TXNIP, a Putative Tumor Suppressor Gene Regulated by Histone Acetylation in Gastric Carcinoma

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Abstract of the entitled:

***TXNIP*, a Putative Tumor Suppressor Gene Regulated by Histone Acetylation in Gastric Carcinoma**

Submitted by TANG Angie

for the degree of Doctor of Philosophy in Anatomical and Cellular Pathology

at The Chinese University of Hong Kong (May 2010)

Acknowledgements

I think the exact wording for "PhD study" should be "Permanent Head Damage Study" instead of the present term as "Philosophy of Doctoral study". I am uncertain if other PhD students have the same feeling and experiences as mine. After this PhD study, I found that I suffered from mildly "Alzheimer disease".

I would like to take this opportunity to express my sincere gratitude to a numbers of peoples who assisted and contributed to my Doctor of Philosophy project. Without their help, the project cannot be completed satisfactorily. I owe many thanks to my chief supervisor Prof. Ka Fai To, Professor of Anatomical and Cellular Pathology, The Chinese University of Hong Kong. He introduced me into the interesting cancer research field at the year of 2003. Since then, he has been supervising my study from MPhil to PhD. In the past 6 years, he gave me numerous of opportunities to take part in different parts of research, from molecular biology to histology. He also encouraged and supported me financially to participate in various international conferences including American Association Cancer Research which allowed me to explore my vision and broaden my horizons. Furthermore, he always allowed me to perform the research in my own way and had faith in me even though when I failed the experiments. His understanding during the preparation period of this thesis should also be highlighted.

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Abstract

Gastric cancer is a common cancer especially in Asian countries and is associated with high morbidity and mortality. Epigenetic inactivation of tumor suppressor is a common mechanism involved in carcinogenesis of a variety of human cancers and recent evidence suggested that targeting epigenetic modifications may be an approach to combat cancer. Our group and others have demonstrated frequent promoter methylation of cancer related genes in gastric cancer. In this study, we aim to identify cancer associated genes regulated by another important epigenetic mechanism, namely histone acetylation.

We first showed that suberoylanilide hydroxamic acid (SAHA), a well known histone deacetylase inhibitor, has anti-proliferative effect in a panel of gastric cancer cell lines (MKN1, MKN7, MKN28, MKN45, SNU1, SNU16, AGS, N87 and KatoIII cells). We compared gene expression profiles of SAHA treated vs control AGS cells to identify a set of genes that were differentially upregulated by SAHA treatment. Based on our microarray analysis in nine gastric cancer cell lines (MKN1, MKN7, MKN28, MKN45, SNU1, SNU16, AGS, N87 and KatoIII) and normal gastric tissues, a set of commonly downregulated genes in gastric cancer cells was elucidated. Analysis of these data sets with subsequent confirmation using real-time PCR analysis, genes that were downregulated in gastric cancer cells but upregulated upon SAHA treatment were identified. Among these selected genes, Thioredoxin Interacting Protein (also known as *VDUP-1/TBP2/TXNIP*) was down-regulated in all cancer cell lines tested, and its protein expression was significantly induced by SAHA treatment in a numbers of gastric cancer cell lines

including AGS, MKN1, MKN45, N87 and KatoIII. Thus, we focused on the *TXNIP* in the subsequent studies.

We analyzed 25 paired gastric cancer and non-cancer gastric mucosa and found that expression of *TXNIP* mRNA level was reduced in 84% of gastric cancer and was significantly downregulated as compared to the paired non-cancer gastric tissues ($p=0.002$). Expression of *TXNIP* protein by western blot was down-regulated in 3 out of 5 cases. Furthermore, by immunohistochemical staining of *TXNIP* in tissue array containing 150 cases of gastric cancer also showed frequent down-regulation of *TXNIP* expression and ~ 26% with complete lack of *TXNIP* expression.

Array-CGH analysis of the gastric cancer cell lines suggested that *TXNIP* loci were intact, suggesting that allelic loss might not be the major mechanism responsible for the downregulation of *TXNIP* in these cells. Furthermore, our data suggested that promoter hypermethylation of *TXNIP* may not be an important epigenetic mechanism that regulate the silencing of this gene. Chromatin immunoprecipitation (ChIP) assay revealed that SAHA induced hyperacetylation of histone H3 and H4 at the 5' flanking region of *TXNIP* gene, suggesting SAHA could promote *TXNIP* gene transcription via modification of histones located at the promoter region. Our data revealed that the loss or reduced expression of *TXNIP* in gastric cancer cells is associated with epigenetic histone acetylation mechanism.

In addition, we demonstrated that over-expression of *TXNIP* significantly reduced cell migration ability and inhibited cell invasiveness in gastric cancer cells.

Furthermore, absence or reduced expression of *TXNIP* in gastric cancer was associated with diffuse-type gastric cancer, advanced stage disease and predicted a poor disease specific survival. The findings supported that *TXNIP* is a functional tumor suppressor gene and may be a potential biomarker in gastric cancer.

摘要

胃癌是亞洲國家常見的癌症，具有高發病率和死亡率。抑癌基因的表遺傳失活是人類癌症病變過程中常見機制。最近研究表明，針對抑癌基因的表遺傳控制可作爲治療癌症的方法。我們和其他研究小組的實驗都證實了啓動子甲基化與胃癌的關係。在本研究中，我們的目標是找出受控於另外一個表遺傳機制，即組蛋白乙酰化的癌症相關基因。

我們發現 suberoylanilide hydroxamic acid (SAHA)，一種組蛋白去乙酰酶抑制劑，對多種胃癌細胞株（MKN1, MKN7, MKN28, MKN45, SNU1, SNU16, AGS, N87 和 KatoIII 細胞）具有抗增生作用。我們比較由 SAHA 處理的 AGS 細胞與對照組的基因表達圖譜，找出由 SAHA 處理後而表達上調的基因。同時我們根據對胃癌細胞株（MKN1, MKN7, MKN28, MKN45, SNU1, SNU16, AGS, N87 和 KatoIII 細胞）和正常胃組織的微陣列分析，確定表達普遍下調的基因。隨後篩選出在胃癌細胞中表達下調但經 SAHA 處理後表達上調的基因群組，再使用定量實時 PCR 驗證了這批基因的表達水平並發現硫氧還原蛋白結合蛋白（即 VDUP-1/TBP2/TXNIP）在所有的胃癌細胞株中表達下調，經 SAHA 處理後其蛋白表達水平最爲明顯上調(AGS, MKN1, MKN45, N87 和 KatoIII)。因此我們選定 TXNIP 作爲研究對象。

我們分析了 TXNIP 在 25 對胃癌及非癌胃粘膜中 mRNA 的表達水平，發現其在 84%的胃癌中表達水平顯著降低（P = 0.002）。在 5 對標本中，我們用 western blot 證實 TXNIP 蛋白表達水平在 3 個病例中下調。此外，用免疫組織化學染色方法對包含 150 例胃癌標本的組織芯片分析，發現 TXNIP 表達普遍下調，其中 26%的病例 TXNIP 表達完全缺失。

胃癌細胞株的 Array-CGH 分析表明，TXNIP DNA 正常，因此排除等位基因的缺失作爲 TXNIP 在胃癌細胞中表達下調的機制。我們的實驗同時證實，啓動子區域甲基化也不是 TXNIP 在胃癌中表達下調的表遺傳學機制。染色質免疫共沈澱（ChIP）實驗表明，SAHA 可引致 TXNIP 基因的 5'區組蛋白

H3 和 H4 乙酰化，表明 SAHA 可通過調節 TXNIP 基因啓動子區域組蛋白控制 TXNIP 的表達。我們的數據表明在胃癌細胞中 TXNIP 表達與表遺傳組蛋白乙酰化的關係。

此外，我們還證實了 TXNIP 表達可顯著降低胃癌細胞的遷移和細胞侵襲能力。而且 TXNIP 表達缺失與彌漫型胃癌及晚期胃癌相關，並可作爲胃癌預後差的標誌物。所有結果表明 TXNIP 在胃癌發病過程中具有腫瘤抑制作用，並可作爲胃癌的生物學標記。

List of Publications

Conference abstract

Angie Tang, K K. So, P S. Leung, H M. Tong, B C. Ko, K F. To. "TXNIP is a potential tumor suppressor gene in gastric cancer cells" in Proceedings of 100th Annual Meeting of American Association for Cancer Research (AACR) on 18-22 April 2009 at San Diego, CA, USA.

List of Abbreviations

5 aza-dR	5-Aza-2′-deoxycytidine
bp	Base pair
BSA	Bovine serum albumin
CDK	Cyclin- dependent kinase
CDKI	Cyclin- dependent kinase inhibitor
cDNA	Complementary DNA
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTPs	Deoxy (G, A, T and C) triphosphates
DTT	Dithiothreitol
EBV	Epstein-Barr virus
EDTA	Ethylenediamine tetraacetic acid
FACS	Fluorescence- activated cell sorting
FBS	Fetal bovine serum
GCA	Gastric adenocarcinoma
HAT	Histone acetyltransferase
HCl	Hydrochloride
HDAC	Histone deacetylase
HP	*Helicobacter pylori*
$MgCl_2$	Magnesium chloride
min	Minute
ml	Milliliter
mRNA	Messenger ribonucleic acid
MTT	3-(4,5-dimthylthiazol-2yl)-2,5-diphenyltetrazolium bromide
NaCl	Sodium chloride
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PI	Propidium iodide
RPMI	Roswell Park Memorial Institute
RNA	Ribonucleic acid

ROS	Reactive oxygen species
RT-PCR	Reverse transcription PCR
SAHA	Suberoylanilide Hydroxamic Acid
SDS	Sodium dodecylsulfate
SDS/PAGE	Sodium dodecylsulfate-polyacrylamide gel electrophoresis
Tris	Tris (hydroxymethyl) aminomethane
TRX	Thioredoxin
TSA	Trichostatin A
TXNIP	Thioredoxin-interacting protein 2
μg	Microgram
μM	Micromolar
μl	Microliter

List of Tables

List of Figures

Table of Contents

Chapter 1 Literature review

Background

Gastric cancer is the fourth most common cancer and the second most common cause of cancer-related death worldwide. It is the leading cancer in many parts of the world particularly in Asia, Eastern Europe, and parts of Central and South America (Brenner et al., 2009). According to the latest statistics (in the year of 2007) published by the Hong Kong Cancer Registry, gastric cancer is the sixth most common cancer locally with a crude incidence rate of 14.5 and a crude mortality rate of 9.2 per 100 000 (Hong Kong Cancer Registry website (www3.ha.org.hk/cancereg/ 1st May 2009).

The overall prognosis of gastric cancer is poor with a 5-year of survival rate below 30% in most countries (Brenner et al., 2009). Primary curative treatment for gastric cancer is surgery. However, gastric cancer is often diagnosed at advanced stage and more than 50% of patients were suffered from unresectable, locally advanced or metastasis disease (Dicken et al., 2005). Unfortunately, the conventional adjunctive treatments such us radiotherapy and chemotherapy for unresectable gastric cancer are unsatisfactory. The response for gastric cancer to chemotherapy treatments remains low. Therefore, there is an urgent need to elucidate the mechanisms of gastric carcinogenesis. The dissection of the carcinogenesis may provide insights in the new therapeutics to combat gastric cancer.

Recent studies suggested that epigenetic mechanisms such as histone acetylation play an important role in tumorigenesis and cancer progression. The acetylation status of histones regulates access of transcription factors to DNA and influences levels of gene expression. It is regulated by the enzyme activities of histone acetyltransferases (HATs) and histone deacetylases (HDACs). The HDAC activity diminishes histone acetylation and causes compaction of the histone/DNA complex. This compaction inhibits gene transcription and differentiation. Such epigenetic mechanism is linked to cancer development by silencing of tumor suppressor genes. The inhibition of HDAC is then associated with chromatin relaxation and gene transcriptions. It provides a rationale for developing HDAC inhibitors for treating human cancer.

At present, a variety of HDAC inhibitors have entered into phase I and/or phase II clinical trials for different types of human cancers. For example, vorinostat (SAHA) has already been approved by United States Food and Drug Administration (FDA) for the treatment of cutaneous T-cell lymphoma (CTCL). Other HDAC inhibitors such as depsipeptide and MGCD0103 have shown to be active against lymphoma, leukemia and solid tumors. To the best of our knowledge, clinical study of the effectiveness of HDAC inhibitors towards gastric cancer has not been reported.

Our previous study showed that HDAC inhibitor SAHA has anti-tumor effect in gastric cancer cell lines. HDAC inhibitor may represent an emerging therapeutic strategy against gastric cancer. However, the underlying mechanisms of HDAC

inhibitors eliciting anti-tumor effects are unclear. In this study, we aim to identify the potential tumor suppressor genes that are silenced by histone acetylation. We hypothesize that the potential target genes should be commonly downregulated/silenced in gastric cancer cells and also could be induced by SAHA treatment. Therefore, potential candidate genes were selected for further characterization with respect to their regulation by histone modifications and anti-tumor effects.

1.1 Gastric cancer-overview

1.1.1 Epidemiology

Gastric cancer (Figure 1.1) is the second most common cancer worldwide and the incidence varies across different geographic locations. The incidence of gastric cancer is particularly high in East Asian countries including Japan, Korea and China (>40 cases per 100 000 men) as compared to countries in west and south Asia, where the incidence is relatively lower (<10 cases per 100 000 men) (Brenner et al., 2009, Dicken et al., 2005, Leung et al., 2008). The incident of gastric cancer in male is twice as that in female (Leung et al., 2008). In Hong Kong, according to the statistics provided by Hong Kong Cancer Registry from Hospital Authority (www3.ha.org.hk/cancereg/ 1st May 2009), there are around 1000 newly registered gastric cancer cases per year. The incidence rate was ranked sixth of all cancer and the mortality rate was ranked fourth of all cancers with a mortality rate of 9.2 per 100 000 at the year 2007. Most patients are diagnosed at median age of 70 and the male to female ratio is 1.6:1.

The vast majority of gastric malignancies are gastric adenocarcinoma which comprises of more than 80% of all malignant gastric tumors. The remainder consists of lymphomas, stromal tumors and other rare tumors (Vogiatzi et al., 2007).

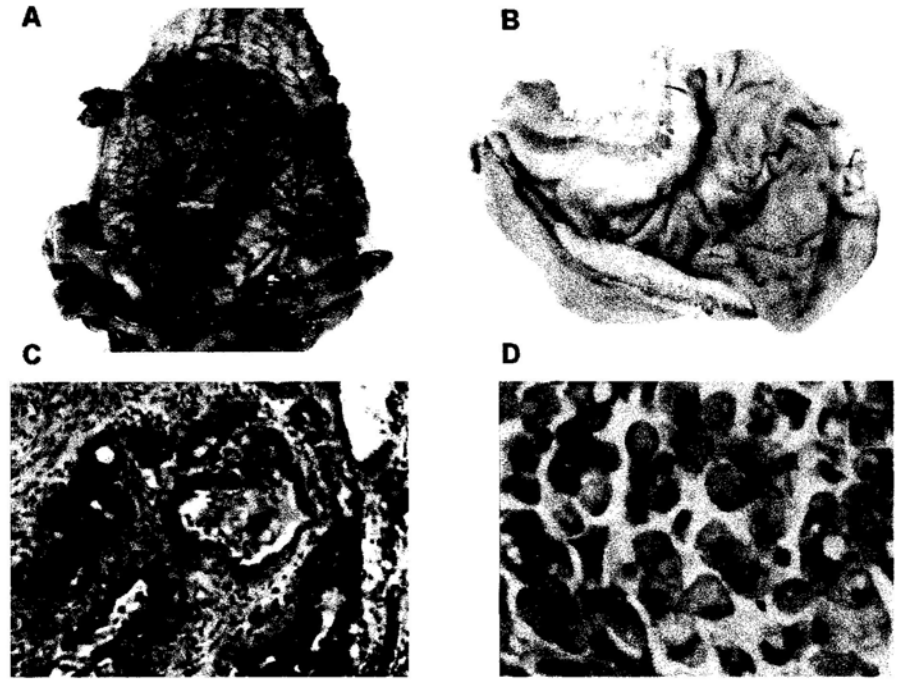


Figure 1.1 Gastric cancer, A) gross photo of a gastric carcinoma forming a large malignant ulcer; B) gross photo of a diffuse-type gastric carcinoma which diffusely infiltrate the gastric wall; C) histology of an intestinal-type adenocarcinoma forming infiltrative malignant glands (H&E staining, 200x original magnification); D) histology of a diffuse-type adenocarcinoma with the typical signet ring cell morphology (H&E staining, 400x original magnification).

1.1.2 Pathogenesis

The development of gastric adenocarinoma is a multi-step process. Gastric adenocarcinoma usually histologically divided into two major types including intestinal- and diffuse-type (based on Lauren classification). Especially for intestinal-type adenocarcinoma, it is believed that it may progress via gastritis to gastric atrophy to intestinal metaplasia to dysplasia and to invasive adenocarcinoma (Vogiatzi et al., 2007).

1.1.2.1 Progression of gastric cancer

1.1.2.1.1 Intestinal metaplasia

Intestinal metaplasia (IM) is the replacement of gastric epithelium by intestinal-type epithelium. The metaplastic changes could be complete or incomplete intestinal metaplasia. In the complete intestinal metaplasia, the metaplastic glandular epithelium consists of goblet cells and intestinal absorptive cells. For the incomplete intestinal metaplasia, the metaplastic glandular epithelium consists of goblet cells and mucin secreting columnar cells (Brunicardi and Schwartz, 2005a). Both of the types of intestinal metaplasia often co-exist in the stomach. IM is believed to be a metaplastic response related chronic inflammation in stomach. IM, especially incomplete IM is considered a condition that may predispose to the gastric cancer development.

1.1.2.1.2 Dysplasia

Gastric glandular dysplasia is considered as pre-malignant condition that may

progress to invasive adenocarcinoma. According to the cytological and architectural atypia, it can divided into low grade and high grade dysplasia (Brunicardi and Schwartz, 2005a). With the advance in endoscopic diagnosis and excision, gastric dysplasia is amendable for endoscopic submucosal dissection or treatment.

1.1.2.1.3 Gastric adenocarcinoma (GCA)

The vast majority of malignant gastric tumors are adenocarcinoma. Gastric adenocarcinoma (GCA) more commonly arising from gastric antrum, followed by gastric body, fundus and cardia. Depend on the depth on invasion into the gastric wall, GCA can be divided into early and advance gastric cancer. Early gastric cancer is defined as GCA that is confined within the mucosa or submucosa of the stomach, regardless of lymph node metastastic status. Accroding to the endoscopic appearance, it can be further subdivided into the protruded, elevated, flat, depressed and excavated subtype (Brunicardi and Schwartz, 2005a). When GCA invades through the submucosal into the muscular propria layer or beyond, it is defined as advanced gastric cancer. Advance gastric cancer has a higher risk of lymph node and distant metastasis and associated with very poor prognosis (Brunicardi and Schwartz, 2005a).

1.1.2.2 Histology

The Lauren classification is the traditional histological classification of gastric adenocarcinoma and remains the most widely used classification. Based on Lauren

histological classification, GCA is subdivided into two main histology types: (1) intestinal-type, and (2) diffuse-type. The intestinal-type GCA forms recognizable glandular architecture and more often associated with chronic atrophic gastritis and intestinal metaplasia (Lochhead and El-Omar, 2008). The diffuse-type composes of dis-cohesive or scattered infiltrative individual cancer cells. Tumor cells contain abundant cytoplasmic mucin compressing the nuclei and producing signet ring appearance are often noted (Lochhead and El-Omar, 2008). However, mixed intestinal- and diffuse-type (mixed-type) is also not uncommon. In addition, there are rare histological types that are not classified into intestinal- or diffuse-type.

1.1.2.3 Staging and Prognosis

Cancer staging refer to the extends of the disease. The most widely used systems for staging of gastric cancer is the tumor-node-metastasis (TNM) staging system. T represents the primary tumor, N represents the degree of spread to regional lymph nodes, and M represents the presence or absence of metastasis (Table 1.1).

