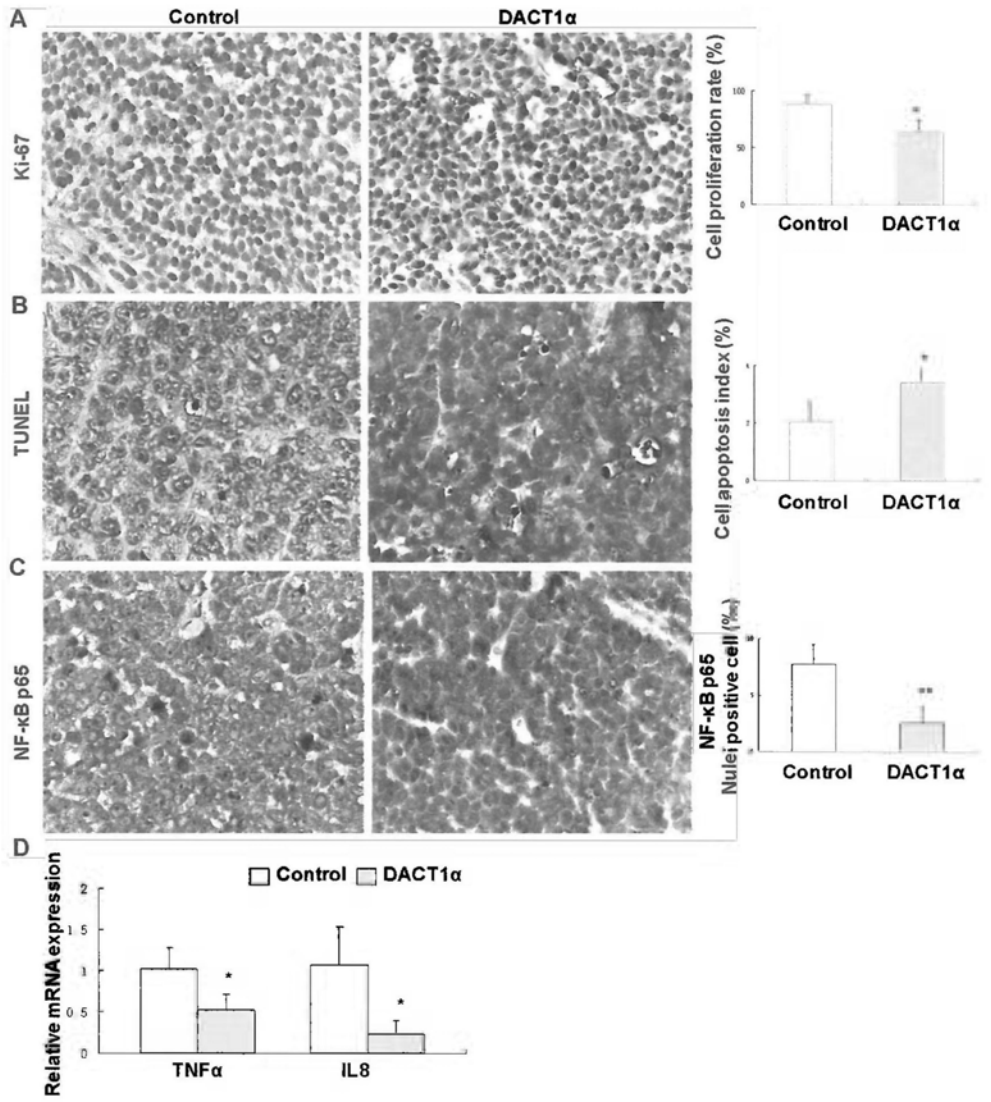

Figure 7. 5. 2. Antitumor effect of DACT1α *in vivo* was attributable to suppression of cell proliferation, induction of apoptosis and inhibiting NF-κB signaling. Representative Ki-67 nuclei staining (A), TUNEL staining (B) and NF-κB p65 nuclei staining (C) of xenografed tumor derived from DACT1α-expressing BGC823 cells or control BGC823 cells. A decrease in the number of proliferative cells (Ki-67 positive nuclei-stained cell) and cells with active NF-κB signaling (p65 positive nuclei-stained cell) and an increase in the number of apoptotic cells (TUNEL positive nuclei-stained cell) were evident in tumors from DACT1α group. Original magnification, ×200, Ki-67 staining; ×400, TUNEL and p65 staining. Quantitative results were shown in the right. (D) The expression of NF-κB downstream effectors TNFα and IL-8 were significantly reduced in tumor tissues from DACT1α group as compared with the control group by real-time PCR. *P<0.05, **P<0.01.

7. 6 DACT1 promoter methylation in primary gastric cancer patients.

7. 6. 1 Association of DACT1 methylation with clinical features of gastric cancer patients.

DACT1 methylation was evaluated in 20 normal gastric biopsies and 205 primary gastric cancer tissues by direct bisulfite genomic sequencing (Figure 7. 6. 1). None was detected to have DACT1 methylation in 20 healthy gastric tissue samples (Figure 7. 6. 1). Among 205 gastric cancer cases, DACT1 promoter partial and dense methylation was observed in 60 (29.3%) cases. DACT1 methylation was significantly associated with advanced tumor size grade (P=0.013) and lymph node metastasis (P=0.015) and distant metastasis (P=0.05) respectively (Table 7. 6. 1). Moreover, DACT1 methylation was observed more frequently in stage III/IV cases (47 of 53 (88.7%) cases) than in stage I/II cases (6 of 53 (11.3%) cases) (P< 0.0005, Table 7. 6. 1).