Various imaging and visualization modalities are developed for staging of gastric cancer. Computed tomography (CT) scan is a commonly use non-invasive technique for staging gastric cancer, though it is not accurate in assessing the depth of tumor invasion and presence of lymph node involvement. Endoscopic ultrasound is developed to better assess the depth of tumor invasion into the gastric wall (Kantarjian et al., 2006). Since cancer cells preferentially accumulate

position-emitting 18^F fluorodeoxyglucose, Position emission tomography (PET) scan is also commonly used as staging procedure and particularly useful in detecting distant metastasis (Brunicardi and Schwartz, 2005a). Gastric carcinoma also prone to spread into the peritoneal cavity and small peritoneal tumor implant and is difficult to detect. Laparoscopic examination may be the only way to pick up those small peritoneal implants (Brunicardi and Schwartz, 2005b, Kantarjian et al., 2006, Brunicardi and Schwartz, 2005a).

Table 1.1 Gastric Carcinoma Staging (6th Edition, AJCC/UICC)

Primary Tumor (T)

TX Cannot be assessed
T0 No evidence of primary tumor
Tis Carcinoma in situ
T1 Tumor invades lamina propria or submucosa
T2 Tumor invades muscularis propria or subserosa
  T2a Tumor invades muscularis propria
  T2b Tumor invades subserosa
T3 Tumor penetrates serosa (visceral peritoneum) without invasion of adjacent structures
T4 Tumor directly invades adjacent structures

Regional Lymph Nodes (N)

NX Cannot be assessed
N0 No regional lymph node metastasis
N1 Metastasis in 1 to 6 perigastric lymph nodes
N2 Metastasis in 7 to 15 perigastric lymph nodes
N3 Metastasis in greater than 15 perigastric lymph nodes

Distant Metastasis (M)

MX Cannot be assessed
M0 No distant metastasis
M1 Distant metastasis

Stage Grouping

Stage 0	Tis	N0	M0
Stage IA	T1	N0	M0
Stage 1B	T1	N1	M0
	T2	N0	M0
Stage II	T1	N2	M0
	T2	N1	M0
	T3	N0	M0
Stage IIIA	T2	N2	M0
	T3	N1	M0
	T4	N0	M0
Stage IIIB	T3	N2	M0
Stage IV	T4	N1-3	M0
	T1-3	N3	M0
	any T	any N	M1

1.1.3 Etiologies

In gastric cancer, a multifactorial etiological model is proposed. Both environmental and genetic susceptibility are implicated. In the following section, both environmental and genetic factors contributing to the development of gastric cancer will be discussed.

1.1.3.1 Environmental factors

1.1.3.1.1 Helicobacter pylori infections

Since the discovery of *Helicobacter pylori* (*H. pylori*) in 1983, *H. pylori* infection is recognized to be etiologically associated with a spectrum of gastric pathologies, including gastritis, gastric ulcer and duodenal ulcer. There are also numerous publications demonstrated the association between gastric cancer and *H. pylori infection*. Epidemiological studies suggested that individuals who are positive carrier for *H. pylori* have at least two times higher risk of developing gastric cancer (Uemura et al., 2001). *H. pylori* infection is associated with both intestinal- and diffuse-type gastric adenocarcinoma. It is also regarded as class I human carcinogen by World Health Organization and the International Agency for Research on Cancer in 1994 (Lochhead and El-Omar, 2008).

H. pylori is a gram-negative bacillus which colonizes in gastric mucosa and is a highly host-adapted bacterial pathogen that establishs a chronic infection in the human stomach without other known animal or environmental reservoirs (Shang

and Pena, 2005). *H. pylori* has the ability to survive in the extreme acidic gastric environment. By utilizing urease enzyme to hydrolyse gastric urea into ammonia and carbon dioxide, it enables *H. pylori* to maintain a constant internal and periplasmic pH under high external acidic environment (Smith et al., 2006).

H. pylori infection alters the expression of genes that encode growth factors, cytokines/chemokines and their receptors, apoptotic proteins, transcription factors, metalloprotease-disintegrin proteins and tissue inhibitors of metalloproteinases (Shang and Pena, 2005). It has been postulated that *H. pylori* induces DNA damage in epithelial cells which in turn leads to apoptosis (Chan et al., 1999). Other studies also suggested that *H. pylori* stimulates the expression of adhesion molecules and neutrophil-recruiting chemokines in endothelial cells, leading to tissue damage and even ulcer (Shang and Pena, 2005). Down-regulation of E-cadherin (*E-CAD*) protein was found to be significantly correlated to *H. pylori* infection in patient's normal gastric mucosa, gastritis, gastric ulcer and duodenal ulcer (Chan et al., 1999). Previous studies indicated that the degree of *H. pylori* infection was proportional to the telomerase RNA and protein levels in gastric carcinoma (Chan et al., 1999). Studies also suggested that *p53* alterations were associated with *H. pylor* infection (Shang and Pena, 2005). These findings suggested the molecular link between *H. pylori* infection and gastric carcinogenesis. Certain strains, like cytotoxin-associated gene A (cagA) producing strains have been implicated as a risk factor for gastric cancer (Shang and Pena, 2005).

H. pylori infection is more common in Asian population and this partly account for the higher incidences of the gastric cancer in Asian. There are also strains variation among different geographic regions and the variations have also been implicated in the risk of gastric cancer (Shang and Pena, 2005). Furthermore, *H. pylori* infection triggers immune responses which involves a complex of inflammatory mediators including cytokines such as interleukin-*1β* and tumor necrosis factor-α. The expressions of these cytokines have also been implicated in gastric carcinogenesis (Smith et al., 2006).

In Asian countries, 40 to 60% of adult population harbors *H. pylori* in the stomach but only a small portion of this population will eventually aquire gastric cancer. Thus, apart from *H. pylori* infection, additional factors may contribute to the development of malignancy. Additional factors may include other risk factors such as environmental factors and genetic factors.

1.1.3.1.2 Epstein-Barr virus (EBV)

Epstein-Barr virus (EBV) is a gamma herpesvirus and was implicated in various human cancer since it's discovery 40 years ago (Sousa et al., 2008). It is etiologically linked to several human malignancies including nasopharyngeal carcinoma and Burkitt's lymphoma (Kuppers, 2003). In gastric cancer, especially in Asian population, a subset of gastric adenocarcinoma (~7% of gastric adenocarcinoma) also harbors EBV. The carcinoma cells harboring latency I EBV infection and clonal EBV proliferation in the carcinoma cells was demonstrated

(Stoicov et al., 2004). Thus, EBV was also etiologically associated with a small subset of gastric carcinoma. How the EBV infects gastric epithelial cells is unclear (Stoicov et al., 2004). Nevertheless, it was shown that EBV positive gastric adenocarcinoma is more often to have multiple gene methylation and associated with a better prognosis (Kang et al., 2002).

1.1.3.1.3 Other factors

Epidemiological studies suggested that the consumption of fruit and vegetable (even in low amounts) was associated with reduced risk of gastric cancer (Kobayashi et al., 2002). However, recent studies reviewed that dietary supplements of antioxidants such as β-carotene, vitamin A and vitamin E do not significantly reduce the incidence of gastrointestinal cancers (Liu and Russell, 2008). Instead, diet rich in lycopene, lycopene products, vitamin C and selenium are associated with reduced gastric cancer risk. Whereas high intake of nitrosamines, processed meat products, salted foods, overweight and obesity are associated with the increased risk (Liu and Russell, 2008).

Smoking is also shown to be associated with gastric cancer risk. Some studies suggested that smoking was associated with higher risk of intestinal-type cancer of distal stomach and others suggested that smoking may be more strongly associated with cardia and upper-third gastric cancers (Sung et al., 2007, Nishino et al., 2006, Leung et al., 2008). Alcohol consumption is also implicated in gastric cancer risk. A study found that there is a greater risk of developing distal gastric cancer in

individual who consumed more that 15g of alcohol per day than non-drinker (Sung et al., 2007). In addition, data from another study suggested that combined exposure to smoking and alcohol increased the risk of gastric non-cardia cancer (Sjodahl et al., 2007).

Some synthetic drugs such as ecstasy may lead to digestive damage or vascular complications in stomach and were shown to be associated with the development of gastric cancer (Dimitrijevic et al., 2008). On the other hand, regular consumption of aspirin may protect against gastric cancer (Brunicardi and Schwartz, 2005a).

1.1.3.2 Genetic events

1.1.3.2.1 Hereditary Gastric Cancer

A small proportion of gastric cancers arise in the background of inherited gastric cancer predisposition syndromes. There are several different inherited gastric cancer predisposition syndromes which include Hereditary Diffuse Gastric Cancer Syndrome (HDGC), Hereditary Nonpolyposis Colorectal Cancer Syndrome (HNPCC), Li-Fraumeni Syndrome (LFS) and Familial Adenomatous Polyposis (FAP). The understanding of the genetic abnormalities underlying these hereditary gastric cancer syndromes not only allows the prevention and surveillance of affected individuals, but also offers clues to the gastric carcinogenesis.

Hereditary Diffuse Gastric Cancer Syndrome (HDGC) was first reported in a

large New Zealand Maori family (Sjodahl et al., 2007). It is a distinctive autosomal dominant inherited gastric cancer susceptibility syndrome and is resulted from germline mutations of E-cadherin *(CDH1)* gene, which is involved in cellular adhesion (Lynch et al., 2005). Defect in E-cadherin has been linked to the diffuse-type gastric cancer (Caldas et al., 1999). Affected individuals inherit one copy of the defective gene and somatic mutation or deletion activates the other copy in the gastric cancer. In addition, *MADH4, CHK2,* and *caspase-10* germline mutations have also been studied in the HDGC families (Lynch et al., 2005).

Hereditary Nonpolyposis Colorectal Cancer Syndrome (HNPCC) is a syndrome caused by germline mutations in mismatch repair genes such as *MSH2, MLH1, MSH6, PMS1, and PMS2*. Defects in these genes would result in microsatellite instability (MSI). Gastric cancers developed in ~11% of HNPCC families and have been shown to occur in families with *MSH2, MLH1 or MSH6* germline mutations (Vasen et al., 1996, Aarnio et al., 1997).

Li-Fraumeni Syndrome (LFS) is originated from Li and Fraumeni's work and characterized by multiple primary malignancies in susceptible families (Li and Fraumeni, 1969). It was initially proposed in 1969 and subsequently confirmed by several epidemiological studies (Li and Fraumeni, 1969, Varley, 2003). It is caused by germline mutations in *TP53* gene that encodes for *p53.* LFS is associated with a variety of human malignancy occurring over a wide age range. Numerous studies identified various component tumors of LFS such as bone and soft-tissue sarcomas, premenopausal breast carcinoma, brain tumors, adrenocortical carcinomas and leukemia (Li and Fraumeni, 1969). Other studies have indicated that tumors such as

Wilms' tumor, lung, gastric and pancreatic carcinoma could occur in LFS families at a higher frequency (Varley, 2003). For gastric cancer, both intestinal- and diffuse-types have been reported in patients with LFS (Varley, 2003).

Familial Adenomatous Polyposis (FAP) is caused by germline mutations in *APC* gene. This disorder is inherited in an autosomal dominant pattern FAP predisposed to the risk of colo-rectal cancer with high penetrance. FAP is associated with gastric fundic gland polyposis and also with increased risks of gastric adenocarcinoma (Hofgartner et al., 1999).

1.1.3.2.2 Genetic polymorphism

Cytokines are important mediators that participated in inflammatory response which associated with both innate and acquired immune responses. Up-regualtion of various inflammatory cytokines, including *IL-1β,* tumor necrosis factor *(TNF-α)* and *INF-γ* is common in *H. pylori* infection. Gastric cancer cells also express a wide variety of growth factors and cytokines which act via autocrine, paracrine and juxtacrine system (Smith et al., 2006). Genetic polymorphisms may alter gene transcription or functions and thereby influence the inflammatory response to infectious diseases (Perez-Perez et al., 2005b). Polymorphisms in human interleukin-*1β (IL-1β)*, interleukin-10 *(IL-10), TNF-A, IFN-G* and *IL-1RN* genes have been reported to influence cytokine expression (Shang and Pena, 2005). El-Omar et al first demonstrated the association between *IL-1β* gene promoter polymorphisms and an increased risk of gastric cancer in the year of 2000 (El-Omar et al., 2000). Interleukin-1 (IL-1) gene encompasses a gene cluster on chromosome 2q. This cluster includes three related genes *IL-1A, IL-1B and IL-1RN* that encode

the pro-inflammatory cytokines, *IL-1α, IL-1β* and their endogeneous receptor antagonist, *IL-1ra* (Roberts-Thomson and Butler, 2005). Polymorphism of *IL-1β* together with *IL-1RN* genes may increase susceptibility of gastric cancer (Shang and Pena, 2005). In addition, several others cytokine polymorphisms including *IL-10* and *TNF-α* have also shown to be associated with gastric cancer (El-Omar et al., 2003). *TNF* is a pro-inflammatory cytokine which inhibits gastric acid secretion (Smith et al., 2006). *TNF-α* is tightly associated with epithelial injury and plays a crucial role in host defense against infection. However, high concentration of *TNF-α* may cause severe adverse effect (Shang and Pena, 2005). The increased concentration of *TNF-α* alters the immune response which confers susceptibility to gastric disease with *H. Pylori*-cagA subtype infection (Shang and Pena, 2005). A single nucleotide polymorphism (SNP) at the position -308 in the *TNF* promoter has been reported to be associated with increased risk of gastric cancer (Smith et al., 2006).

Other cellular mediators or growth factors are also implicated in gastric carcinogenesis. However, the genetic polymorphism has not been shown to be associated with gastric cancer risk. For example, EGF family which includes *EGF, TGF-α, IGF-II* and *bFEF* are commonly over-expressed in intestinal-type gastric cancer, whereas *TGF-β, IGF-II* and *bFGF* are commonly over-expressed in the diffuse-type gastric cancer (Smith et al., 2006). The growth factor *TGF-β* is predominantly over-expressed in diffuse-type gastric cancer with fibrosis (Smith et al., 2006). Angiogeneic factors including vascular endothelial growth factor (*VEGF*), basic fibroblast growth factor (*bFGF*) and *IL-8* are produced by cancer cells and believed to play a role in neovascularisation (Smith et al., 2006). *IL-1α*

also acts as autocrine growth factor in gastric cancer cells and is required for *EGF* and *EGF* receptor expression (Smith et al., 2006).

1.1.3.3 Molecular pathogenesis of gastric cancer

The development and progression of gastric cancer is believed to be resulted from accumulation of genetic and epigenetic alterations. Intestinal- and diffuse-type gastric carcinomas may be derived from different carcinogenetic pathways (Figure 1.2). There is a proposal suggesting that intestinal-type gastric carcinogenesis may be developed along three pathways: 1) an intestinal metaplasia–adenoma /dysplasia-carcinoma sequence, 2) an intestinal metaplasia-carcinoma sequence and 3) *de novo* pathway (Tahara, 2004). For intestinal metaplasia, it was shown to have genetic instability of D1S191 (1q) and DNA hypermethylation. Gastric adenoma/dysplasia may displaced APC and p53 mutation, pS2 (a gastric-specific trefoil factor) reduction, p16 and hMLH1 loss, and loss of heterozygosity (LOH) of p73 (Vogiatzi et al., 2007, Smith et al., 2006). The *de novo* pathway of intestinal type gastric cancer involves LOH and abnormal expression of *p73* that is associated with foveolar-type gastric cancers (Smith et al., 2006). Other genetic changes included 7q LOH, loss of RUNX3, amplification of cyclin E and *C-erbB2* expression, growth factor overexpression, and aberrant CD44 transcripts were also described (Vogiatzi et al., 2007, Smith et al., 2006).

Human telomerase reverse transcriptase (hTERT) also plays an important role in progression of intestinal-type gastric carcinoma. It regulates the telomerase activity by catalyzing the telomerase DNA synthesis. The majority of

intestinal-type gastric carcinomas have shortened telomere length and exhibit high levels of telomerase activity and high expression of hTERT (Yasui et al., 1998). Previous studies found that more than 50% of the intestinal metaplasias have low levels of telomerase activity. Whereas majority of the intestinal-type gastric carcinomas have high expression of hTERT and the expression of hTERT was absent in normal gastric mucosa. Futhermore, some scientists also suggested that human telomerase reverse transcriptase (hTERT)-positive epithelial cells in normal gastric mucosa, intestinal metaplasia and gastric adenoma are referred as epithelial "stem cell". Infection by *Heliobacter pylori* in these hTERT-epithelial "stem cell" in intestinal metaplasia may induce chronic mitogenesis which facilitates the carcinogenesis of gastric cancer (Tahara, 2004, Vogiatzi et al., 2007).

For diffuse-type gastric cancer, genetic instability, 17q21 LOH, LOH or mutation of p53, hTERT expression with telomerase reactivation, LOH or mutation of E-cadherin, loss of retinoic acid receptor β (*RAR-β*), and over-expression of cyclin E and CDC25B, growth factor, aberrant CD44 transcripts, 7q LOH, loss of RUNX3 expression and reduction of p27 expression and amplication of K-sam (KatoIII cell-derived stomach cancer amplified) oncogene, c-met and cyclin E were reported (Smith et al., 2006, Vogiatzi et al., 2007). Nevertheless, mixed intestinal- and diffuse-type gastric carcinoma is not uncommon. Thus, it suggested that molecular pathways are more complicated and complex than one may expected (Vogiatzi et al., 2007, Tahara, 2004).

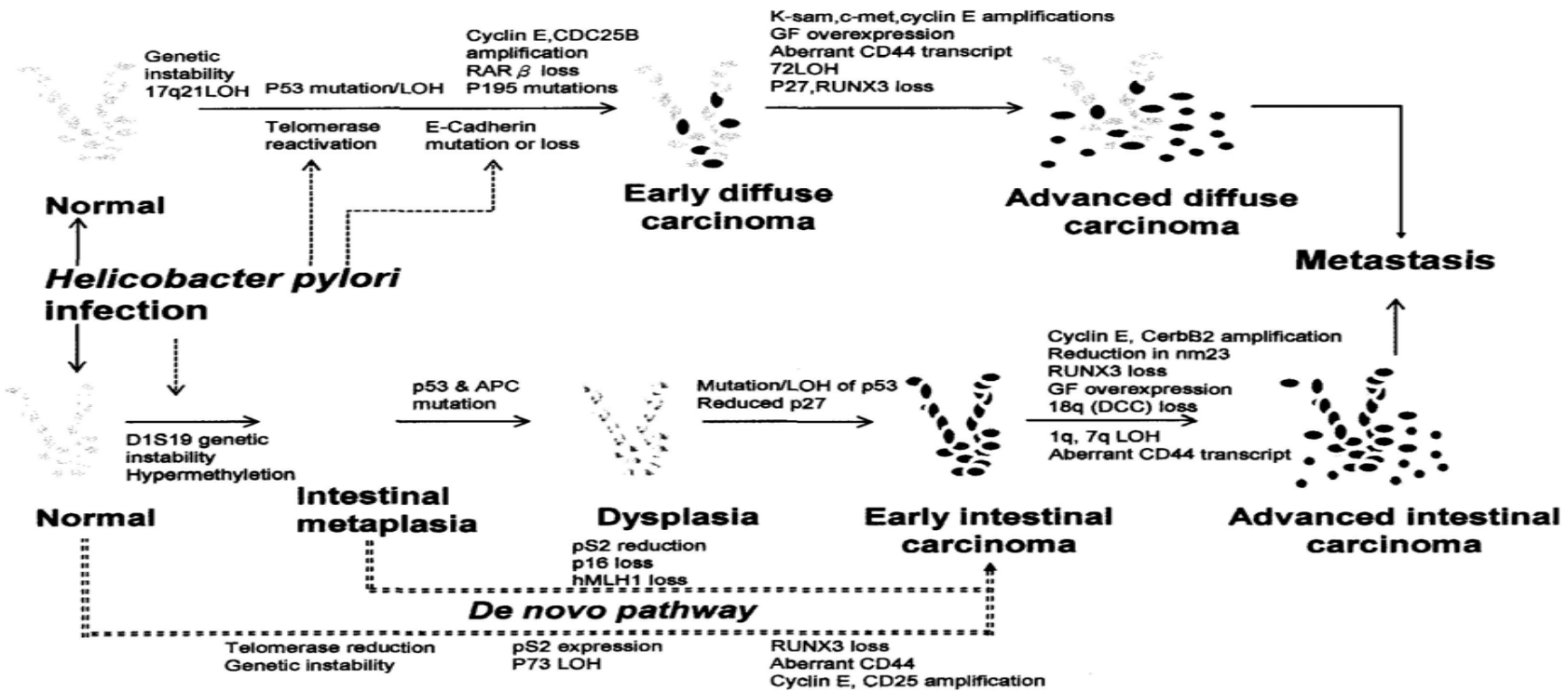


Figure 1.2 This diagram summarizes the involvement of *Heliobacter pylori* and the possible molecular pathways in the development of diffuse- and intestinal-types of gastric carcinoma. Abbreviation: GF, growth factor. [Modified according to (Chan et al., 1999, Vogiatzi et al., 2007, Smith et al., 2006)].