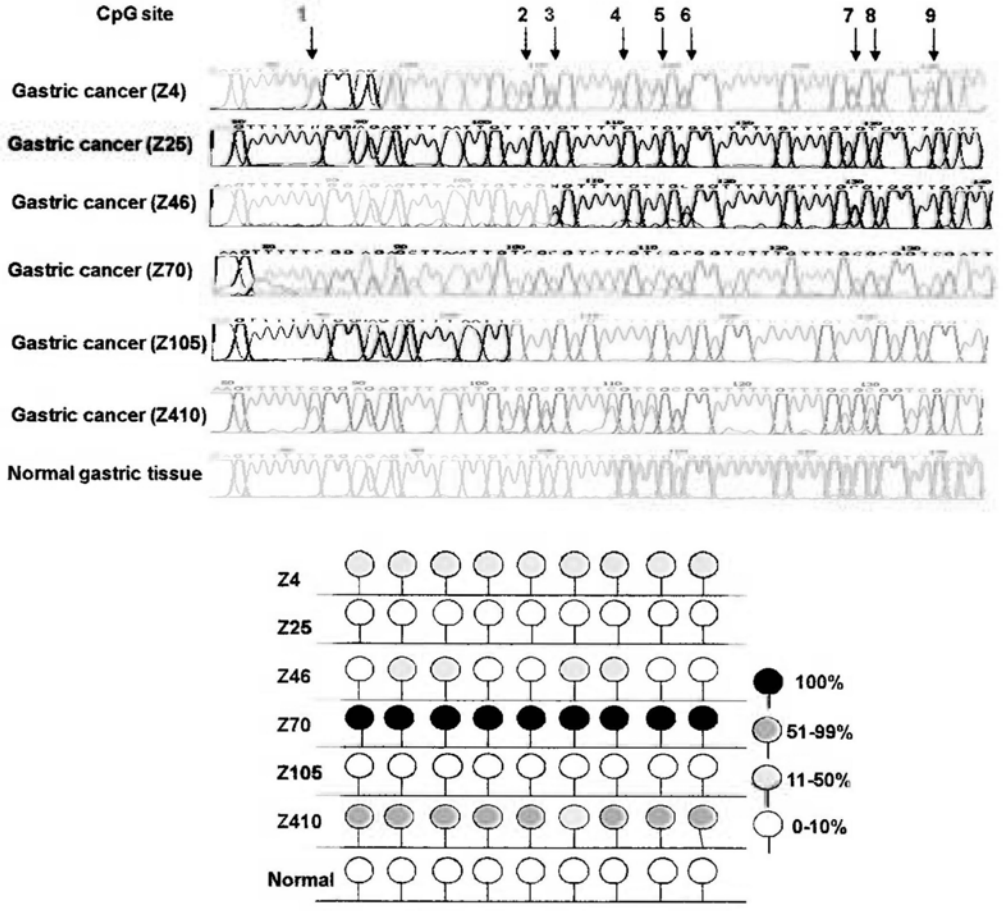

Figure 7. 6. 1. DACT1 was frequently methylated in primary gastric cancers. Representative images of DACT1 methylation status in primary gastric cancers and adjacent non-tumor tissues by direct BGS.

Table 7. 6. 1. Clinicopathologic features of patients with gastric cancer according to the methylation status of the DACT1 promoter.

Variable	Non- or slightly methylated (*n* =145)	%	Partially- or densely methylated (*n* =60)	%	*P* value
Age					
Mean ± SD	58 ± 11.5		55 ± 13.9		0.07
Gender					
M	102	70.3	37	61.7	0.23
F	43	29.7	23	38.3	
***H. pylori* infection**					
Negative	66	66.7	20	64.5	0.83
Positive	33	33.3	11	35.5	
Lauren					
Diffuse or Mixed	25	17.4	8	13.6	0.51
Intestinal	119	82.6	51	86.4	
Location					
Cardiac	30	20.7	11	18.3	0.70
Non-cardiac	115	79.3	49	81.7	
Differentiation					
Poor differentiation	78	64.5	40	76.9	0.11
Well or moderate	43	35.5	12	23.1	
Lymph node status					
Negative	37	31.1	5	11.9	0.015*
Positive	82	68.9	37	88.1	
Tumor Size					
T1/T2	34	29.6	4	10.0	0.013*
T3/T4	81	70.4	36	90.0	
Distant Metastasis					

Negative	95	77.9	27	62.8	0.05*
Positive	27	22.1	16	37.2	
TNM stage					
I-II	51	38.1	6	11.3	<0.0005*
III-IV	83	61.9	47	88.7	

7. 6. 2 Association of DACT1 methylation with the survival of gastric cancer patients.

Overall survival of the gastric cancer patients was analyzed for dependence on DACT1 promoter methylation using Kaplan-Meier survival curves. The overall survival of patients with DACT1 methylation was significantly shorter than that of other gastric cancer patients (P=0.007, Figure 7. 6. 2). Using univariate Cox regression analysis, DACT1 methylation was associated with a significantly increased risk of cancer-related death (P=0.007, Table 7. 6. 2. 1). In the multivariate Cox regression analysis, TNM stage were significantly associated with the survival of gastric cancer patients (Table 7. 6. 2. 2). However, DACT1 methylation were not associated with the outcome in gastric cancer patients (Table 7. 6. 2. 2).

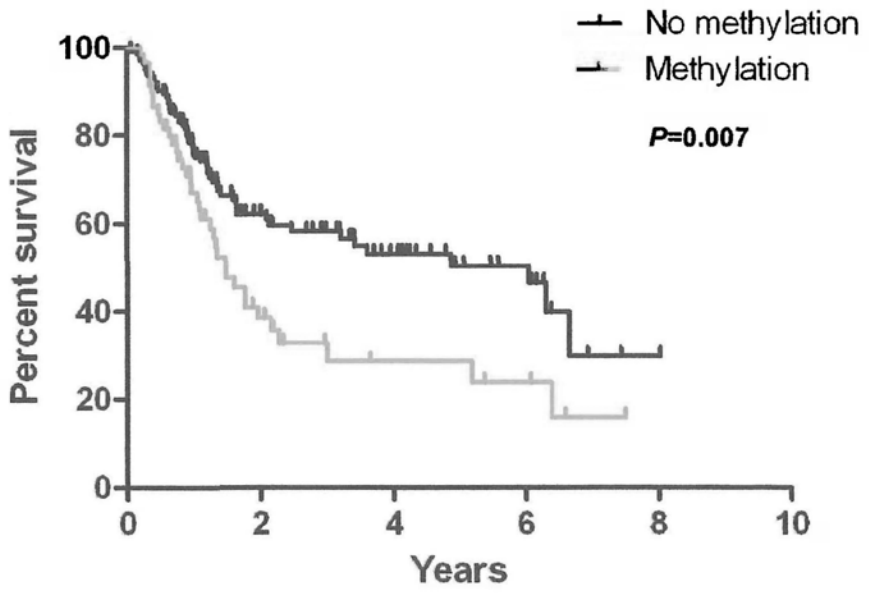

Figure 7. 6. 2. Kaplan-Meier survival curves show that gastric cancer patients with DACT1 methylation had poorer survival than those without DACT1 methylation. This difference is statistically significant based on log-rank test (*P*=0.007).

Table 7. 6. 2. 1. Univariate Cox regression analysis of potential prognostic factors for gastric cancer patients

Variable	HR (95% CI)	*P* value
Age	1.01 (0.99 - 1.03)	0.50
Gender		
Male	1.07 (0.70 - 1.64)	0.77
Female	1.00	
***H. pylori* infection**		
Negative	1.46 (0.80 - 2.68)	0.22
Positive	1.00	
Lauren		
Intestinal	1.25 (0.66-2.52)	0.53
Diffuse	1.83 (0.72 – 4.66)	0.20
Mixed	1.00	
TNM stage		
I	0.08 (0.02 - 0.24)	<0.0005
II	0.14 (0.06 - 0.32)	<0.0005
III	0.27 (0.16 - 0.44)	<0.0005
IV	1.00	
***DACT1* methylation**		
Partially- or densely methylated	1.76 (1.03 – 2.72)	0.007
Non- or slightly methylated	1.00	

Table 7. 6. 2. 2. Multivariate Cox regression analysis of potential prognostic factors for gastric cancer patients

Variable	HR (95% CI)	*P* value
Age	1.01 (0.99-1.03)	0.33
Gender		
Male	1.39 (0.87 – 2.24)	0.073
Female	1.00	
TNM stage		
I	0.07 (0.02 - 0.21)	<0.0005
II	0.12 (0.05 - 0.29)	<0.0005
III	0.24 (0.14 – 0.40)	<0.0005
IV	1.00	
***DACT1* methylation**		
Partially- or densely methylated	1.13 (0.70 – 1.82)	0.63
Non- or slightly methylated	1.00	

Chapter 8 DISCUSSION

8. 1. Silence or down-regulation of DACT1 mediated mainly by promoter methylation in gastric cancer

In this study, we found that the mRNA expression of DACT1 was silenced or reduced in 70% (7 of 10) gastric cancer cell lines. The down-regulation of DACT1 expression was also seen in primary gastric cancer tissues by immunostaining. This down-regulation was mostly attributed to promoter methylation as demonstrated by MSP, which was further confirmed by bisulfite sequencing. Restored DACT1 exppression was achieved by demethylation treatment. These results suggested that promoter methylation was a major mechanism for the transcriptional silencing of DACT1 in gastric cancer. In addition, there were also unmethylated alleles in 20% (2 of 10) gastric cancer cell lines with no DACT1 expression (MKN28 and BGC823 cell lines), Genomic copy number loss in two locus of DACT1 gene was observed in MKN28 cell line by Array-CGH, implying that apart from promoter methylation, other genetic alteration such as breakage of a chromosome, a non-reciprocal translocation event or loss of heterozygosity, might play a role in the down-regulation of DACT1 in gastric cancer, It had been reported that loss of heterozygosity or genomic copy number loss at DACT1 locus was found in liver cancer, gastrointestinal stromal tumor, oligodendroglial tumor and head and neck squamous cell carcinomas (Yau TO et al 2005, Felsberg J et al 2006, Pehlivan D et al