1.1.3.3.1 Activation of proto-oncogenes

Several proto-oncogenes have been implicated in gastric carcinogenesis. For example, three genes located at 17q21 locus, namely *HER2/NEU, TOP2A,* and *DARPP32* are over-expressed in human primary gastric cancers (Varis et al., 2004). *HER2/NEU* is amplified in a subset of gastric cancer, *C-erbB2* is preferentially amplified in intestinal-type gastric cancers and correlate with poorer prognosis and liver metastases (Yokota et al., 1988). *TOP2A* over-expression was preferentially detected in diffuse-type carcinoma, whereas over-expression of *DARPP32* was detected in both histological subtypes (Varis et al., 2004). Increased expression of *Ras* oncogene has also been demonstrated in gastric cancers (Noguchi et al., 1986) and more often in intestinal-type GCA and the precursor lesions, intestinal metaplasia and adenomas (Yoshida et al., 1988, Isogaki et al., 1999).

Other proto-oncogenes including *C-met, K-sam,* and *C-myc* gene have also reported to be activated in gastric cancer. *C-met* encodes a receptor for hepatocyte growth factor is amplified in 19% of intestinal-type and 39% in diffuse-type of gastric cancer (Kuniyasu et al., 1992). *K-sam* (KATO-III cell-derived stomach cancer amplified) oncogene is frequently activated in gastric cancer and preferentially amplified in advanced diffuse-type carcinoma (Hattori et al., 1990). Over-expression of *c-myc* gene has been reported in gastric cancer (Shibuya et al., 1985) and also correlated with the stage of disease, depth of invasion and peritoneal dissemination (Ninomiya et al., 1991).

Genes that involved in cell cycle regulation have been reported to be over-expressed in gastric cancer such as *Cyclin E*. *Cyclin E* is a cell cycle regulator which is amplified and over-expressed in ~15-20% of gastric cancer. Studies have also found that the amplification and over-expression of the corresponding genes are correlated with aggressiveness and lymph node metastasis (Akama et al., 1995).

1.1.3.3.2 Silencing of tumor suppressor genes

Tumor suppressor genes may be altered by mutation and/or silenced by deletion and/or epigenetic mechanisms.

1.1.3.3.2.1 Gene mutation and alteration

(i) p53

p53 gene is located at 17p13.1 which encodes a nuclear protein that involves in regulation of cell growth, initiation of programmed cell death or apoptosis. Studies have found that *p53* is frequently inactivated by loss of heterozygosity (LOH), missense mutations and frame shift deletions that caused allelic deletions in more than 60% of gastric cancers (Chan et al., 1999). *p53* mutations is one of the most prevalent genetic alternations discovered in human cancers. Investigators have suggested that *p53* mutation would be an early event in intestinal-type gastric cancer (Wu et al., 1997). Some studies showed that the mutations seem to predominantly in intestinal-type gastric cancer while other studies showed that the incidence of mutations is similar in both intestinal- and diffuse-type gastric cancer (Panani, 2008). G:C→A:T transitions at CpG sites are the most common type of mutation. Recently, mutations in the codon 72 of exon 4 have been reported to be associated with distal gastric cancer (Perez-Perez et al., 2005a). Some studies also

suggested that aberrant *p53* expression correlated with the proliferative rate of the gastric cancer cells and *p53* mutations may contribute to the increased expression of cyclooxygenase-2 (*COX-2*) in gastric cancer (Leung et al., 2001).

(ii)Adenomatous polyposis gene (APC)

Mutation of *APC* gene is involved in familial polyposis coli and has been observed in over 50% of the intestinal-type gastric cancer cases (Kinzler et al., 1991). Somatic mutations are observed in 20-40% of gastric adenomas and 6% of intestinal metaplasias (Nakatsuru et al., 1993). Furthermore, *APC* gene may also be silenced by promoter methylation. *APC* protein is located in the cytoplasm and interacts with E-cadherin and β-catenin and plays an important role in cell signaling involved in the development of gastric cancer (Chan et al., 1999)..

(iii) Bcl-2

Bcl-2 gene alterations have been frequently found in gastric cancer. Over-expression of *Bcl-2* inhibits the cellular proliferation and is correlated with less aggressive behavior in gastric cancer (Lee et al., 2003, Aizawa et al., 1999). Other studies have also demonstrated a significant negative correlation between the *Bcl-2* expression versus the depth of invasion and lymph node metastasis in gastric cancer (Lee et al., 2003). In addition, *p53* and *Bcl-2* positive gastric cancers have a better survival rate (Lee et al., 2003).

(iv) Deletion in colon cancer (DCC)

DCC is a tumor suppressor gene that also implicated in gastric carcinogenesis. This tumor suppressor gene is located on chromosome 18q. This gene could be

inactivated by allelic deletion and promoter hypermethylation. *DCC* expression is more often down-regulated in well-differentiated as compare to poorly-differentiated gastric adenocarcinoma (Chan et al., 1999).

(v) RUNX3

RUNX3 is a runt-domain transcription factor and is normally expressed in gastric epithelia. In an animal model experiment, *RUNX3* knockout mice exhibited gastric epithelial hyperplasia with suppression of apoptosis and stimulation of proliferation (Li et al., 2002). *RUNX3* is frequently inactivated in gastric cancer cell lines and primary gastric cancer tissues by hemizygous deletion or promoter hypermethylation (Li et al., 2002). The loss of *RUNX3* affects the clinical outcome of gastric cancer patients and has been suggested to be related to tumor growth and metastasis (Wei et al., 2005). *RUNX3* is post-translationally regulated through acetylation and ubiquitination via TGF-β signaling pathway (Jin et al., 2004). Recent studies demonstrated that hypoxia silenced the expression of *RUNX3* via epigenetic histone regulation in human gastric cancer cells (Lee et al., 2009).

(vi) Cadherin gene (CDH1)

The cadherin gene is a homophilic cell adhesion molecule belongs to a superfamily of cell-cell adhesions molecules. *CDH1* plays an important role in intercellular adhesion by establishing cell polarity, maintaining tissue morphology and cellular differentiation in normal cells (Wijnhoven et al., 2000, Smith and Pignatelli, 1997). E-cadherin (*E-CAD*) is one of the members of cadherins which not only acts as an adhesion molecule but also involves in growth development and carcinogenesis. In gastric cancer, *E-CAD* mutations have been reported to be

preferentially observed in diffuse-type gastric cancer (Becker et al., 1994). In addition, germline mutations of *E-CAD* have been identified as the major cause of hereditary diffuse gastric cancer (HDGC) (Lynch et al., 2005).

(vii) p27

p27 is a cyclin dependent kinase (CDK) inhibitor and was reported to be frequently down-regulated in advanced gastric cancer (Yasui et al., 1999).

1.2 Epigenetics

In 1942, Biologist Waddington defined epigenetics as "the branch of biology which studies the causal interactions between genes and their products which bring the phenotype into being" (Slack, 2002). However, in modern view, epigenetic is defined as "heritable changes in gene expression that does not involve changes in the sequence of nuclear DNA and can persist through one or more than one generations and are reversible" (Lengauer and Issa, 1998, Laird, 2005, Mai et al., 2005). In recent years, rapid progress has been made in understanding epigenetic mechanisms in regulation of gene expression. Epigenetic regulations involve changes in the structure (remodeling) of chromatin through covalent modifications of histone proteins and DNA methylation (Bolden et al., 2006). Such epigenetic control mechanisms are important in normal development as well as in various diseases. Epigenetics also play an important role in biological processes, including X chromosome inactivation (Avner and Heard, 2001), genomic imprinting (Feinberg, 2001), developmental abnormalities (Feinberg, 2001), activity of mobile genetic elements (Hagan and Rudin, 2002) and also implicated in cancer biology, viral latency (Robertson, 2000, Takacs et al., 2001), somatic gene therapy (Rideout et al., 2001, El-Osta and Wolffe, 2000), cloning and transgenic technologies. The two best-known epigenetic mechanisms, gene promoter hypermethylation and histone modifications will be further discussed in the following sections.

1.2.1 DNA methylation

In human somatic cells, 5-methylcytosine accounts for approximately 1% of total DNA bases and is widely spread-out in genome that accounts for

approximately 70-80% of all CpG dinucleotides in the genome (Worm and Guldberg, 2002). 5-methylcytosine tends to mutate to T by deamination which is believed to be the reason why bulk human DNA CpG dinucleotides occur about five times less frequently than expected (Bird, 1980, Jones et al., 1992). The most important feature of DNA methylation patterns is the presence of CpG islands. CpG islands are unmethylated GC-rich regions that are possess with high relative densities of CpG at the 5'end of many human genes (Bird, 1986). Usually CpG islands overlay the promoter region and extend about 1kb downstream of the transcriptional start site. The usual formal definition of a CpG island is a region (1) with at least 200 bp, (2) with a GC percentage that is greater than 50%, and (3) with an observed/expected CpG ratio that is greater than 60% (Gardiner-Garden and Frommer, 1987). While CpG islands are unmethylated in normal cells, they can become methyalted in special developmental circumstances or in abnormal cells (cancer cells) and lead to transcriptional silencing (Bird and Wolffe, 1999).

DNA methylation is a complex process which affects CpG dinucleotide. DNA methyltransferases (*DNMTs*) catalyze the addition of a methyl group from the universal methyl donor S-adenosyl-L-methionine to the 5-carbon position of cytosine. There are three major catalytically active mammalian DNMTs, include *DNMT 1, DNMT 3a and DNMT 3b*. *DNMT1* is referred as the "maintenance" methylase which is essential for maintaining DNA methylation patterns in proliferating cells (Li et al., 1992). *DNMT 3a and DNMT 3b* are considered to be *de novo* methylases as they are responsible for the initiation of *de novo* methylation *in vivo* and for establishing new DNA methylation patterns during development (Okano et al., 1999). However, cooperative function has been observed in all three

DNMTs. Tthus, it was suggested that the functional differences among the methylases may due to the genomic regions that they act upon (Liang et al., 2002).

DNA hypermethylation plays a critical role in the gastric cancer progression via transcriptional silencing of cancer related genes by promoter hypermethylation (Figure 1.3) (Worm and Guldberg, 2002). Cancer-related or tumor suppressor gene silenced by gene promoter hypermethylation is common in human cancer. Previous studies suggested that one may classified the methylation pattern in gastric cancer into five different classes: (1) genes are only methylated in cancers (such as *GSTP1* and *RASSF1A*); (2) genes are present with low methyaltion frequency in chronic gastritis, intestinal metaplasia and gastric adenoma but have significantly higher methylation frequency in cancers (such as Cox-2, hMLH1 and p16); (3) genes with low and similar methylation frequency in all different stages of cancer development (such as MGMT); (4) genes with high and similar methylation frequency in all different stages of cancer development; and (5) genes with increased methylation frequency in the progression of cancer (such as DAPkinase, p14, THBS1 and TIMP-3) (Kang et al., 2003). Some of the well known hypermethylated genes in gastric cancer will be further discussed in details in the next paragraph.

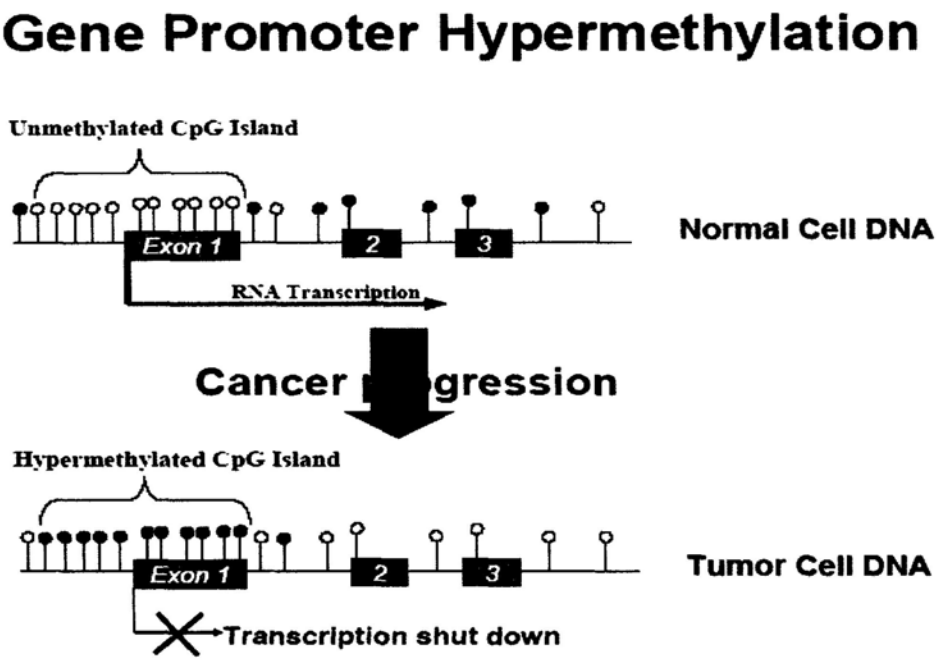


Figure 1.3 Gene promoter hypermethylation associated with cancer progression. Completely unmethylated state of a promoter CpG island (white dots) is associated with transcriptional activity, whereas hypermethylation (black dots) causes transcriptional silencing [modified from (Worm and Guldberg, 2002)].

1.2.1.1 Hypermethylation genes in gastric cancer

p14^{ARF}, *p15*^{INK4B} and *p16*^{INK4a} (CDKN2A) are well know hypermethylated genes in gastric cancer. They all located on chromosome 9p21. Chromosome 9p21 demonstrated a high frequency of loss of heterozygosity in gastric cancers (Iida et al., 2000). The region encodes *p14*^{ARF} and *p16*^{INK4a} which shares an exon in different reading frames. Inactivation of *p16*^{INK4a} by mutations, homozygous deletions or gene methylation is observed in many human cancers. *p16*^{INK4a} specifically inhibits the interactions between Cdk4/Cdk6 and cyclin D1 which resulted in blockage of the progression of cell cycle from G1 to S phase. In our previous study, we found that the frequency of *p16*^{INK4a} methylation in gastric cancer is ~ 45% (To et al., 2002).

p14^{ARF} functions as a stabilizer of the tumor suppressor protein p53 as it can interact and induce degradation of the MDM2 protein that is responsible for the degradation of p53 (Iida et al., 2000). Methylation of *p14*^{ARF} has been reported in gastric cancer, with a frequency of ~32 % in our previous study (To et al., 2002). Another studies demonstrated that *p14*^{ARF} gene is more frequently inactivated by homomozygous deletion or methylation in diffuse-type than in intestinal-type gastric cancer cells. Furthermore, the study also showed that the methylation frequency of *p14*^{ARF} gene is higher in diffuse-type (46%) than in intestinal-type primary gastric cancers (25%) (Iida et al., 2000).

p15^{INK4B} gene is an inhibitor of cyclin-dependent kinase 4 which is an important mediator of cell cycle control especially in the pathway stimulated by transforming growth factor. In our previous study, the frequency of

hypermethylation of $p15^{INK4B}$ was ~ 48% in gastric carcinomas (To et al., 2002). The finding suggested that aberrant methylation of $p14^{ARF}$, $p15^{INK4B}$ and $p16^{INK4a}$ was frequently detected in gastric cancer and may involved in the multistep gastric carcinogenesis.

Some studies have observed aberrant DNA methylation in DNA mismatch-repair gene, like Human MutL Homolog 1 (*hMLH1*). Mutation and promoter hymethylation of *hMLH1* was found to contribute to a substantial proportion of sporadic gastric cancer which displays microsatellite instability phenotypes (To et al., 2002). It was found to be 100% methylated in MSI-H sporadic gastric cancer (Leung et al., 1999). Our previous studies also demonstrated that *hMLH1* had comparable methylation frequency in gastric cancer (29%; 9 of 31) and intestinal metaplasia (22%; 8 of 36) (To et al., 2002).

Increasing evidences have demonstrated that there is an interaction between DNA methylation and histone modifications (Figure 1.4). For example, Methyl-CpG binding protein (MeCP2) has been found to be associated with HDAC3 which histone modification can be induced by DNA methylation changes (Jones et al., 1998). Thus, understanding the relationship between DNA methylation and histone modification may provide further insights in the process of carcinogenesis.

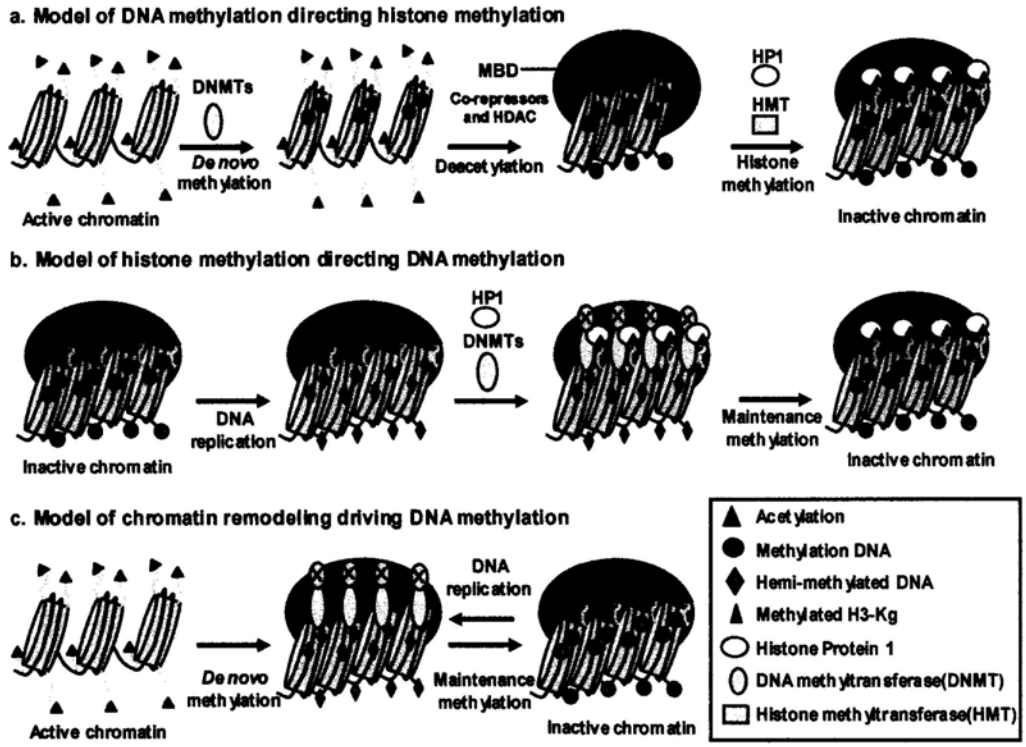


Figure 1.4 Links between DNA methylation, histone modification and chromatin remodelling. In mammalian cells, both DNA methylation and histone modification are involved in chromatin remodelling. Three possible models of how they might influence each other are shown (a) A model of DNA methylation directing histone methylation. DNA methylation patterns are established through *de novo* methylation by DNA methyltransferase DNMT3a and DNMT3b, and are maintained by DNMT1. Methyl-CpG-binding proteins (MBD) and histone deacetylase (HDAC) complexes, such as the MECP2–Sin3a–HDAC complex, are believed to be recruited to the methylated region to induce histone deacetylation (Nan et al., 1998, Jones et al., 1998). The chromatin then attracts histone methyltransferases (HMTs) which methylate the lysine 9 residue on histone H3 (H3-K9) and stabilize the inactive state of the chromatin (Peters et al., 2001, Tachibana et al., 2002). (b) A model of histone methylation directing DNA methylation. Methyl H3-K9 acts as a signal for inactive chromatin by recruiting HP1 to methylated histones, which might in turn recruit DNA methyltransferases directly or indirectly (via unknown factor X) to the silent chromatin to maintain DNA methylation and stabilize the inactive chromatin (Tamaru and Selker, 2001,

Jackson et al., 2002). (c) A model of chromatin remodelling driving DNA methylation. The ATP-dependent chromatin-remodelling and DNA-helicase activities of proteins might facilitate DNA methylation and histone modification by unwinding nucleosomal DNA to increase its accessibility to DNMTs, HDACs and HMTs. The disrupted function of these proteins impairs both DNA methylation and histone methylation, as has been shown in plants (Gendrel et al., 2002, Jeddeloh et al., 1999). The chromatin-remodelling protein (CRP) that is involved in *de novo* methylation has yet to be identified [modified from (Li, 2002)].