2008, Astolfi A et al 2010),

8. 2 The biological function of DACT1α as a tumor suppressor in gastric cancer

Since evident down-regulation of DACT1 expression was observed in gastric cancer cell lines and primary tissues, whereas it was robustly expressed in normal gastric tissues. Thus, DACT1α may function as a potential tumor suppressor and its downregulation could have some role in the development of gastric cancer. We therefore tested the putative tumor suppressor function of DACT1α in gastric cancer cell lines both *in vitro* and *in vivo*. Ectopic expression of DACT1α in silenced gastric cancer cell lines (AGS, BGC823 and MGC803) dramatically suppressed their growth and clonogenicity by inhibiting cell proliferation and inducing apoptosis. The diminution of tumor growth in DACT1α-reexpressed cells was further confirmed in the tumorigenesity in nude mice. The observation of decreased cell proliferation by Ki-67 nuclei immunostaining and increased apoptosis by TUNEL staining induced by DACT1α *in vivo* was entirely consistent with the *in vitro* effects, adding further weight to the potential significance of these findings. On the other hand, shRNA mediated knockdown of DACT1 in normal gastric epithelial GES1 cell significantly increased clonogenicity.

Moreover, DACT1α led to a significant decrease of cell spreading ability in BGC823 and MGC803 cells. At the beginning of cell spreading, most of

DACT1α-reexpressed cells maintained a spherical shape whereas a large population of control cells had flatten out and spread onto the matrix. Cell spreading is the first step for cancer cell migrating and invading neighboring tissue. In order for cells to move and invade, they must adhere, flatten out and spread onto the extracellular matrix with extended protrusions. In keeping with reduced cell spreading ability, DACT1α significantly dampened cell migration and invasion as demonstrated by wound healing assay and trans-well invasion assay.

Collectively, these results indicated for the first time that DACT1α functions as a tumor suppressor in gastric cancer.

8. 3 Molecular mechanism of DACT1α as a tumor suppressor gene

8. 3. 1 Induction of apoptosis by DACT1α was mediated by intrinsic caspase-dependent pathway

Apoptosis is frequently dysregulated in human cancers. There are two biochemically distinct apoptotic pathways, namely, mitochondria (intrinsic)- and death receptor (extrinsic)-mediated apoptotic cascades. The underling mechanism(s) for inhibition of cell apoptosis by DACT1α was investigated in this study. We demonstrated that the induction of apoptosis by DACT1α was mediated through caspase-dependent pathway including activation of caspase-9, followed by cleavage

of downstream caspase effectors caspase-3 and caspase-7, ultimately stimulating the activation of PARP and cellular disassembly and apoptosis. By Cancer Pathway cDNA microarray, we found that DACT1α mediated apoptosis occurred through down-regulation of anti-apoptotic genes BCL2 and BCL-XL, both of which prevent mitochondrial apoptosis in response to a wide range of apoptotic stimuli through inhibition of mitochondrial cytochrome c release or apoptotic proteins.