1.2.2 Histone modification

1.2.2.1 Chromatin structure

In eukaryotic cells, genomic DNA is packaged with histone proteins and non-histone proteins into precise regulatory complexes known as chromatin (Kornberg, 1974). The fundamental repeating unit of chromatin is the nucleosome. Nucleosomes composed of approximately 146 bp of double-stranded DNA wrapped around an octamer core consisting 4 pairs of small and highly basic histones (H2A, H2B, H3 and H4) in tight 1.75 left-handed superhelical turns (Figure 1.5) (Luger et al., 1997, Kornberg and Lorch, 1999, Kornberg, 1974). The fifth class of histone, Histone H1, lies on the outer portion of the nucleosome and occurs in the chromatin in approximately half the amount of the others types of histones (Marks et al., 2000). Histone H1/DNA complex links nucleosomes to form the primary structure of chromatin while secondary inter-nucleosomal and other DNA-protein interactions form higher order chromosomal structures (Verdin, 2006).

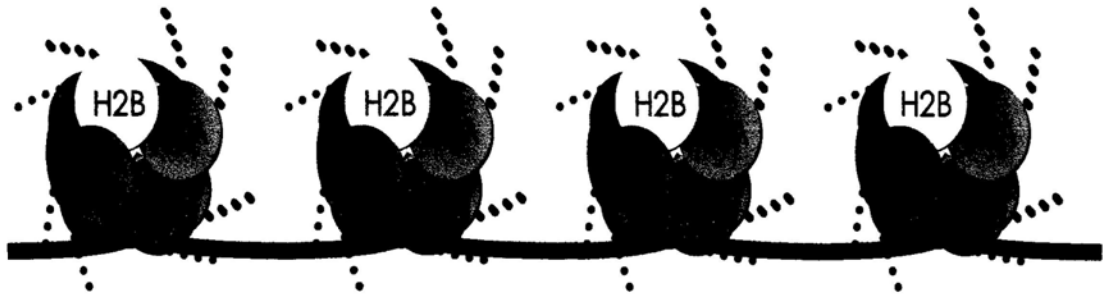


Figure 1.5 Schematic structure of histones in nucleosomes. The core proteins of nucleosomes are designated H2A (histone 2A), H2B (histone 2B), H3 (histone 3) and H4 (histone 4).

Chromatin is organized into two domains which are euchromatin and heterochromatin, based on the chromosomal architecture, transcriptional activity and replication timing (Verdin, 2006, Li, 2002). Heterochromatin domains are largely inaccessible to transcription factors whereas the euchromatin domains are more transcriptionally active (Verdin, 2006). Chromatin structure can be regulated either locally which involves single genes or globally which involves a large domain or entire chromatin (Verdin, 2006). Chromatin contains various proteins that are required for chromatin assembly and packaging, DNA replication, DNA and histone modification and transcription activity (Mai et al., 2005). The mechanisms involve in regulating the chromatin include re-organization of nucleosome position by ATP-dependent remodeling complexes that alter post-translational modification of histones (Narlikar et al., 2002, Lusser and Kadonaga, 2003) (Verdin, 2006).

1.2.2.1.1 Histone code

Histone proteins are composed of two domains, globular carboxyl C-terminal domain and flexible, highly basic amino N-terminal tail domain. C-terminal domain forms the core of the nucleosome while the N-terminal domain located outside of the core particle and is accessible to various modifying agents (Verdin, 2006, Luger et al., 1997). In old days, histones were viewed as inert packaging materials. Recent studies have shown that N-terminal tail protrudes from the nucleosome contain large numbers of sites where multiple post-translational modifications can occur. The pattern of modifications has been termed as histone code (Van Lint et al., 1996, Strahl and Allis, 2000, Turner, 2000). The N-terminal tails serve as the targets for post-translational modifications including acetylation

of lysines by histone acetyltransferases (HATs), methylation of lysines and arginines by histone methyltransferases (HMTs) and phosphorylation of serines by histone kinases (HKs) (Figure 1.6) (Marks et al., 2004, Spotswood and Turner, 2002, Camporeale et al., 2004, Verdin, 2006, Espino et al., 2005, Biel et al., 2005). The histone code hypothesis proposed that multiple histone modifications serve as domains for interaction with specific proteins and such interactions compartmentalize chromatin into heterochromatin and euchromatin patterns that provide a framework for activation or silencing of gene expression (Verdin, 2006). In general, histone modifications are reversible (Verdin, 2006). The modifications can be act in a combinatorial or sequential fashion on one or multiple histone tails which specify unique gene expression patterns (Strahl and Allis, 2000).

Histones are synthesized in cytosol and then assembled into nucleosomes. Some of the modifications of histones occur just after their synthesis, but before their assembly. These histones modifications are added and removed by the action of enzymes that reside in the nucleus, for example, acetyl groups are added to the histone by histone acetyltransferases (HATs) and taken off by histone deacetylases (HDACs). These posttranslational modifications determine not only the structure but also the pattern of chromatin condensation and histone code that imparts gene expression, epigenetic silencing or centromeric functions (Strahl and Allis, 2000, Struhl, 1998).

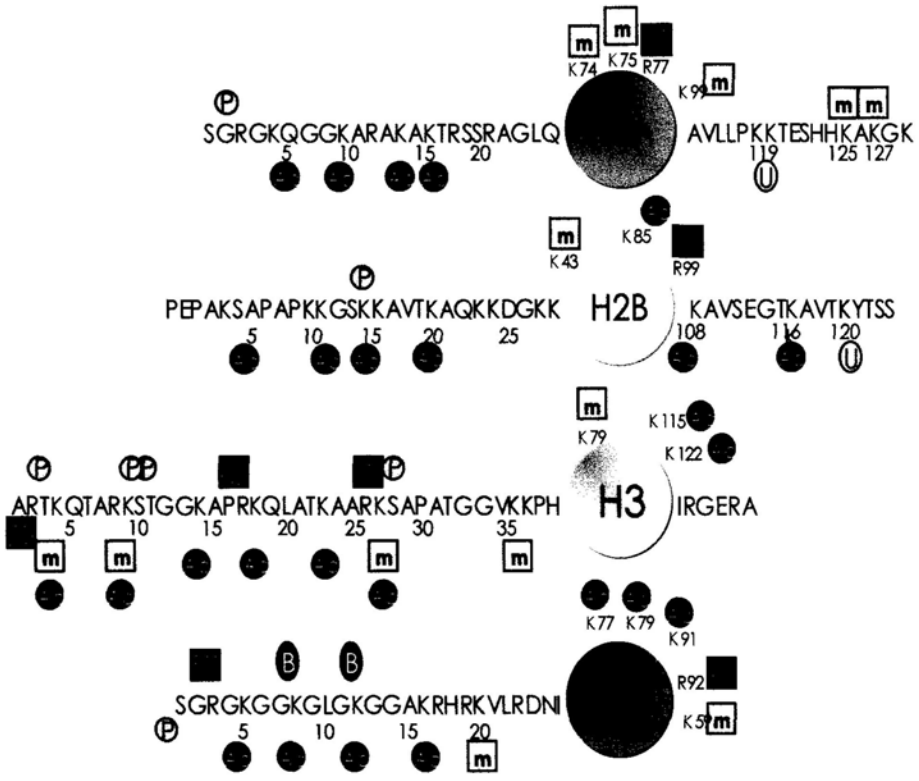


Figure 1.6 Schematic structure of modifications histones at the amino-terminal tails. Lysines (K) in the amino-terminal tails of histones H2A, H2B, H3 and H4 are potential acetylation/deacetylation sites for histone acetyltransferases (HATs) and histone deacetylases (HDACs). Acetylation neutralizes the charge on lysines. The modifications include methylation (M), acetylation (Ac), phosphorylation (P), ubiquitination (U), and biotinylation (B) [modified from (Espino et al., 2005)].

1.2.2.2 Histone acetylation

Since the discovery of reversible acetylation of histone proteins by Vince Allfrey's group in 1964, many studies have been conducted to investigate the role of histone acetylation in transcriptional regulation (Allfrey et al., 1964). Among all the N-terminal histone tail modifications, histone acetylation remains as one of the most abundant and intensely investigated. In euchromatin, acetylation of histones has found to be associated with actively transcribed regions of chromatin. On the other hand, histones at transcriptionally silent heterochromatin regions are frequently deacetylated (Espino et al., 2005, Struhl, 1998). The N-terminal of the histones contains positively charged lysine groups that undergo reversible acetylation and deacetylation.

Histone acetylation is the process of modification of chromatin structure by attachment of acetyl groups to core histones whereas histone deacetylation is the removal of the attached acetyl groups. Histone acetyltransferases (HATs) and histone deacetylases (HDACs) are the two opposing enzymes responsible for the histone acetylation and deacetylation respectively. In unacetylated histones, the N-terminal lysines are positively charged and interact strongly with DNA phosphates of the DNA backbone, increasing the affinity of DNA for the nucleosome surface. This strong interaction may prevent access of transcription factors to promoter regions. However, in the acetylated form, the positive charge of the lysine-amino groups are neutralized and theirs interaction with DNA phosphate group are eliminated. Therefore, it reduces the affinity of histones for DNA and facilitates the binding of transcription factors to promoter regions (Van Lint et al., 1996). This increased accessibility of binding factors and transcriptional machinery

to access to the DNA. Thus, histone deacetylation plays an important role in chromatin-mediated gene repression.

1.2.2.3 Other histone modifications

Other histone modifications include histone methylation, histone phosphorylation, histone ubiquitylation, histone sumoylation and histone poly-ADP ribosylation.

Unlike histone acetylation, histone methylation is a rather stable epigenetic mark which does not change the charges of the histone tails (Rice and Allis, 2001). Histone methyltransferases (HMTases) direct the site-specific methylation of lysine residues in the N-terminal of histone. Recent findings suggested that site-specific methylation is associated with various biological processes ranging from transcriptional regulation and epigenetic silencing via heterochromatin assembly (Rice and Allis, 2001).

Histone phosphorylation was initially reported in the 1960s (Gutierrez and Hnilica, 1967). The well known phosphorylation sites such as H3-S10 and H3-S28 were identified to be associated with cell cycle processes during mitosis and meiosis, as well as in gene activation during interphase (Gurley et al., 1978). All phosphorylated serine residues are located within highly conserved amino acid sequence, Ala-Arg-Lys-Ser (Biel et al., 2005).

Both ubiquitylation and de-ubiquitylation are responsible for gene activation. Ubiquitin is a highly conserved protein that consists of 76 amino acids and related

to proteasome-dependent degradation (Pickart, 2004). The exact function of this modification is still under investigation. In usual circumstance, ubiquitinylation serves as a sign for the proteolytic, ATP-dependent protein degradation by proteasome (Hershko, 1988). During the process, ubiquitin units are transferred to the target protein by a succession of three enzymatic steps (Hershko, 1988).

Sumoylation refers to the post-translational modification by small ubiquitin-like modifier which is a small group of proteins that are related to ubiquitin through secondary and tertiary structure elements (Melchior, 2000). The mechanism of attachment to the target protein is similar to that of ubiquitinylation. Different from ubiquitinylation, sumoylation does not represent a mark for protein degradation (Hay, 2001). The exact functional role of sumoylation remains unclear.

Poly-ADP ribosylation is the attachment of anionic ADP-ribose monomers to the histone tails to generate poly ADP-ribose (PAR) chains. Seven individual PAR polymerases (PARPs) have been discovered in mammals and PARP-1 is dominantly expressed (Hassa and Hottiger, 2002). Beside the formation of poly ADP-ribose, the PARP-1 is also involved in DNA repair, replication and transcription reactions (D'Amours et al., 1999). It also participates directly in the assembly of transcription complexes at enhancers and promoters (Kraus and Wong, 2002).

1.3 HAT, HDAC and HDAC inhibitors

The acetylation of histones is held at equilibrium by the action of the enzyme, histone acetyltransferase (HAT) and histone deacetylase (HDAC) superfamilies. An imbalance of one or the other enzymes can lead to phenotype changes in the cells (Figure 1.7). In general, transcriptional active genes are associated with highly acetylated histones whereas transcriptional repression is associated with low levels of histone acetylation. Within the nucleosome, positively charged hypoacetylated histones are tightly bound to the phosphate backbone of DNA and maintain chromatin in a transcriptionally silent state. Histone acetylation by HATs neutralizes the charges of the histones, disrupts higher-order structures in chromatin and thereby favors the accessibility for transcription factors, transcriptional regulatory complexes, and RNA polymerases to the DNA elements. On the other hand, histone deacetylation by HDACs restores positive charges on lysine residues of core histones and leads to packaging of chromatin into a condensed, tightly supercoiled, repressed structure and thereby, prevent the contact of transcription factors and leading to transcriptionally silent state (Verdin, 2006).

The reversible histone acetylation effects are normally localized to specific portions of DNA and both HATs and HDACs can be associated with specific transcription factors or modulators and being guided to specific coding regions (Emanuele et al., 2008). Thus, the reversible histone acetylation plays a critical role in the modification of chromatin structure, chromatin function and regulation of gene expression. Despite the fact that there are only a small subset of encoding genes (~2-5%) could be transcriptionally regulated by the histone acetylation status (Emanuele et al., 2008), many of these genes exert important roles in cell cycle

control, differentiation and apoptosis (Mai et al., 2005). Thus, altered expression or mutation of genes that encode HATs, HDACs or their binding and recruiting partners can lead to aberrant expression of genes that regulate cellular differentiation, cell cycle, and apoptosis (Mai et al., 2005).

Beside HDACs and HATs activities, other factors include methyl-CpG binding protein and adenosine triphosphate (ATP)-dependent chromatin-remodeling complexes are also involved in the regulation of chromatin structure. Studies have found that these chromatin-modifying complexes interact with HAT and HDAC to regulate transcriptional activity of genes (Bird and Wolffe, 1999, Vignali et al., 2000). Furthermore, HATs and HDACs may also involve in acetylation and deacetylation of non-histone proteins that control cell-cycle progression, differentiation, and/or apoptosis (Mai et al., 2005). Thus, modulating HAT/HDAC activity using specific inhibitors (such as HDAC inhibitors) represents a novel approach to cancer therapy.

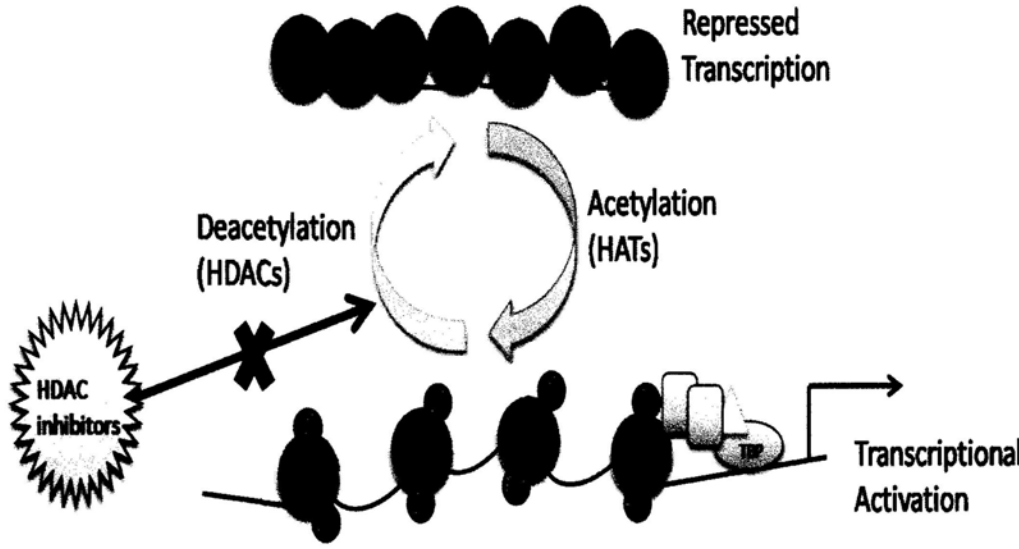


Figure 1.7 Modulation of chromatin structure by histone acetylation and histone deacetylation affects transcription of genes. The core histones interact with other histones and with DNA in the nucleosome. The N-terminal tails of the core histones are modified by the addition of acetyl groups to the side chains of specific lysine residues [modified from (Verdin, 2006)].

1.3.1 Histone acetyltransferase (HAT)

Histone acetyltransferase (HAT) transfer an acetyl group from acetyl CoA to lysine residues in the histone tails and thereby forming ε-N-acetyl lysine (Marmorstein and Roth, 2001). Vince Allfrey and his colleagues were the first group to observe reversible acetylation of histone proteins and hypothesized that acetylation of histone code was related to the regulation of gene transcription (Allfrey et al., 1964). The role of histone acetylation remained controversial until the first HAT was identified in *Tetrahymena thermophila* and showed that it is homologous to the putative transcription adaptor GCN5 characterized in yeast (Brownell et al., 1996). GCN5 has been previously identified in yeast using a genetic screen to search for transcriptional regulators (Verdin, 2006). There are now approximately twenty HAT proteins have been identified from yeast to human. Mammalian HATs are subdivided into two types, type A and B, based on their specific roles. Type A HATs play a key role in the regulation of gene expression as behaving like transcriptional coactivators. While type B HATs are responsible for the recruitment of newly synthesized histones into chromosomes (Emanuele et al., 2008). Furthermore, the HATs are classes into different families based on equence homologies and similarities in biological functions (Roth et al., 2001). Mainly, there are three major families of HATs : 1) generally control non-depressible 5 (GCN5)-related N-acetyltransferase (GNAT), 2) cAMP response element binding protein (CBP/p300) and 3) MYST families (Marmorstein, 2001). GNAT family is the first superfamily that has been identified which includes GCN5, p300/CEP-associated factor (PCAF), Elp3 and activating transcription factor-2 (ATF-2). This family contains a C-terminal catalytic HAT domain and a bromo

domain for specific interaction with acetylated lysine groups (Dhalluin et al., 1999). The CBP/p300 family includes p300 and cyclic adenosine monophosphate response element (CREB)-binding proteins (CBP). CBP and p300 are less substrate-specific and serve as global modulators for transcription (Biel et al., 2005). The members in this family have a 500 residue catalytic HAT domain, a bromo domain and a cysteine-rich motif which may associate with protein-protein interactions (Marmorstein, 2001). Members of the MYST family include monocyte leukemia zinc-finger protein (MOZ), Ybf2/Sas3, Sas2, Tip60, Esa1 and MOF. They have a 250 amino acid catalytic-rich motif and an N-terminal chromo domain for the specific recognition of methylated lysine groups and participated in a much broader range of biological process (Utley and Cote, 2003).

1.3.2 Histone deacetylase (HDAC)

In the early 1990s, a Japanese group screened for compounds that could return spindle-like transformed NIH3T3 cells to normal fibroblast-like morphology and they identified an epoxyketone-containing cyclic tetrapeptide called trapoxin (Kijima et al., 1993). Later, trapoxin was found to inhibit the histone deacetylation and induced the accumulation of hyperacetylated core histones in living cells at low nanomolar concentrations (Kijima et al., 1993). Since then, it was becoming much clear that reversible acetylation of histone plays a critical role in the regulation of chromatin structure and transcriptional activity. The first histone deacetylase (HDAC) was subsequently identified in 1996 when the first HAT was also identified at the same year (Taunton et al., 1996). So far, there are eighteen human HDACs have been identified. Human HDACs are classified into three major

classes based on the sequence homology with the yeast *Saccharomyces cerevisiae* histone deacetylases Rpd3, Hda1 and Sir2 - Class I (HDAC 1-3, HDAC 8 and HDAC 11); Class II (HDAC 4-7 and HDAC 9-10) and Class III (SIRT 1-7)(Verdin, 2006).

1.3.2.1 Class I

The first histone deacetylase that identified in mammals was HDAC 1 and it was considered as the prototype of this large family of enzymes. Class I human HDACs members include HDAC1, HDAC2, HDAC3, HDAC8 and HDAC 11, they all have homology to a yeast protein called reduced potassium dependency 3 (Rpd3). The members of this class are around 400 to 500 amino acids long and are primarily located in the nucleus (except for HDAC 3) (Mai et al., 2005). They cleave the acetamide bond by activating a water molecule with a zinc atom coupled to a histidine-aspartate charge-relay system (Finnin et al., 1999). They are ubiquitously expressed in many human cell lines and tissues and the molecular masses are approximately 22-55 kDa (Marks et al., 2004). HDAC enzymes from this family access specific regions of DNA through interaction with a numbers of transcription and chromatin-related factors that recruit class I HDACs to specific chromosomal regions. Thus, class I HDACs need to interact with DNA binding protein to facilitate its access to chromatin for transcriptional repression (Verdin, 2006). Comparing to class II HDACs, class I HDACs seem to interact with a different set of proteins *in vivo* (Mai et al., 2005).

HDAC 1 and HDAC 2 have a C-terminal retinoblastoma (Rb) binding motif adjacent to a basic region and are associated with other multiprotein complexes

(Thiagalingam et al., 2003). HDAC 1 and HDAC 2 together with histone chaperoned retinoblastoma associated proteins (RbAp) 46 and 48 form the core complex. This core complex can interact with Sin3 and SAP30 to form Sin3 corepressor complex or interact with MBD3, MTA2 and Mi2 to form NuRD corepressor complex. HDACs provide enzymatic activity whereas Sin3 coordinates the interaction between HDACs and sequence-specific DNA binding proteins in the complex. RbAp proteins seem to play a role in stabilizing the interaction between the complex and the core histones located within nucleosomes (Verdin, 2006).

Similar to HDAC 1 and HDAC 2, HDAC3 also involved in transcriptional repression but seem to be involved in repression of a specific group of genes, especially for genes that are involved in nuclear receptor signaling (Verdin, 2006). HDAC8 is the second shortest human HDAC after HDAC 11, as it contains a shorter C-terminal extension compared to other class 1 HDACs (de Ruijter et al., 2003). HDAC 8 lack of some C-terminal domains that take part in the recruitment of the enzyme to the protein complexes. Unlike other class 1 HDACs, HDAC8 can be phosphorylated in the N-terminal (Hu et al., 2000). HDAC 11 has the properties of both class I and II HDACs, so it was suggested to be classified into a new class, class IV (Gao et al., 2002). All the members of this family have shown to be sensitive to histone deacetylase inhibitors, such as Trichostatin A (TSA) and Suberoylanilide Hydroxamin Acid (SAHA).

1.3.2.2 Class II

Class II HDACs include HDAC4, HDAC5, HDAC6, HDAC7, HDAC 9, and HDAC 10 and are homologous to the budding yeast Hda1 (Gray and Ekstrom,

2001) . In humans, class II HDACs can be further subdivided into two subclasses, IIa (HDAC 4, -5, -7 and -9) and IIb (HDAC 6 and -10), based on the sequence homology among the deacetylase domains (Fischle et al., 2001). The molecular masses of class II deacetylases are 120-135 kDa and they are larger than the members in class I family (Mai et al., 2005). Class I and II HDACs do share some conserved sequence homology among their deacetylase domains (Mai et al., 2005). In general, class II HDACs members have a COOH-terminus catalytic domain and a functional important non-catalytic N-terminal domain. Class IIa HDACs have a striking feature which is their ability to shuttle between the nucleus and the cytoplasm (Fischle et al., 2001). The non-catalytic N-terminal domain mediates both the recruitment of class IIa HDACs to specific promoters and signal-dependent shuttling between the nucleus and the cytoplasm (Fischle et al., 2001). For HDAC 6, it contains two catalytic domains in the NH_2 terminus (Verdin, 2006). HDAC 10 consists of a NH_2 terminus catalytic domain and a COOH-terminus that shared homology with the catalytic domain (Thiagalingam et al., 2003). HDAC 10 is more similar to HDAC 6 and therefore, HDAC 6 and HDAC 10 are classified as subclass IIb (Verdin, 2006, Mai et al., 2005).

As mentioned before, class I and II HDACs seem to interact with a different set of proteins *in vivo*. Class II HDACs are associated with DNA binding transcriptional factors (such as MEF2, BCL2, PLZF and TR2), with transcriptional co-repressors (such as N-CoR, SMRT, B-CoR and CtBP) and with heterochromatin protein 1 (HP1) (Bertos et al., 2001, Mai et al., 2005). These transcriptional factors play an important role in recruiting HDAC-containing multiprotein complexes to specific promoter regions. Initially, it is believed that class II HDACs display

tissue-specific expression in human instead of ubiquitouly expressed. However, recent studies have found that when comparing the ESTs from different tissue libraries like NCBI Unigene resource, class IIa HDACs are widely expressed. Nevertheless, despite they are widely expressed, they have higher expression in a limited subset of cell types (Bertos et al., 2001, Verdin, 2006). Class II HDACs are also are sensitive to histone deacetylase inhibitors such as Trichostatin A (TSA), Suberoylanilide Hydroxamin Acid (SAHA) and related compounds.

1.3.2.3 Class III

Class III HDACs were identified based on the similarity to the yeast protein NAD^+-dependent HDAC silent information regulator 2 (Sir2), which is involved in gene silencing via the generation of heterochromatin-like compacted chromatin that is hypoacetylated in histone H3 and H4 tails (Verdin, 2006). In humans, class III HDACs are called sirtuins. The mechanism of enzymatic reactions of sirtuins is very different from class I and II HDACs (Mai et al., 2005). Thus, the inhibitors and mechanism of inhibition of this class are also different from other classes of HDACs. Class III HDACs appear not to act on histones and are insensitive to HDAC inhibitors such as Trichostatin A (TSA) and Suberoylanilide Hydroxamin Acid (SAHA) (Marks et al., 2001). They are required for gene silencing at the telomerase, the silent mating-type loci and ribosomal DNA loci (Guarente, 2000, Imai et al., 2000, Verdin, 2006).

1.3.2.4 Regulation of HDAC activity

HDACs activities are precisely regulated by multiple mechanisms, which includes protein-protein interactions, post-translational modifications, subcellular

localization, availability of cofactors and proteolytic processing (Sengupta and Seto, 2004).

Protein-protein interactions

Regarding to protein-protein interactions, for example, HDAC 1 and HDAC 2 together with RbAp 46 and 48 form a core complex that can interact with Sin3 and SAP30 or MBD3, MTA2 and Mi2 to form a corepressor complex. Studies have showed that HDAC activity of the core complex was severely compromised as compared to the holo-complex (Zhang et al., 1999). Another protein-protein interaction example is the interaction of HDAC3 and SMRT/N-CoR which resulted in the stimulation of HDAC3 enzymatic activity (Zhang et al., 2002).

Post-translational modifications

Phosphorylation of HDAC1 represents an example of the regulation by post-translational modification. The phosphorylation of HDAC1 alter the HDAC conformation into a more favorable enzymatically active form (Sengupta and Seto, 2004). Site-directed mutation of particular phosphorylated serine to alanine or deletion of C-terminal in HDAC1 would affect the binding with RbAp 48, MT2A, mSin3A and CoREST and reduced the enzymatic activity and transcriptional repression (Pflum et al., 2001).

Subcellular localization

HDACs must reside in the nucleus in order to deacetylase histone. Class II HDAC 4, -5, -7 and -9 shuttle between the nucleus and the cytoplasm and involve the interaction with 14-3-3 protein. When N-terminal serine residues of HDACs are

phosphorylated, HDACs will bind to 14-3-3 proteins, which resulted in cytoplasmic subcellular localization of HDACs (Verdin, 2006).

Availability of cofactors

Class III HDACs require the NAD^+ for catalytic activity. The exact physiological regulator of the SIR2 enzymes has not been fully understood. However, two alternative models of SIR2 have been proposed (1) increase NAD+/NADH ratio or (2) decreasing the level of nicotinamide, an inhibitory product of SIR2 (Bitterman et al., 2002). The requirement of NAD^+ for SIR2 activity can regulate class III HDACs in response to the metabolic status of the cells (Sengupta and Seto, 2004). Human SIRT3 is an inactive precursor which is imported into the mitochondria and subsequently cleaved by matrix processing peptidase to form enzymatically active form (Sengupta and Seto, 2004).

Proteolytic processing

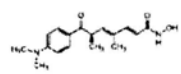
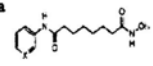
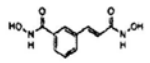
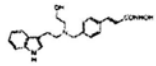
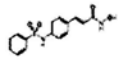
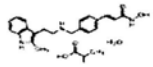
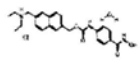
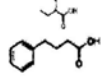
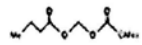
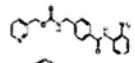
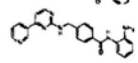
Proteosome-mediated degradation of HDAC1 of the HDAC1/mSin3A corepressor complex during steroid-induced pre-adipocyte differentiation would resulted in complex depletion (Wiper-Bergeron et al., 2003). N-CoR which is a HDAC3 associated protein can be proteolysis via mSiah2. Thus, targeted proteolysis of N-CoR could be a potential indirect mechanism for controlling HDAC3 activity (Zhang et al., 1998).

1.3.3 HDAC inhibitors

Sodium butyrate is the first identified HDAC inhibitor. In 1978, scientists

discovered that sodium butyrate could inhibit histone deacetylase in cultured cells (Candido et al., 1978). Since the discovery of sodium butyrate, a numbers of HDAC inhibitors have been identified (Table 1.2) that could inhibit deacetylation of both histone and non-histone proteins. HDAC inhibitor causes the accumulations of acetylated histones in the nucleosomes which loosen chromatin structure and resulted in re-expression of specific genes. They can be divided into two major classes, natural and synthetic. The classes of the compounds can be further sub-divided based on the structure, including hydroxamates (such as SAHA, pyroxamide, TSA, oxamflatin and CHAPs), cyclic peptides (such as trapoxin, apicidin and depsipeptide), aliphatic acids (such as valproic acid) and benzamides (such as MS-275). HDAC inhibitors not only contributed to the discovery of HDAC enzyme and explore their functions but also emerged as potential promising therapeutic agents against diseases including cancer. Several HDAC inhibitors are currently being investigated in clinical trials. For example, SAHA has already approved by the FDA department in United States in 2006 to be used for treatment of cutaneous T-cell lymphoma (CTCL) (Verdin, 2006).

Table 1.2 Histone deacetylase inhibitor (partial list) [reference from (Dokmanovic et al., 2007)

Class	Compound	Structure	HDAC Target (Potency)	Effects on Transformed Cells	Stage of Development
Hydroxamates	TSA		Class I and II (nmol/L)	TD;GA;A;AI;AE	N/A
	SAHA, Zolinza Vorinostat		Class I and II (μmol/L)	TD;GA;AI;AE;MF;AU;S;PP;ROS-CD	Merck Food and Drug Administration approved for CTCL
	CBHA		N/A (μmol/L)	GA;A;AI;AE	Merck
	LAQ-824		Class I and II (nmol/L)	GA;A;AI	Novartis phase I
	PDX-101		Class I and II (μmol/L)	GA; A	TopoTarget phase II
	LBH-589		Class I and II (nmol/L)	GA;A;ROS-CD	Novartis phase I
	ITF2357		Class I and II (nmol/L)	GA;A;AI	Italfarmaco phase I
	PCI-24781	N/A	Class I and II (NA)	N/A	Pharmacyclics phase I
Cyclic peptide	Depsipeptide		Class I (nmol/L)	TD;GA;A;AI;AE;MF;ROS-CD	Gloucester Pharmaceuticals phase IIb
Aliphatic acids	Valproic acid		Class I and IIa (mmol/L)	TD;GA;A;S	Abbot phase II
	Phenyl butyrate		Class I and IIa (mmol/L)	TD;GA;A;AI;AE	Phase II
	Butyrate		Class I and IIa (mmol/L)	TD;GA;A;AI;AE	Phase II
	AN-9		N/A (μmol/L)	TD;GA;A	Titan Pharmaceuticals phase II
Benzamides	MS-275		HDAC-1, -2, -3 (μmol/L)	TD;GA;A;AI;AE;ROS-CD	Schering AG phase II
Miscellaneous	MGCD0103		Class I (μmol/L)	TD;GA;A	Methylgene phase II

GA, growth arrest; TD, terminal differentiation; A, apoptosis; AI, cell death by activating intrinsic apoptotic pathway; AE, cell death by activating extrinsic apoptotic pathway; MF, mitotic failure; AU, autophagic cell death; S, senescence; PP, polyploidy; ROS-CD, reactive oxygen species facilitated cell death; N/A, unknown; CBHA, M-carboxycinnamic acid bishydroxamate; CTCL, cutaneous T-cell lymphoma; PTCL, peripheral T-cell lymphoma.

1.3.3.1 Class I/II natural inhibitors

As mentioned above, sodium butyrate is the first known HDAC inhibitor. Butyrate, a carboxylate, is a product of fatty acid metabolism and bacterial fermentation of fiber in colon. Butyrate has a carboxylate in the metal binding moiety and this class has generally poorer HDAC inhibitory activity in comparison with other HDAC inhibitors. Other members of this class include phenyl butyrate and valproic acid. Sodium butyrate possesses some selectivity that poorly inhibits class IIb HDACs, such as HDAC6 and HDAC10. The poor inhibition activity of butyrate may due to the weak coordination with zinc ions when compared to other functional groups such as hydroxamic acid (Verdin, 2006). Previous studies of sodium butyrate in cancer cells showed that it could reduce cellular proliferation, enhance differentiation and induce apoptosis (Chopin et al., 2002). However, the effective concentration for butyrate is relative high, exceeds milimolar levels, as compared to other HDAC inhibitors. Butyrate treatment may also affect other enzymes, cytoskeleton and cell membrane. Thus, these may limited the potential clinical usage of this compound. Pivaloyloxymethyl butyrate (AN-9), an acyloxyalkyl ester pro-drug of butyrate has a higher cell permeability and greater ability to inhibit cancer cell proliferation than butyrate (Zimra et al., 1997). The possible clinical usage of AN-9 required further investigations.

Trichostatin A (TSA) was the first natural hydroxamate compound discovered to inhibit specific HDACs (Yoshida et al., 1990). It belongs to the hydroxamic acids subclass. The hydroxamic acid coordinates the zinc present at the bottom of the binding pocket of HDAC through its carbonyl and hydroxyl groups which resulted in the formation of a penta-coordinted zinc. TSA and its derivative trichostatin C

were originally isolated from metabolites of *Streptomyces hygroscopicus* as a new antifungal antibiotic against *Trichophyton* (Tsuji et al., 1976). Later, it was rediscovered as a strong differentiation inducer in murine erythroleukemia (MEL) cells (Yoshida and Beppu, 1988). Trichostatins found to have the ability to inhibit cell growth and enhance differentiation at low concentrations. TSA was also shown to induce cell cycle arrest at both G1 and G2 phases in a number of normal and cancer cell lines (Yoshida and Beppu, 1988).

Trapoxin (TPX) is belongs to the subclass of electrophilic ketones. It was originally isolated from fungus *Helioma amibien* and was originally identified as a fungal metabolite. It is a cyclic tetrapeptide containing a unique amino acid which could return spindle-like transformed NIH3T3 cells to normal fibroblast-like morphology (Kijima et al., 1993). Trapoxins induce the accumulation of hyperacetyated histones by irreversibly inhibited the deacetylation of acetylated histone in living cells at low nanomolar concentrations (Kijima et al., 1993). The epoxy ketone group of the unique amino acid seems to play a role in forming a covalent bond between TPX and the active sites residues of HDAC enzymes (Kijima et al., 1993).

In addition, there are others natural products have been isolated such as apicidin, depsipeptide and depudecin. Apicidin is a cyclic tetrapeptide metabolite isolated from *Fusarium pallodoroseum* and is under the electrophilic ketone subclass (Verdin, 2006). It is a potent and broad-spectrum antiprotozoal agent that can reversibly inhibit HDAC activity. Apicidin contains an ethyl ketone moiety instead of an epoxy ketone (Verdin, 2006). Depsipeptide (also known as FK228 and

FR-901228) was a cyclic peptide that isolated from *Chromobacterium violaceum* as a bacterial metabolite. The chemical structure of this HDAC inhibitor is different from others as it does not contain a functional group that interacts with the zinc ion of the HDAC binding pocket. However, depsipeptide showed strong HDAC-inhibitory activity and is one of the first potent compounds entering into clinical trials (Nakajima et al., 1998). In recent study, it was found that the depsipeptide was activated by chemical reduction that generates two free thiol groups. One of the thiol groups is accessible to the catalytic zinc of the HDAC enzymes. Thus, depsipeptide is under the subclass of thiols and is currently under phase II clinical trial (Verdin, 2006). Depudecin was isolated from the fungus *Alternaria brassicicola* and could induce the morphological reversion of transformed NIH3T3 cells. Depudecin is a structurally novel natural product and can irreversibly inhibit HDAC activity effectively both *in vivo* and *in vitro* (Kwon et al., 1998).

1.3.3.2 Class I/II synthetic inhibitors

Most of the identified synthetic inhibitors are under the hydroxamic acid subclass, such as oxamflatin and suberoylanilide hydroxamic acid (SAHA). These inhibitors contain hydroxamic acid as the functional group. The discovery of SAHA began with an observation that dimethylsulfoxide (DMSO) caused terminal differentiation of the virus-transformed cells, murine erythroleukemia cells (Friend et al., 1971). Later, it was found that the polar group of DMSO is responsible for the induction of differentiation of murine erythroleukemia cells. Then, it lead to the discovery of hexamethylene bisacetamide (HMBA) and a series of bishydroxamic acids which can induced growth arrest, cell death and differentiation of various

transformed cells and selectively altered the expression of a subset of genes (Marks and Rifkind, 1978, Richon et al., 1996, Marks, 2007). SAHA was one of the compounds of bishydroxamic acids. SAHA is HDAC inhibitor that is active at nanomolar concentration. It inhibits both class I and II HDAC enzymes. X-ray crystallographic studies have shown that the hydroxamic acid group of SAHA interacts with a zinc atom at the rim, walls and bottom of the catalytic site inside the enzyme pocket of a histone deacetylase-like protein (HDLP) (Finnin et al., 1999). The polymethylene chain of SAHA extended down to a narrow tube-like tunnel to get to the zinc atom whereas the phenyl group was on the hydrophobic surface of the enzyme. This interaction between the SAHA and the catalytic site of the enzyme pocket eventually block the substrate in accessing to the zinc atom (Marks, 2007). The active catalytic site of the HDLP is based on the HDAC homologue from the hyperthermophilic bacterium, *Aquifex aeolicus* that shares 35% identity with human HDAC1 over 375 residues (Finnin et al., 1999). In addition, other hydroxamate inhibitors such as TSA, similarly insert into the catalytic pocket and binds to the zinc ion of HDACs to prevent the binding of the substrate and deacetylation (Finnin et al., 1999). Currently, a numbers of clinical trials with SAHA are ongoing. SAHA has been administrated intravenously to patients with refractory hematologic and solid tumors and the drug showed significant anticancer activity in a numbers of human cancers (Kelly et al., 2003). For oral administrative, SAHA also showed good oral availability and favorable pharmacokinetics (Kelly et al., 2005). The side effects of SAHA included fatigue, diarrhea, anorexia and dehydration. However, the side effects were well tolerated by patients and were all reversible on cessation of therapy for 4-7 days (Kelly et al., 2005). SAHA has been approved by the Food and Drug Administration (FDA) for a new drug application

for the treatment of cutaneous T-Cell lymphoma (CTCL). SAHA is commercialised as "vorinostat" by Merck. To date, the National Cancer Institute has listed 36 clinical trials using SAHA for monotherapy and in combination with other agents (Marks, 2007).