8. 3. 2 Molecular basis of the inhibition of proliferation by DACT1α

We found that the anti-proliferative effect derived by DACT1α was at least owing to the down-regulation of oncogenic cytokines TNF-α, IL-8, PDGFB and VEGFA. As a tumor-promoting factor, TNFα induced a range of inflammatory mediators, cytokines, chemokines and growth factors through JNK and NFκB pathway activation, which resulted in enhancing malignant cell survival, remodeling of the extracellular matrix, leukocyte infiltration and angiogenesis (Balkwill F et al 2009). IL-8, PDGFB and VEGFA were also potent chemoattractant and angiogenic factors and mediated tumor growth, angiogenesis and metastasis (Waugh DJ et al 2008, Huang S et al 2000, Huang S et al 2001, Karashima T et al 2003, Sparmann A et al 2004, Roskoski R Jr et al 2007, Govindarajan B et al 2005). Therefore, down-regulation of TNFα, IL-8, PDGFB and VEGFA may explain the anti-proliferative effect exerted by DACT1α in gastric cancer cells.

8. 3. 3 Cellular signaling pathways affected by DACT1α

To elucidate the downstream signaling pathways modulated by DACT1α in tumor inhibition, we performed promoter-luciferase activity assays using three pathway luciferase reporters including NF-κB-Luc, AP-1-Luc and TOPFlash in AGS and BGC823 cells. Our data showed that DACT1α inhibited NF-κB activity in both cell lines by luciferase reporter activity assay, which was further confirmed by NF-κB transcription factor binding activity assay and NF-κB p65 immunostaining in nude mice. NFκB signaling pathway, a key link between inflammation and cancer, plays a critical role in cancer development and progression by regulating the transcription of genes involved in cell proliferation, angiogenesis, metastasis and suppression of apoptosis (Baud V et al 2009, Karin M et al 2006). Genetic inactivation of NFκB decreases tumor multiplicity or size in inflammation-driven cancer mice models by downregulating anti-apoptotic genes expression and dampened production of growth-stimulating cytokines (Greten FR et al 2004, Pikarsky E et al 2004). In keeping with this, ectopic expression of DACT1α inhibited the mRNA expressions of four NF-κB downstream factors: anti-apoptotic genes BCL2 and BCL-XL and oncogenic cytokines TNF-α and IL-8.

BCL2 and BCL-XL are two critical anti-apoptotic genes induced by NF-κB activation in cancer development (Karin M et al 2006). The promoter of BCL2 and BCL-XL could be directly activated by NFκB, contributing to decreased apoptosis

and enhanced cell survival (Khoshnan A et al 2000, Lee HH et al 1999, Tamatani M et al 1999, Grossmann M et al 2000). In the mouse model of colitis-associated cancer, NFκB inactivation in epithelial cells reduced tumor incidence by increasing apoptosis with remarkable induction of BCL2 and BCL-XL (Greten FR et al 2004). Thus, DACT1α induced apoptosis was mediated by NFκB-dependent up-regulation of BCL2 and BCL-XL.

TNFα and IL-8 were key mediators in NFκB-associated cancer progression and angiogenesis (Baud V et al 2009, Karin M et al 2006, Pikarsky E et al 2004, Popivanova BK et al 2008, Popivanova BK et al 2008, Waugh DJ et al 2008, Huang S et al 2000, Huang S et al 2001, Karashima T et al 2003, Sparmann A et al 2004). Greten FR *et al* revealed crucial involvement of NFκB activation in the colitis-associated colorectal cancer mice model (Greten FR et al 2004) whereas TNFR1-deficient mice showed reduced infiltration of macrophages and neutrophils and attenuated tumor formation compared with wild type mice in this model (Popivanova BK et al 2008). Interference with TNFα or genetic blockade of NFκB inhibited tumor cell invasiveness (Hagemann T et al 2005) and dampened tumor growth with increased apoptosis of transformed hepatocytes in Mdr2-knockout mice liver cancer model, suggesting that TNFα facilitated NFκB-dependent anti-apoptosis, inflammation, invasion and cell growth (Pikarsky E et al 2004). Ras induced IL-8 secretion through NFκB signaling, contributing to neoplastic growth and tumor vascularizton (Sparmann A et al 2004). Furthermore, both TNFα and IL-8 served as

not only NFκB downstream responsive genes but also potent inducer of NFκB signaling activation, thus augmenting NFκB signaling in an autocrine manner. Collectively, through modulating NF-κB signaling pathway and its downstream target genes, DACT1α could functions as a tumor suppressor gene by promoting apoptosis and growth inhibition.