Others synthetic compounds include Cyclic hydroxamic acid-containing peptide (CHAP), MS-275 and CI-994. CHAP is a hybrid of TPX and TSA in which the epoxyketone is replaced with hydroxamic acid and is a member of hydroxamic acid subclass. Although CHAP is very potent, it has been prohibited from clinical trials because of the high toxicity. MS-275 is a member of anilide class of inhibitors and it includes bezamides with a phenylene diamine as the functional group. This class of HDAC inhibitors interacts with HDAC through the two-substituted anilide moiety. MS-275 has been shown to inhibit cell growth of various solid cancer cell lines and so in human xenografts (Saito et al., 1999). This class showed weak inhibitory effect on HDAC as compared with hydroxamic acid subclass (Verdin, 2006). CI-994 is an analog of MS-275 and was originally identified as an angiogenesis inhibitor. Both CI-994 and MS-275 have already entered into phase II clinical trail as a potential anticancer drug (Verdin, 2006).

1.3.3.3 Anti-tumor activity of HDAC inhibitors

Growing evidences have been shown that HDAC inhibitors have the potential to activate differentiation programs, inhibit cell proliferation and induce apoptosis in transformed cells, including soild (neuroblastoma, glioma, melanoma, breast, ovary, colon, bladder, gastric, pancreas, lung and prostate) and hematological malignancy (leukemia, lymphoma and multiple myeloma) (Verdin, 2006).

Selectively knockout certain HDACs in mouse embryonic stem cells could also resulted in embryonic lethality (Lagger et al., 2002, Glaser et al., 2003a). This may correlated with an increase in histone acetylation and activation of selected group of genes transcription.

The general mechanism by which HDAC inhibitors exhibit anti-tumor activity is still unclear. It is believed that the cellular effect of individual HDAC inhibitors depend on the concentration, the duration of exposure and is cell-line specific (Mei et al., 2004). The suggested mechanisms of anti-cancer effects which HDAC inhibitors exert biological activities are through the deacetylase inhibition of histone and non-histone proteins (Figure 1.8). As HDAC can bind to, deacetylate and regulate the activity of both histone and non-histone proteins, thus inhibition of HDAC can initiate various gene transcriptional and non-gene transcriptional effects (Bolden et al., 2006).

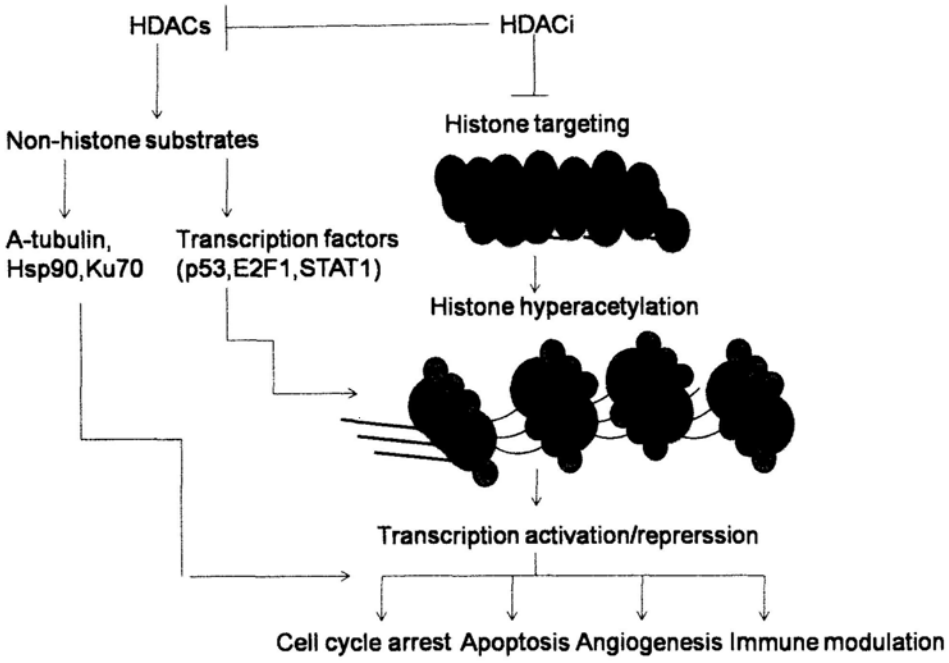


Figure 1.8 Effects of histone deacetylase inhibition on histone proteins and non-histone proteins. **a)** HDAC can bind to, deacetylate and regulate the activity of a number of other proteins, including transcription factors and proteins with diverse biological functions (α-tubulin, Ku70 and heat-shock protein 90 (Hsp90)). Hyperacetylation of transcription factors with HDAC inhibitors can augment their gene-regulatory activities and contribute to the changes in gene expression observed following direct HDAC inhibitor-mediated histone hyperacetylation. Hyperacetylation of proteins such as Ku70 and Hsp90 by HDAC inhibitor might have no direct or indirect effect on gene expression but could be important for certain biological effects of HDACi, in particular the induction of apoptosis and cellcycle arrest. **b)** Antitumour effects of HDAC inhibitor. HDACi can affect tumour cell growth and survival through multiple biological effects. HDACi induce cell-cycle arrest at the G1/S boundary through upregulation of p21 and/or through downregulation of cyclins. HDACi can suppress angiogenesis through decreased expression of pro-angiogenic factors such as VEGF, HIF1α and chemokine (C-X-C motif) receptor 4 (CXCR4). HDACi can also have immuno modulatory effects by enhancing tumour cell antigenicity (upregulation of major histocompatibility complex class I and II, MICA,CD40) and by altering the expression of key cytokines including tumour-necrosis factor-α, interleukin-1 and interferon-γ [modified from (Bolden et al., 2006)].

1.3.3.3.1 Gene transcription effects of HDAC inhibitors

HDAC inhibitors modulate gene expression through histone hyperacetylation on both histones and non-histones proteins. Based on the microarray results of HDAC inhibitors on gene expression, the effects of HDAC inhibitors seem to be highly selective and affect approximately only 1 to 2 % of the total genes (Van Lint et al., 1996). For example, using differential display to compare the differences in gene expression on lymphoid cell lines found that only 2% of genes out of 340 were affected after TSA treatment (Van Lint et al., 1996). In another studies, high-throughput microarray analysis of gene expression profiles using three different HDAC inhibitors (SAHA, TSA and MS-27-275), showed similar differential expression patterns (Glaser et al., 2003b). Subsequent microarray studies were also showed that only a selected set of genes were altered that often involved in cell cycling, apoptosis, signal transduction, metabolism, transcription, cytoskeletal structure and adhesion. This suggested that HDACs are recruited to specific promoters and regulate a sub-group of genes. However, the underlying mechanism that regulates the specific gene expression profile is not well understood (Verdin, 2006)

For example, $p21^{WAF1}$ is one the target of HDAC inhibitor and up-regulation of $p21^{WAF1}$ would cause G1 cell cycle arrest (further discussed in detail in cellular effects section). In additional to $p21^{WAF1}$, other genes being reported to be up-regualted by HDAC inhibitors include *CDKN2A, CDKN1B, CDKN1C*, $p27^{KIP}$, RARβ, cyclin E, thioredoxin and thioredoxin binding protein 2 (*TBP2/TXNIP*) (Marks, 2007) (Table 1.3). SAHA has been reported to up-regulate the expression of vitamin D upregulated protein 1/thioredoxin-binding protein-2

(*TBP-2/VDUP-1/TXNIP*) and down-regulate the expression thioredoxin. TXNIP will be further discussed in Results and Discussions section. However, most of these genes were only shown to be up-regulated upon treatment of HDAC inhibitors, and whether theses genes are directly regulated hy histone acetylation are not well demonstrated.

Table 1.3 Genes reported to be transcriptionally regulated by HDAC inhibitors (Marks, 2007).

Induced		***Repressed***	
Cell cycle	p1 and cyclin E	Cell cycle	Cyclin D1 and A, and thymidylate synthase
Proapoptotic	Bak, BAX, CD95, and its ligand gelsolin GADD45β, p53, Apaf-1 DFF45α, Bim BAD, TRAIL, DR5, Fas and its ligand, and Caspase 9, -8 and -3	Antiapoptotic	Bcl-2, Bcl-XL, c-FLIP, survivin, XIAP,
Redox Components	Thioredoxin-binding protein-1, thioredoxin, glutaredoxin and methallothionein 1L	Angiogenic factor	Vascular endothelial growth factor and HIF-Loc
Chromatin structure	Histone H2B	Lipopolysaccharide-induced inflammatory cytokines	TNF-α, IFN-γ and IL-1β and -6
Retinoic acid pathway	RARβ	Signal transducer and activator of transcription 5-controlled genes	STAT5

Abbreviations: IL-1β ,interleukin-1β , IFN-γ, interferon-y; TNF-α, tumor necrosis factor-α.

1.3.3.3.2 Non-transcriptional effects of HDAC inhibitors

In recent years, it was found that non-histone acetylases modify the lysine residues of various transcription factors. Theses include p53, E2F1, T-cell factor (TCF), GATA1, nuclear factor-κB and hypoxia-inducible factor-1α (HIF-1α) and their functions (DNA binding, protein-protein interaction, cellular localization and proteasomal degradation) are affected (Table 1.4) (Verdin, 2006). HDAC inhibitors increased the acetylated form of p53 which then increased $p21^{WAF1}$ levels and blocked cell cycle progression. Increased acetylated form of E2F1 may promote retinoblastoma protein (Rb)-mediated differentiation (Martinez-Balbas et al., 2000, Gu and Roeder, 1997, Verdin, 2006). Another studies regarding the nuclear factor-κB (NF-κB) also showed that acetylated NF-κB promote prosurvival transcription proficiency. Inhibitor of NF-κB was unable to bind to the acetylated form of NF-κB (Chen et al., 2001).

Studies indicated that HDAC inhibitors increased acetylated heat shock protein (Hsp90). The chaperone activity of Hsp90 was inactivated and leaded to destabilization of various protein including AKT, mutant p53 and Her-2/neu (Fuino et al., 2003). For example when human breast cancer cells were exposed to HDAC inhibitor NVP-LAQ824, it increased the acetylation of Hsp90 which leaded to inefficient binding of ATP. This resulted in Her-2/neu oncoprotein destabilization and facilitated the polyubiquitination and proteasomal degradation (Fuino et al., 2003). Studies have also suggested that p53 was regulated by various degree of protein acetylation and mutant p53 was depleted upon HDAC inhibitor treatment (Verdin, 2006).

Table 1.4 Non-histone proteins that regulated by HDAC inhibitors (partial list) (Marks, 2007).

Protein	***Function***	***Protein***	***Function***
Bcl-6	Oncoprotein	LEF/TCF	Lymphoid enhancer factor
P53	Tumor suppressor	Ku70	Autoantigen with multiple function, including DNA repair
		H1F-1α	angiogenesis
GATA-1	Transcription factor	WRN	Werner helicase
E2F-1	Transcription factor	Smad7	Transcription factor
Rb	Tumor suppressor	TFIIF	Transcription machinery
c-Jun	Transcription factor	α-Tubulin	Structural protein
HMGI(Y)	Chromatin structure	ACTR	Nuclear receptor coactivator
Androgen Receptor	Signal transduction	EKLF	Erythroid kruppel-like factor
YY-1	Transcription factor	NF-$^{\kappa}$B(RelA)	Transcription factor
MyoD	Transcription factor	Importin α7	Nuclear pore protein
Hsp90	Chaperone protein	TFIIE	Transcription machinery
β-Catenin	Signal transduction	TFJB	Transcription factor

Abbreviations: ACTR, nuclear receptor co-activator; E2F, erythroid kruppel-like factor; HIF, -1α, hypoxia inducible factor; HMG(Y), high –mobility group protein; LEF, lymphoid-enhancer factor/T-cell factor; TFII, Transcription factor II; WRN, Werner helicase; YY-1, Yin Yang 1 transcription factor.

1.3.3.3.3 Anti-cancer effects of HDAC inhibitors

Induced apoptosis

HDAC inhibitors could induce apoptosis in various tumor cells but the molecular mechanisms underlying their anticancer effects are poorly understood. Evidences have indicated that HDAC inhibitors can activate both "extrinsic" death receptor pathway and "intrinsic" mitochondrial pathway (Peart et al., 2003). The death receptor pathway involves binding of ligands to their cognate death receptors (such as CD95/Fas, tumor necrosis factor receptor, and TRAIL receptor), resulting in binding of adaptor proteins, recruitment and activation of membrane caspases (such as caspase-8 and caspase-10). These enzymes subsequently activate effector caspases (such as caspase-3), resulting in apoptosis by the cleavage of a number of nuclear and cytoplasmic substrates (Peart et al., 2003). The intrinsic pathway is initiated by stress stimuli such as chemotherapeutic drugs, ionizing radiation, hypoxia, oncoproteins and growth factor withdrawal. Stress stimuli disrupts the mitochondrial membrane and induces the release of mitochondrial proteins, which then induces activation of caspase 9 via assembly of the apoptosome (Bolden et al., 2006). Studies have found that both butyrate and TSA could convert the caspase-3 of intrinsic pathway to catalytically active protease which resulted in apoptotic cell death (Mei et al., 2004). Other studies have demonstrated that SAHA and oxamflatin were able to activate the intrinsic pathway through cleavage and activation of caspase-2 and Bid in leukemia and colon carcinoma cells (Peart et al., 2003). Suberic bishydroxamic acid (SBHA) has also been reported to induce apoptosis through a caspase-dependent and a mitochondrial pathway in melanoma cell lines (Zhang et al., 2003).

Cell cycle alteration

HDAC inhibitors could induce either G1 and/or G2/M phase cell cycle arrests. Cell cycle progression is controlled by the orderly activation of the members of cyclin dependent kinases (cdks). These kinases are regulated in different manner, such as association with regulatory cyclin subunit, phosphorylation of both the cdk and cyclin subunits and association with inhibitory protein subunits (cdk inhibitors) (Verdin, 2006). For example, in order to progress form G1 into replicative S phase, it requires sequential activation of cyclin D/cdk4 or cdk6 and cyclin E/cdk2 complexes which thereby phosphorylate retinoblastoma protein (Rb) (Verdin, 2006). One of the cell cycle gene targets of HDAC inhibitors is CDKN1A, which encodes the cyclin-dependent kinase (cdk) inhibitor $p21^{WAF1}$. $p21^{WAF1}$ gene is localized on chromosome 6p21.2 and is a well studied tumor suppression gene. Treatment of HDAC inhibitor increases histone acetylation in the promoter region of the $p21^{WAF1}$ gene which increases the accessibility of Sp1/Sp3 transcriptional factors (Richon et al., 2000). The induction of $p21^{WAF1}$ by HDAC inhibitor is regulated by the degree of acetylation of histones and indicated that $p21^{WAF1}$ is a direct target for HDAC inhibitors(Richon et al., 2000, Gui et al., 2004). HDAC inhibitors not only up-regulate the expression of $p21^{WAF1}$ but also up-regulate the cdk4 inhibitor $p19^{INK4D}$ and down-regulating cyclin D and cyclin A levels. These result in blockage of retinoblastoma protein (Rb) phosphorylation and promoting G1 cell cycle arrest. (Sandor et al., 2000). The induction of $p21^{WAF1}$ expression and result in G1 arrest only requires relatively low, non-cytotoxic doses of HDAC inhibitor (Richon et al., 2000).

Although it is a rarer event than G1/M arrest, histone deacetylase inhibitors

can also mediate G2/M arrest by activating a G2 phase checkpoint mechanism. Interestingly, treatment with different HDAC inhibitors may associated with different patterns of gene expressions and resulted in either G1/S (most commonly) or G2/M arrest in the same treated cell line (Verdin, 2006). G2/M arrest is usually observed at higher doses of histone deacetylase inhibitor than required for G1/M arrest (Qiu et al., 2000, Burgess et al., 2001, Richon et al., 2000). During normal progression from S phase to mitosis, G2 checkpoints and mitosis remain latent unless specific stresses activate them to block further progression. Normal cells have an intact and functional checkpoint which can block progression into mitosis and remain G2 arrested until they can resume normal progression after drug removal. Most cancer cells have a defective G2 phase checkpoint and undergo aberrant mitosis which may result in apoptosis (Peart et al., 2003). Normal cells are resistant to killing even at high doses of histone deacetylase inhibitors and may be related to the presence of functional G2 phase checkpoint. Therefore the cance cells are more sensitive to histone deacetylase inhibitor treatment. Thus, this G2 phase checkpoint may determine the sensitivity to histone deacetylase inhibitor-induced apoptosis. However, the precise underlying mechanisms responsible for histone deacetylase inhibitor-mediated G2 arrest still remain unclear. Some studies have proposed that the induction of G2 phase checkpoint response is associated with hyperacetylation of pericentric heterochromatin and loss of this checkpoint can result in abnormal chromosomal segregation and nuclear fragmentation (Taddei et al., 2005).

Inhibition of angiogenesis

Angiogenesis is considered to be critical for tumor growth and progression.

Tumors induce angiogenesis by secreting various growth factors such as vascular endothelial growth factor (*VEGF*). Most HDAC inhibitors are capable to inhibit angiogenesis by reducing *VEGF* expression in cancer cell and inhibit the endothelial cells migration and proliferation (Kim et al., 2001, Bolden et al., 2006). HDACs are also involved in oxygen-dependent gene expression and hypoxia-induced angiogenesis. Hypoxia is a common feature of malignant tumors and more often detected in the central regions of the solid tumors. Hypoxia-inducible factor (HIF)-1α regulated by hypoxia controls hypoxia-inducible angiogenic factors such as *VEGF*. Under hypoxic condition, HDAC inhibitors such as TSA inhibited *VEGF* and HIF-1α expressions and concurrently induced the expression of angiogenic-inhibiting factors *p53* (Kim et al., 2001).

HDAC inhibitor effects in xenograft tumor models

HDAC inhibitors including SAHA and TSA, have been identified to inhibit proliferation and induce differentiation and/or apoptosis of cancer cells in animal models with little or no toxicity (Marks et al., 2004, Dokmanovic and Marks, 2005). The tumor models include both solid and hematological malignhancy, including human breast, prostate, lung and stomach cancers, neuroblastoma, medullablastoma, multiple myeloma and leukemia. HDAC inhibitors also caused an accumulation of acetylated histones in both normal and tumor tissues which include spleen, liver and peripheral mononuclear cells. The increased accumulation of acetylated histones is a useful marker of HDAC biological activity and has been used to monitor dosing in patients in clinical trials (Dokmanovic and Marks, 2005).