8. 3. 4 Molecular mechanisms of DACT1α inhibited gastric cancer cell spreading, migration and invasion

The reduced cell motility and spreading effect caused by DACT1α in gastric cancer cells was revealed to be at least associated with altered PCP signaling pathwy and JNK activity. DACT1α promoted central PCP component Dvl-2 degradation and suppressed PCP downstream pathway JNK activity in AGS cells. Consistently, DACT1 knockout mice showed disregulated PCP signaling pathways by post-translationally antaganizing Dvl2 and Vangl2 (Cheyette BN et al 2002, Zhang L et al 2006, Suriben R et al 2009, Wen J et al 2010). JNK pathway and RhoA pathway are two main PCP pathways affected by DACT1, both of which controlled cytoskeleton reorganization in cell movements and polarity (Suriben R et al 2009, Wen J et al 2010). Less active RhoA but more active JNK was found in mouse embryonic fibroblast derived from DACT1 knockout mice (Suriben R et al 2009, Wen J et al 2010), Notably, it has been well accepted that RhoA normally allowed the cell to maintain a spherical shape in the process of spreading whereas RhoA-depleted

cells showed enhanced elongated and extended protrusions, permitting cells to flatten out, spread and invade much faster (Arthur WT et al 2001, Gong H et al 2010, Vega FM et al 2011). Therefore, DACT1α over-expression modulated PCP pathways, contributing to decreased cell spreading activity, F-actin assembly, cell migration and invasion abilities.

Cancer Pathway cDNA microarray analysis revealed that DACT1α down-regulated cell adhesion receptors from the integrin family including ITGA1, ITGA2, ITGA3 and ITGB3. Integrins spanned the cell membrane and controlled adhesion to the extracellular matrix. Each integrin consisted of two subunits: α and β. One of the most well-studied integrins in cancer was oncogenic integrin $\alpha_v\beta_3$, of which ITGB3 (also known as β3 integrin) was a key component (Desgrosellier JS et al 2010). Integrin $\alpha_v\beta_3$ was usually expressed at low or undetectable levels in most adult epithelia, but can be highly upregulated on the most aggressive tumor cells in a variety of cancer (Desgrosellier JS et al 2010). In melanoma, the expression of ITGB3 was restricted exclusively to cells within vertical growth phase and metastatic melanomas (Albelda SM et al 1990) and was correlated with shorter survival (Hieken TJ et al 1999). Several integrin $\alpha_v\beta_3$ antagonists have been proved to be efficient in reducing tumor growth and metastasis in many mouse models and clinical studies (Desgrosellier JS et al 2010). In a mouse model of spontaneous tumorigenesis caused by WNT1 overexpression, the luminal epithelial progenitor marker ITGB3 identified a highly tumorigenic cancer stem cell population (Vaillant

F et al 2008). By recruiting c-Src to ITGB3 cytoplasmic tail, integrin $\alpha_v\beta_3$ activated c-Src, substantially increased anchorage-independent tumor cell survival *in vitro* and metastasis *in vivo* (Desgrosellier JS et al 2009). In addition, DACT1α decreased the mRNA expression of ITGA1 and ITGA2, which were components of collagen receptor integrin $\alpha_1\beta_1$ and $\alpha_2\beta_1$. Integrin $\alpha_1\beta_1$ and $\alpha_2\beta_1$ were found to be important regulators in cell invasion in liver and colon cancer and melanoma (Yang C et al 2003, Staniszewska I et al 2009, Van Slambrouck S et al 2007). Taken together, those down-regulated integrin molecules by DACT1α may at least offer partial explanation for impaired cell adhesion spreading ability, migration and invasion.