As for SAHA, the efficacy *in vivo* was first demonstrated in nude mice xenograft model bearing CWR222 human prostate cancer cells. Intraperitoneal administration of SAHA caused significant suppression of tumor growth with a 97% reduction in the mean final tumor volume when compared with the controls. Furthermore, the dosages of SAHA treatment used have no observable toxic effects on normal cells (Butler et al., 2000). Administration of SAHA either parenterally or orally also shown to be effective in suppressing the tumor growth in a carcinogen-induced mammary tumor in rats, human neuroblastoma xenograft in mice, a transgenic mouse model of therapy resistant- acute promyelocytic leukemia and a carcinogen-induced lung cancer in mice (Dokmanovic and Marks, 2005).

Administration of CBHA induced apoptosis of human neuroblastoma cells both alone and in combination with all-trans retinoic acid (atRA) in a severe combined immunodeficiency-mouse xenograft model in a dose-dependent fashion (Coffey et al., 2001). Kim and his group observed that TSA specifically inhibited hypoxia-induced angiogenesis by reducing the vessels inside the hypoxic regions of tumors in the Lewis lung carcinoma model *in vivo* (Kim et al., 2001). Orally administered MS-275 strongly inhibited the growth in seven out of eight tumor lines implanted into nude mice (Saito et al., 1999).

1.3.3.4 Combination of HDACi with other agents

There are growing number of studies demonstrated that various treatment modalities, including radiation therapy, chemotherapy, differentiation agents, other epigenetic therapy, new target agents may have synergistic or additive effect with HDAC inhibitors (Dokmanovic et al., 2007, Bolden et al., 2006, Dokmanovic and

Marks, 2005). HDAC inhibitors may act synergistically with the demethylating agent, 5-aza-2'-deoxycytidine (5 aza-dR), in the re-activation of tumor suppressor genes. 5 aza-dR in combination with HDAC inhibitors FK-228 act synergistically in inducing cell differentiation and/or cell growth arrest, apoptosis and cytotoxicity (Primeau et al., 2003)

Chemotherapeutic agents, including anti-tubulin agent docetaxel, topoisomerase II inhibitors, doxorubicin, etoposide and ellipticine and DNA cross-linking reagent cisplatin may also act synergistically or additively with HDAC inhibitors (Bolden et al., 2006, Dokmanovic and Marks, 2005, Dokmanovic et al., 2007). In addition, combining with new target agents (e.g: Bcr-abl inhibitor imatinib, heat shock protein-90 inhibitor 17-N-allylamino-17 -demethoxygeldanamycin, proteasome inhibitor bortezomib and Her2 receptor inhibitor trastuzumab) may also deserved for further investigations (Rahmani et al., 2005, Yu et al., 2003, Bolden et al., 2006). By exploring the underlying downstream pathways altered by HDAC inhibitors may provide futher insights into the development of novel therapeutic strategies.

1.3.3.5 HDAC inhibitor treatment and genes regulated by histone acetylation in gastric cancer

Although there are numbers of studies demonstrated the anti-cancer effects of HDAC inhibitors in various cancers, data of the effect in gastric cancer cells is limited. HDAC inhibitors (such as TSA and butyrate), have reported to have anti-cancer effects in gastric cancer cells (Schauber et al., 2004, Kim et al., 2008, Kondo et al., 2005). Huang et al also reported that treatment of SAHA would inhibit

cell growth, induce cell cycle arrest and apoptosis in gastric cancer cells (Huang et al., 2007).

Some cancer related genes have been reported to be up-regualted upon treatment with HDAC inhibitors. Cathelicidin LL37 expression was induced after the treatment of TSA or butyrate in gastrointestinal cells including gastric and hepatocellular cells (Schauber et al., 2004). Aberrant methylation was observed in harakin (HRK) in gastric cancer and it's expression was restored by 5 aza-dR and further enhanced by HDAC inhibitor TSA or depsipeptide treatment (Obata et al., 2003).

Several studies have demonstrated the up-regualted genes upone treatment with HDAC inhitors were related to histone acetylation. These putative target genes regulated by histone acetylation genes include *p21*, *LRRC3B*, *PINX*, Death-associated protein kinase (*DAPK*) and *RUNX3*. Previous studies have reported that *p21* expression was induced by TSA and the induction was associated with hyperacetylation of histone H3 in the *p21* promoter region and coding region in gastric carcinoma (Mitani et al., 2005). Leucine-rich repeat-containing 3B (*LRRC3B*) is a putative tumor suppressor gene in gastric cancer and it's expression could be induced by 5 aza-dR and/or TSA treatment (Kim et al., 2008). Kim et al found that TSA was more effective to induce *LRRC3B* expression than 5 aza-dR and combination treatment of both resulted in syngergistic activation of *LRRC3B* (Kim et al., 2008). Using ChIP assay, they showed that acetylation levels of histones H3 and H4 at the *LRRC3B* CpG island were elevated when the CpG island was unmethylated or transcriptionally active in gastric cancer cell (Kim et al., 2008).

Another putative tumor suppressor gene, Pin2/TRF1-binding protein (*PINX1*), was commonly down-regulated in gastric cancer cell. TSA treatment could induce *PINX1* expression and enhanced the acetylation levels of histone H4 in the 5'UTR of *PINX1* (Kondo et al., 2005). Death-associated protein kinase (DAPK) was found to be densely methylated in CpG island in gastric cancer cells. In addition, acetylation of histones H3 and H4 in the 5'region of the gene was found to be directly correlated with DAPK expression and inversely with DNA methylation (Satoh et al., 2002). However, in regarding to SAHA, the only reported putative target gene in gastric cancer was RUNX3 (Huang et al., 2007). Huang et al reported the treatment of SAHA induced the accumulation of acetylated histones and upregulated the expression of the tumor suppressor gene *RUNX3* (Huang et al., 2007). Chromatin immunoprecipitation was performed and showed that SAHA treatment remarkably increased *RUNX3* transcriptional activity at the downstream of transcriptional start site (Huang et al., 2007). Thus, data regarding the putative target genes regulated by the epigenetic mechanism, histone acetylation in gastric cancer cells is limited.

1.3.3.6 Clinical trials of HDAC inhibitors

Increasing numbers of HDAC inhibitors include SAHA, butyrate, MS-275, AN-9 and valproic acid, have shown impressive antitumor activity *in vivo* with remarkably little toxicity in preclinical studies and have entered into phase I and/or II clinical trials (Table 1.2).

SAHA (Vorinostat) is the first of the new HDAC inhibitor to be approved by the FDA of the United States for use in patients with cutaneous T-cell lymphoma

(CTCL). In phase I studies, administrated of SAHA intravenously to patients with either hematological or solid tumors showed that the drug was well tolerated and it also inhibit histone deacetylase in normal and malignant cells (Kelly et al., 2005, Kelly et al., 2003). In order to improved the drug delivery, oral administration was developed in phase II studies for patients with hematological malignancy including Hodgkin's disease, non-Hodgkin's lymphomas and cutaneous T-cell lymphoma (CTCL) and in patients with solid tumors including thyroid, renal cell, mesothelioma, laryngeal and urothelial carcinomas (Kelly et al., 2005). Oral administration of SAHA showed good oral availability and favorable pharmacokinetics. According to the prescribing information of Zolinza™ (Vorinostat, SAHA) from The Merck & Co., Inc., website (http://www.zolinza.com/vorinostat/zolinza/hcp/index.jsp 1st May 2009), patients with refractory CTCL orally administrated with 400mg of Zolinza™ once daily, showed a significant response rate to the drug and 30% patients have objective response to the treatment. The median time to response was approximately 55 days in both overall population and in patients with advanced CTCL. The most common adverse events observed in patients with Zolinza™, regardless of causality, were fatigue, diarrhea, nausea, dysgeusia, thrombocytopenia, anorexia, decreased weight, and muscle spasms. Most common serious adverse reactions reported include pulmonary embolism, anemia and deep vein thrombosis. Therefore, patients should be monitor for pertinent signs, symptoms, platelet counts and hemoglobin, particularly in patients with a history of thromboembolic events. Currently, there are more than 50 clinical trails with combination therapy with Vorinostat and various agents include carboplatin, paclitaxel, 5-fluorouracil, in patients with advanced hematologic and solid tumors (http://clinicaltrials.gov;

http://cancer.gov/clinicaltrials 1st May 2009). However, in regarding to gastric cancer, there is currently no clinical trail.

Some others HDAC inhibitors, like Depsipeptide (FR-901228 or FK-228) is currently in phase II study in a pivotal trial in CTCL and peripheral T-cell lymphomas (Piekarz et al., 2007). In the clinical study, objective response was observed in 10 out of 28 patients with an overall response rate of 36%. The most common adverse events include myelotoxicity, nausea, vomiting and cardiac dysrhythmias. Depsipeptide has been used as monotherapy and in combination therapy with different agents in clinical trials in patients with hematologic and solid tumors (Piekarz et al., 2007). Oral administration of MS-275 impressively suppressed the growth of tumors such as neuroblastoma and undifferentiated sarcoma (Marks et al., 2004).

On the other hand, Apliphatic acids based HDAC inhibitors are relatively weak inhibitors for HDAC enzymes compared to hydroxamic acid or cyclic peptide HDAC inhibitors. Both valproic acid and phenylbutyrate are commercially available in the market for non-oncological treatment. Phenylacetate (PA) is a metabolite of phenylbutyrate following beta-oxidation in the liver and kidney and approved to be used in children with urea cycle disorders and in patients with portal encephalopathy and chemotherapy-induced hyperammonemia (Marks et al., 2004). Clinical trials with phenylacetate have shown little anticancer activity (Dokmanovic et al., 2007). Both Valproic acid and phenylbutyrate (PB) showed some therapeutic effect as monotherapy in patients with myelodysplastic syndrome

and/or acute myeloid leukemia and were well tolerated clinically (Dokmanovic et al., 2007).

Chapter 2 Aims of the study

Gastric cancer is a common cancer especially in Asian countries and is associated with high morbidity and mortality. Epigenetic inactivation of tumor suppressor is a common mechanism involved in carcinogenesis of a variety of human cancers and recent evidence suggested that targeting epigenetic modifications may be an approach to combat cancer. Our group and others have demonstrated frequent promoter methylation of cancer related genes in gastric cancer. In this study, we aim to identify cancer associated genes regulated by another important epigenetic mechanism, namely histone acetylation.

1. To identify a potential tumor suppressor gene that is regulated by histone acetylation in gastric cancer cells.

a. We first investigated suberoylanilide hydroxamic acid (SAHA), a well known histone deacetylase inhibitor, has anti-proliferative effect in a panel of gastric cancer cell lines. Then we compared gene expression profiles of SAHA treated vs control AGS cells to identify a set of genes that were differentially upregulated by SAHA treatment. Based on our microarray analysis in gastric cancer cell lines and normal gastric tissues, a set of commonly down-regulated genes in gastric cancer cells was elucidated. Analysis of these data sets with subsequent confirmation using real-time PCR analysis, genes that down-regulated in gastric cancer cells and unregulated upon SAHA treatment were identified. Then among these genes, a selected gene will be subjected for in-depth study.

b. To elucidated the down-regulation mechanism(s) in the selected gene.

c. To verify the down-regulation of the selected gene in gastric cancer by RT-PCR, western blot and immunohistochemical study.

2. **To characterize the possible functional role of the selected putative tumor suppressor gene in gastric cancer.**

a. The possible functional roles will be investigated through a series of experiments in gastric cancer cells.

b. Expression of the selected gene will be correlated with the clinical-pathological parameters and assess the possible association with the clinical-pathological features.

Chapter 3 Materials and Methods

3.1 Cell culture

Nine human gastric cell line including MKN1, MKN7, MKN28, MKN45, AGS, N87, KatoIII, SNU1 and SNU16 were purchased either from American Type Culture Collection (ATCC) (Rockville, Maryland, USA) or Japanese Collection of Research Bioresources (JCRB) (Japan). The cell lines were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and 10% of 100mM of Glutamine, all were purchased from Invitrogen (Invitrogen Corporation, Carlsbad, CA, USA). After retrieval of cell pellets from the liquid nitrogen tank, cells pellets were dispersed with 10 ml culture medium in 10 cm culture plate (Greiner Bio-One, Frinkenhausen). Cells were then incubated in a 37°C incubator, supplied with humidified atmosphere containing 5 % CO_2 and 95% air. Cells were sub-cultured or harvested at around 70% confluency. For cell harvest, cells were washed with 5 ml phosphate-buffered saline (PBS), followed with the treatment of 1 x trypsin-EDTA (Invitrogen Corporation, Carlsbad, CA, USA) for 5-15 minutes at 37°C incubator to detach the cells from the bottom of the flasks. Cells were collected by centrifugation at 1000 rpm for 3 minutes. The supernatant was discarded and the cell pellets were stored at -80°C.

3.2 Drug treatment

3.2.1 Suberoylanilide Hydroxamic Acid treatment

Histone deacetylase (HDAC) inhibitor, Suberoylanilide Hydroxamic Acid (SAHA; BioVision, Mountain View, CA, USA) was used in the study. A stock solution of SAHA was dissolved in DMSO and aliquoted to 20 mM and stored at -20°C. Nine gastric cancer cell lines were seeded in different cell numbers from 1-3 x 10^6 cells on 10cm culture plates (Becton Dickinson Labware, Franklin Lakes, NJ, USA) one day before the treatment. Drug treatment was started the next day. Cells were treated with various concentrations (2 μM or 4 μM) of SAHA for different time points. Culture cells without SAHA treatment were used as control.

3.2.2 5-aza-2'deoxycytidine treatment

5-Aza-2′-deoxycytidine (5aza-dR; Sigma-Aldrich Co., St. Louis, MO, USA) is a demethylating agent that inhibits DNA methyltransferase activity and causes DNA demethylation. A stock solution of 5aza-dR was dissolved in milli-Q water (50mg/ml) and aliquoted into 5μl per eppendorf and stored at -20°C. Gastric cancer cells were seeded at 50-70% confluency on 10 cm culture plates. Drug treatment was started on the next day and 5 μM of 5aza-dR was added into growth medium freshly and daily for 3 consecutive days. Culture cells without 5aza-dR treatment were used as control.

3.2.3 Combine treatment

For the sequential treatment with the demethylating agent and HDAC inhibitor, the cells were cultured in medium supplemented with 5 μM of 5aza-dR for the first three consecutive days (72 hours), then with 4 μM SAHA for the next 24 hours.

3.3 DNA, RNA and Protein extraction

3.3.1 DNA extraction

For the extraction of DNA, cells were washed with PBS, trypsinized and collected by centrifugation at 1000 rpm for 3 minutes. DNA from the cell pellet samples was retrieved by High Pure PCR Template Preparation Kit (Roche, Mannheim, Germany) according to manufacturer's protocol. The cell pellets were resuspended in 200 μl of PBS and 40 μl of 1 mg/ml proteinase K was added into each sample and incubated at 56°C for few hours. After complete digestion of the samples, 200 μl of binding buffer was added and incubated at 72°C for 10 minutes. The reaction mixture was then mixed with 100 μl of isopropanol and transferred to the High Pure filter column (combined with collection tube) and centrifuged at 8,000 rpm for 1 minute. The flow through and the collection tube were discarded and 500 μl of washing buffer was added to the column followed by centrifugation at 8, 000 rpm for 1 minute. The washing procedure was repeated for the second time. The column was centrifuged at 12,000 rpm for 10 seconds to remove the residual wash buffer. The elution buffer was pre-warmed at 70°C for few minutes before the elution procedure. Lastly, the column was placed into a new 1.5 ml eppendorf tube and DNA was eluted with 200 μl of pre-warmed elution buffer. The concentration

of DNA was measured by NanoDrop ND-1000 UV-VIS (Thermo Scientific,Wilmington, DE, USA) at 260nm wavelength.

3.3.2 Total RNA extraction

For RNA extraction, cells pellets were washed with 5 ml of cold PBS. Total RNA was extracted using RNeasy kit (Qiagen, Germantown, MD) according to manufacturer's protocol. Freshly prepared 350 µl of RLT (1% of β-Mercaptoethanol was freshly added) was added to the cell pellet. The cell lysate was then passed through a 21-gauge syringe (Becton Dickinson Labware, NJ, USA). 350 µl of 70% ethanol was added to the homogenized lysate. The samples were mixed and transferred to an RNeasy mini column placed in a 2 ml collection tubes and centrifuged for 30 seconds at 13,000 rpm. The flow through was discarded and 700 µl of RW1 buffer was added to the column and centrifuged at 13,000 rpm for 30 seconds. The columns were transferred to a new collection tube and added 500 µl of RPE Buffer to wash the column twice. The columns were centrifuged for 30 seconds at 13,000 rpm and discard the flow through afterwards. In order to avoid RPE buffer carryover, the columns were centrifuged for 2 minutes at 13,000 rpm. The total RNA was eluted by adding 30 µl of RNase-free water directly onto the RNeasy silica-gel membrane and incubated for 5 mins and then centrifuged for 2 minute at 13,000 rpm. Total cellular RNA concentrations were measured by NanoDrop ND-1000 UV-VIS (Thermo Scientific,Wilmington, DE, USA) to read the absorbance at wavelength 280 nm. The RNA samples were stored at -80°C for subsequent analysis.

3.3.3 Protein extraction

Culture cells were harvested at various time points after the drug treatments or transient transfection. Medium was discarded and sub-confluent dishes of cells were washed with 1 x PBS. Protein lysis buffer containing 50mM Tris-HCl pH 7.9, 150mM NaCl, 1% Triton X-100, 0.1% sodium dodecyl sulfate (SDS), 2mM EDTA, 2mM EGTA, 20mM β-glycrophosphate, 20mM sodium pyrophosphate and 1mM sodium orthovanadate was freshly prepared with adding of 1 × Protease Inhibitor Cocktail (Roche, Indianapolis, IN), 1mM PMSF and 1mM DTT (most of the chemicals were purchased either from USB or Sigma). Cold protein lysis buffer was added into the dishes to lyse the cells and collected by cell scrapter. Whole cell lysates were then incubated on ice for 20 min and centrifuged at 13,000 rpm in a microcentrifuge for another 10 min at 4°C. The supernatant was collected and aliquot in new 1.5 eppendorf tubes and store at -80°C for further analysis.

3.4 Microarray analysis

3.4.1 Sample preparation for microarray

Total cellular RNA was isolated from the culture cells using RNeasy kit (Qiagen, Germantown, MD) according to the manufacturer's instructions. RNA concentration was assessed by NanoDrop (Thermo Scientific,Wilmington, DE, USA) at 260nm wavelength and quality was assessed by gel electrophoresis. RNA used for microarray chip analysis should have a minimum purity and integrity standards for total RNA quality i.e. OD 260/280 > 1.8, DNA contamination < 5%. CodeLink™ UniSet Human 20K Gene Expression Array (Amersham Bioscience,

Chandler, USA) contains a collection of approximately 20,289 probes and 19,881 numbers of genes was used to perform the bioarray hybridization. Whereas Agilent 4 x 44k Whole Human Genome Microarray kit (Agilent Technologies, Inc., Santa Clara, CA, USA) contains approximately 41,000 unique human genes and transcripts (all with public domain annotations) was used to perform two-color hybridization. The total RNA of normal gastric sample that purchased from Ambion (Am7996; Applied Biosystems Inc, Foster City, CA, USA) was used as the control for gastric gene expression array.

CodeLink™ UniSet human 20K gene expression array

2ug of total cellular RNA was used to synthesize the first-strand cDNA according to the manufacturer's protocol. Six bacterial mRNA controls were prepared freshly before the first-stand cDNA reaction and act as fiducial markers for the cDNA synthesis and *in vitro* transcription reactions. After the second-strand cDNA synthesis, the double-stranded cDNA was purified using GFX-PCR purification kit (Amersham Bioscience, Chandler, USA) and concentrated. cDNA serves as the template for an *in vitro* transcription reaction to produce the target cRNA. The *in vitro* transcription reaction was incubated in a thermal cycler at 37°C for 15 h and the target cRNA was labeled by the biotin-11-UTP (Perkin Elmer, Boston, MA). Though this amplification method, 1000-5000 fold of the input poly(A)$^+$ RNA could be produced. Biotin-11-UTP labeled cRNA was recovered using an RNeasy Mini kit (Qiagen Germantown, MD). Quantitation of cRNA was performed by UV spectrophotometry (BioPhotometer, Eppendorf, Hamburg, Germany) at 260 nm and the concentration and purity were assessed. The cRNA OD 260/280 ratio of the samples all were within 1.8-2.1 and the results indicated

the samples were pure.