Other cell migration and invasion molecules regulated by DACT1α included PLAU, MCAM and MMP9. PLAU played important functions in cell migration, invasion and survival (Harvey et al 2010). Elevated PLAU expression was found in many human cancers including gastric cancer, breast cancer and colorectal cancer and correlated with the clinical features of biologically aggressive cancer and poor outcome of cancer patients (Nekarda H et al 1994, Kaneko T et al 2003, Look MP et al 2002, Van den Eynden GG et al 2009). By binding urokinase-type plasminogen activator receptor (uPAR), PLAU promoted extracellular matrix (ECM) proteolysis and generated the protease plasmin which cleaved a range of ECM components and activated growth factors and matrix metalloproteases such as MMP9 (Harvey et al 2010). Moreover, activated uPAR signals interacted with ITBG3 and triggered intracellular Rac signaling pathway for cell migration, leading to increased F-actin

assembly and membrane protrusion (Harvey et al 2010). Interestingly, both PLAU and MMP9 played an important role in mediating NFκB-induced tumor promotion and metastasis (Baud V et al 2009, Karin M et al 2006, Chan CF et al 2004, Huang S et al 2001). Osteopontin stimulated cell motility and growth through NFκB-dependent PLAU secretion (Das R et al 2003). NFκB activated PLAU and MMP9 expression directly through binding NFκB-responsive element in their promoters (Mahabeleshwar GH et al 2003, Rhee JW et al 2006). Thus, down-regulation of PLAU and MMP9 expression by DACT1α contributed to dampened cell adhesion spreading and invasion ability probably via decreased NFκB activity.

8. 4 Clinical significance of DACT1 methylation in primary gastric cancer

To assess the clinical application of DACT1 in gastric cancer, we examined the promoter methylation of DACT1 by direct BGS in 205 primary gastric cancer patients and 20 normal controls. We found that promoter methylation of DACT1 was detected in 29.3% (60/205) of gastric cancers, but not in normal controls. This result indicated that methylation mediated silencing of DACT1 is a frequent event in gastric cancer. Moreover, in keeping with the dampened migration and invasive ability of gastric cancer cells by DACT1 *in vitro*, DACT1 methylation was associated with lymph node metastasis and distant metastasis and was more frequently in advanced stage of gastric cancers. This referred that epigenetic

inactivation of DACT1 more likely occur in late stages of gastric cancer and contribute to tumor progression and metastasis during gastric cancer development.

In regard to the associations with patient outcome, DACT1 methylation was significantly correlated with patient survival by Kaplan–Meier curves and univariate Cox regression analysis. However, multivariate Cox regression analysis revealed that after adjustment for TNM stage, age and gender, gastric cancer patients with DACT1 methylation showed no poorer survival. This is probably because DACT1 methylation mainly occurs in advanced tumor stage (III/IV) whereas cancer patients showed poorer survival with the increase of TNM staging.

Chapter 9 CONCLUSION AND FUTURE WORKS

We have identified a novel functional tumor suppressor gene DACT1 mainly inactivated by promoter methylation in gastric cancer. Besides, other genetic alteration such as loss of copy number of DACT1 may also mediate transcriptional repression of DACT1 in gastric cancer.

DACT1α contributes to the suppression of tumorigenesis by promoting cell apoptosis, decreasing cell proliferation and dampening tumor cell motility and invasion through inhibiting NF-κB signaling pathway and modulating PCP pathway.

DACT1 methylation is associated with lymph node metastasis, distant metastasis and advanced stage of primary gastric cancers. DACT1 methylation is associated with poorer survival of gastric cancer patients.

Future works on DACT1:

1) The molecular mechanism how DACT1α inhibited cell spreading through modulating PCP pathways needs to be investigated.

2) The molecular mechanism how DACT1α inhibited NF-κB signaling pathway in gastric cancer needs to be explored.

3) Genomic DNA copy number loss at DACT1 locus should be examined in primary gastric cancer samples.

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