Agilent 4 x 44k whole human genome microarray

50 to 5000 ng of total RNA (must be 8.3 µl or less but at least 2µl) and 1.2 µl of T7 Promoter primer from the Aglient Low RNA Input Linear Amplification Kit PLUS (Agilent Technologies, Inc., Santa Clara, CA, USA) was added to the 1.5ml eppendorf and the volume was top up to 11.5 µl with nuclease-free water. Denaturing was performed to denature the primer and template by incubating the sample tube at 65°C for 10 mins. Then incubated the sample tube on ice for 5 mins and freshly prepared the master mix by mixing the following components: 4µl of 5 x first strand buffer, 2 µl of 0.1M DTT, 1 µl of 10mM dNTP mix, 1µl MMLV-RT and 0.5µl of RNaseOut per reaction. Added the 8.5µl freshly prepared master mix into each sample and gently mixed the sample. Incubated the sample tube at 40°C for 2 hours and then at 65°C for 15 mins. Incubated the sample tube on ice for few mins and freshly prepared the transcription master mix (60 µl) by mixing the following components: 15.3µl nuclease-free water, 20 µl 4 x transcription buffer, 6 µl 0.1M DTT, 8 µl NTP mix, 6.4 µl 50% PEG (per-warm at 40°C for 1 min before use), 0.5 µl RNaseOut, 0.6 µl inorganic pyrophosphatase, 0.8 µl T7 RNA polymerase and 2.4 µl Cyanine 3-CTP or Cyanine 5-CTP. Added the 60 µl of transcription master mix to each sample tube and gently mixed the sample by pipetting. The sample tube was then incubated at 40°C for 2 hours. Purification was performed on the labeled and amplified DNA by using Qiagen RNeasy kit (Qiagen, Germantown, MD, USA) according to manufacturer's instructions. Finally elute the cRNA with 30 µl of RNase-free water into new 1.5 eppendorf. After elution, cRNA sample tube must be kept on ice. The quantity of the labeled and purified cRNA was measured by NanoDrop (Thermo Scientific,Wilmington, DE, USA) at

280nm wavelength. The quality of the cRNA was analyzed by Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA, USA) before hybridization.

3.4.2 Hybridization

CodeLink™ UniSet human 20K gene expression array

20 ug of cRNA was fragmented and mixed with the hybridization buffer. The hybridization reaction mixture was then slowly injected into the array chamber. Hybridization was performed in a 37°C shaking incubator for 18 hours at 300 rpm. Post-hybridization, the bioarray was subjected to a stringent wash to remove the unbound and non-specific hybridization. The bioarray was stained with Cy5-streptavidin dye (Amersham Bioscience, Chandler, USA) conjugate in the dark. Unbound conjugate was removed by several non-stringent washes at room temperature (1×TNT, 5 min × 4; 0.05% Tween 20, 30 seconds) and dried by low speed centrifugation.

Agilent 4 x 44k whole human genome microarray

Freshly prepared the 2-color fragmentation mix for hybridization by mixing the following components per reaction: 825 ng of each Cyanine 3-labelled and Cyanine 5-labelled linearly amplified cRNA, 11 µl of 10 x blocking agent, 2.2 µl of 25 x fragmentation buffer and finally top up the volume to 55 µl with nuclease-free water. Added 55 µl of the fragmentation mix into the sample tube and mix the sample gently. Incubated the sample tube at 60°C for 30 mins in exact in order to fragment the RNA. The fragmentation process was stopped by adding 55µl of 2 x

GE hybridization buffer HI-RPM (GE Healthcare, Chandler, USA). Mixed the sample slowly by pipetting up and down to avoid forming bubbles. Then load the sample onto the array platform as soon as possible and placed onto the chamber slide according to the manufacturer's protocol. The assembled slide chamber was placed in a hybridization oven set at 65°C at 10 rpm for 17 hours. After hybridization, the assembled slide chamber was washed extensively according to the manufacturer's protocol before scanning.

3.4.3 Scanning and data processing

CodeLink™ UniSet human 20K gene expression array

Bioarray slides were scanned by 4000B GenePix™ scanner (Axon Instruments, Foster City, CA) calibrated prior to each use. Laser scanning parameters were 635 nm, PMT voltage at 600, and 10 u resolution of 10 μm and the scan area adjusted for the entire array. The bioarray was quantitated by Codelink Scanning and Expression Analysis software (version 2.3; Amersham Bioscience, Chandler, USA). The program generated raw outputs for each spot by segmentation and detection of the spot intensity versus surrounding background. The data were determined by using the raw intensity per pixel within each probe zone subtracted by the local background. Each spot was evaluated by comparison to local array signal to noise ratios. The data was generated as both raw intensity values and normalized for the large dynamic range by dividing each probes by the overall median array intensity value. The spot with manufacturing error or poor spot profiles were removed from computation. Each batch of bioarray have approximately 1% manufacturing errors that designated by the CodeLink

manufacturing spot report.

Agilent 4 x 44k whole human genome microarray

The slides were scanned by Agilent DNA Microarray Scanner (Agilent Technologies, Inc., Santa Clara, CA, USA). Feature Extraction v 9.5.3.1, LOWESS was applied for normalization to allow the correction of strong non-linear dye distortion of Cy3 and Cy5 signals and 'spatial-dependent' dye bias. Further data normalization was applied in GeneSpring v 7.3.1 by dividing all measurements on each chip using 50^{th} percentile value and comparing the relative change in gene expression levels. Control fibroblast (derived from cardinal ligaments) was used as an internal control for each array and normal gastric RNA was use as normalization and fold change calculation.

3.4.4 Data analysis

CodeLink™ UniSet human 20K gene expression array

The normalized data would be analyzed by significance analysis of microarrays (SAM) method simultaneously in order to identify differential gene expressions with statistical significance. The SAM method automatically calculated the false discovery rate (FDR) using a permutation test. The FDR provides information of the expected proportion of genes that are false-positive. The gene was considered as significantly induced if the FDR was controlled to be less than 5% and was induced at least a 2-fold changed upon SAHA treatment as compared to the control.

Agilent 4 x 44k whole human genome microarray

Only the expression of TXNIP in the nine gastric cancer cell lines were observed. The expression profile data of the nine gastric cancer cell lines were compared with the normal gastric control.

3.5 Array comparative genomic hybridization

1 μg of genomic DNA (gDNA) of the nine gastric cancer cell lines and 1 μg Human Genomic DNA purchased from Promega (G147A 21927801, Promega, Madison, WI, USA) were digested with AluI and RsaI for 2 hours at 37°C incubator. Fluorescent labeling was performed using Aglient Genomic DNA labeling Kit PLUS (Agilent Technologies, Inc., Santa Clara, CA, USA) according to the manufacturer's manual. The labeled genomic DNA was then purified by adding 430 μl 1 x TE buffer pH 8 to the samples and loaded the samples through Micron YM-30 filter (Millipore, Corporatem Billerica, MA, USA) at 8000 x g for 10 mins. Discarded the flow through and repeat the step once by adding another 480 μl of 1 x TE buffer into the filter. Transferred the filter into a new 1.5 ml eppendorf and eluted the gDNA with 80.5μl of 1 x TE buffer. 1 μl of the labeled gDNA samples were then subjected to Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA, USA) to analyze the quality of the gDNA and determine the enzymes digestion efficiency. 1 μl of the labeled gDNA samples were used to determine the yield and specific activity by NanoDrop ND-1000 UV-VIS spectrophotometer (Thermo Scientific,Wilmington, DE, USA). The specific activity of Cy3- and Cy5-labelled gDNA should be approximately 20-40 pmol/μg for 5-7 μg of gDNA.

Agilent Oligo aCGH Hybridization Kit (Agilent Technologies, Inc., Santa Clara, CA, USA) was used for hybridization according to manufacturer's procedures. 490 µl of hybridization sample mixture was loaded onto the gasket well of the Agilent SureHyb chamber. The assembled slide chamber was then placed in a rotator rack in hybridization oven which rotate at 20rpm at 65°C for 40 mins. After hybridization, the slides were washed with Aglient Oligo aCGH wash buffer according to manufacturer's procedures. The slides were scanned by Agilent Microarray Scanner Model no. G2505B and analyzed by CGH Analytics 3.4.2 software.

3.6 Polymerase Chain Reaction

3.6.1 Primer design

The primers were designed using web software Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) provide by Whitehead Institute/MIT Center for Genome Research.

3.6.2 Reverse transcription

The reverse transcription of total RNA to complementary DNA (cDNA) was performed using SuperScriptTM III reverse transcriptase for RT-PCR kit (Invitrogen, Carlsbad, CA, USA). For each reaction, 1-2 ug of total RNA was mixed with 1 µl of 0.3 µg/µl random primers (50-250 ng) and l µl of 10 mM dNTP Mix (10 mM each dATP, dGTP, dCTP and dTTP at neutral pH). Sterile and distilled water was added to bring a final volume of 13 µl. The mixture was incubated at

65°C for 5 minutes and chilled on ice for at least 1 minute. Other reaction components including 4 µl of 5 x First-Strand buffer (250 mM Tris-HCL (pH 8.3), 375 mM KCL, 15 mM MgCl2), 1 µl of 0.1 M DTT and 1 µl of SuperScriptTM III RT (200 units/µl) were added to the mixture and incubated at 25°C for 5 minutes and 50°C for 1 hour to synthesize the first strand cDNA. The reverse transcription reaction was inactivated by heating at 70°C for 15 minutes. The first strand cDNA was stored at -20°C until subsequent PCR reaction.

3.6.3 Quantitative RT-PCR

Quantitative real time PCR was performed using Taqman® probe purchased from Applied Biosystem (Applied Biosystems, Foster city, CA, USA). The house-keeping gene, *β–actin* and GAPDH, were also included as control for each QRT-PCR. Each 10 µl of PCR reaction mix was prepared as followed: 0.4µl of cDNA, 5 µl of 2X Taqman SuperMix (Applied Biosystems, Foster city, CA, USA), 0.5 µl of 20x Taqman® probe and appropriate amounts of milli-Q water to top up the volume. For the chromatin immunoprecipitation (ChIP) assay, DNA was amplified by using 4 pairs of primers which located on different regions around the TXNIP promoter using SYBR Green system. Each 10 µl of PCR reaction mix was prepared as followed: 4 ul of purified DNA, 5 µl of 2X SYBR Green SuperMix (Applied Biosystems, Foster city, CA, USA), 0.25 µl of each 10 pmole/µl forward and reverse primers and appropriate amounts of milli-Q water to top up the volume. All reaction was performed on the ABI 7900 HT Fast Real time PCR System (Applied Biosystems, Foster city, CA, USA). The default thermal cycler condition was used for Taqman® reaction. The SYBR PCR reactions were performed under

the following conditions: 95°C for 10 minutes, 45 cycles of 95°C for 30 seconds, 58°C for 30 seconds and 72°C for 20 seconds. The fold induction for ChIP assay was calculated by the cycle threshold (CT) of the SAHA treatment versus no treatment and normalized by the expression of the input of the same samples.

3.7 Western blotting

Protein concentration was measured using the Bradford D*c* protein assay kit (Biorad, Hercules, CA, USA) and bovine serum albumin (BSA; Promega, Madison, WI, USA) was used as a standard. The concentrations of protein samples were measured by 96-well plate reader spectrophotometer to read the absorbance at 595 nm wavelength (Victor^{TM} X Multilabel plate reader, Perkin Elmer, Waltham, Massachusetts, USA). Cell protein extracts were denatured in 6 x sample buffer containing 350mM Tris-HCL pH 6.8, 10% (w/v) SDS, 30% (v/v) glycerol, 0.6M DTT, 0.012% (w/v) bromophenol blue (all, except SDS, were purchased from Sigma, St Louis, MO). The protein samples were heated to 95°C for 10 minutes. Samples were then electrophoresed through a 10% SDS-polyacrylamide gel (SDS/PAGE) at 120V for 1.5 hr at room temperature. Rainbow marker SeeBlue Plus 2 from Invitrogen (Carlsbad, California, USA) was used as a size marker.

The proteins on the separating gel were transferred onto PVDF membranes by electroblotting. Membranes were then incubated overnight at 4°C with blocking solution [5% (w/v) low-fat milk in TBS-T (10mM Tris pH 7.5, 100mM NaCl and 0.1% Tween 20)]. Antibodies against Histone acetylated H3 (1:1000) (Millipore, Billerica, MA, USA), Histone acetylated H4 (1:1000) (Millipore, Billerica, MA, USA), TXNIP (1:1000) (MBL International, Woburn, MA) and monoclonal

anti-β-actin antibody (Sigma, St. Louis, MO, USA) were used. All antibodies were incubated for 1 hr at room temperature on a shaker except for TXNIP for which the incubation time was 2 hrs. Following incubation with the primary antibody, blots were washed in TBS-T × 3 times for 5 min each. Secondary antibody linked to horseradish peroxidase conjugated anti-rabbit HRP (Dako, Glostrup, Denmark) or anti-mouse HRP (Dako, Glostrup, Denmark) was incubated with the blots at 1:10000 dilution for 1 hr at room temperature on a shaker. Blots were then washed as previously described and developed with a detection kit (Millipore, Billerica, MA, USA). Immunodectection was recorded by Fujifilm (Toyko, Japan).

3.8 Epigenetic study

3.8.1 Bisulfite modification

Treatment of genomic DNA (gDNA) samples with sodium bisulfite converts all unmethylated cytosines (but not methylcytosines) to uracils, which then further converted to thymidine during the subsequent PCR step. Bisulfite modification allows the subsequent differentiation of methylated and unmethylated sequences by Bisulfite sequencing. Bisulfite modification was carried out by EZ DNA Methylation-Gold™ kit (Zymo Research Corporation, CA, USA) using 2 ug of input DNA according to the manufacturer's description. Genomic DNA was amplified by PCR using primers, forward 5'-GGAGAAGACATCGGTCCT-3' and reverse 5'-CATGATGGAACTGAGTTGGT-3'. For bisulfite sequencing analyses, sodium bisulfate-treated gDNA was amplified by two pairs of methylation-specific primers (2 rounds of PCR). Bisulfite sequencing was performed using 10 pmole of sequencing primers and 2 μl of BigDye® Terminator v1.1 Cycle Sequencing Kit

(Applied Biosystems Inc, Foster City, CA, USA) according to the manufacturer's protocol and sequenced by ABI 3130 Genetic Analyzer (Applied Biosystems Inc, Foster City, CA, USA).

First round PCR:

-TATA box (forward-5'-GGTTTTAGGGTTAGTGGGA -3'),

(reverse-5'-AAAAACCTTCTTTCCCCCAA -3');

-exon 1 (forward-5'-GGAAAGAAGGTTTTTTTTTGA -3'),

(reverse-5'-CCACTTACCTATTAACTAATCT -3');

Second round PCR:

-TATA box (forward-5'-TTTATTGGATTTGGGAGAA -3'),

(reverse-5'-ATCCAATCTCCACAAACACTCC -3');

-exon 1 (forward-5'-TGATTTTGTTTAGTGTAATTAG -3'),

(reverse-5'-CCTATTAACTAATCTTCCAA -3');

3.8.2 Chromatin immunoprecipitation (ChIP)

Human gastric cancer cell line MKN45 was used in this experiment. 5 x 10^6 of MKN45 was seeded on three separate 10 cm culture dish one day before SAHA treatment. On the next day, 4 μM of SAHA was added to the culture dishes and incubated for 20 hours. After 20 hours of drug treatment, culture dishes were washed twice with 1 x cold PBS and cells were collected on ice with cell scrapter. Cells were harvested into 15 ml falcon and centrifuged 1000 rpm for 8 mins at 4°C. All the supernatant was discarded completely by inverting the 15 ml falcon on a

tissue towel. SDS lysis buffer was prepared (4 ml of 10% SDS, 800 µl of 0.5 M EDTA pH8, 2 ml of 1 M Tris pH8.1 and top up the volume to 40 ml with Milli-Q) freshly and was added with 4.2 µl of 100 mM PMSF and 16.8 µl of 25 x Roche protease inhibitor cocktail into 420 µl of SDS lysis buffer at room temperature. Cell pellet was resuspended with 420 µl SDS lysis buffer and transferred into two separate 1.5 ml eppendorf each with 210 µl per tube. Pre-cool the Bioruptor™ sonicator (Diagenode Inc., New York, NY, USA) with ice 15 mins before the sonication. Appropriate amount of ddH2O and a hand-full of ice were added into the sonicator. The 210 µl sample tubes were sonicated for 35 mins (30 seconds on and 30 seconds off) and fresh ice was added into the sonicator every 3 mins. After sonication, the samples (2 x 210 µl per tube) were grouped back into new 1.5 ml eppendorf and centrifuged 13500 rpm for 10 mins at 4°C. The supernatant was collected into new eppendorf and stored at -80°C. The sonicated supernatant was diluted 10 x with ChIP dilution buffer (0.2 ml of 10% SDS, 2.2 ml of Triton X-100, 0.48 ml of 0.5 M EDTA pH8, 3.34 ml of 1M Tris pH 8.1, 6.68ml of 5 M NaCl and top up the volume to 200 ml with Milli-Q). 180µl of sonicated samples and diluted with 1.8 ml ChIP dilution buffer. To normalize the ChIP results, 60 µl of each diluted sonicated supernatant sample was kept in another 1.5 ml eppendorf as PCR input control.

For immunoprecipitation, we first pre-clear the diluted sonicated samples with 80 µl of Salmon Sperm DNA/Protein A Agarose-50% slurry (vortex) (Upstate, Millipore Corporatem Billerica, MA, USA) for 30 mins at 4°C in a shaker or rotator. The tubes were centrifuged at 1000 rpm for 1 mins at 4°C to pellet the agarose. 200 µl of supernatant was transferred into a 2 ml eppendorf (total 9 times) and then 30 µl

of diluted acetyl-Histone H3 or acetyl-Histone H4 antibody (diluted 8 µl of antibody with 22 µl of ChIP dilution buffer) was added into each ChIP sample. The ChIP sample tubes were then inverted 8 times and incubated at 4°C (with or without rotation). On the next day, 60 µl of Salmon Sperm DNA/Protein A Agarose-50% slurry (vortex) was added into each ChIP sample tubes and was incubate at 4°C with rotation for an hour to collect the histone-DNA/antibody complex. One then pellet the complex by centrifugation at 1000 rpm for 1 mins at 4°C and the supernatant was carefully removed without disrupting the pellet. The agarose pellet was washed extensively with each wash buffer for 5 mins at 4°C with rotation and then one pellet the agarose by centrifugion at 1000 rpm for 1 min at 4°C according to the following order: 1 ml cold low salt immune complex wash buffer; 1 ml cold high salt immune complex wash buffer; 1 ml cold LiCl immune complex wash buffer and finally 1 ml TE buffer x 2 times at room temperature. All the washing buffers were included in the Chromatin Immunoprecipitation Kit from Upstate (Millipore Corporatem Billerica, MA, USA).

The elution buffer was freshly prepared (1% SDS, 0.1M $NaHCO_3$) by mixing 50 µl of 10% SDS, 50 µl of 1M $NaHCO_3$ and top up to 500 µl with Milli-Q water per ChIP sample. 200 µl of elution buffer was added to each ChIP sample, gently mixed and incubated at room temperature with rotation for 10 mins. The tube was centrifuged at 4000 rpm for 1 min and the eluted supernatant was collected into a new 1.5ml eppendorf. The elution procedure was repeated with 200 µl of elution buffer once. At the last elution, the pellet was resuspend with the final 100 µl elution buffer and transferred all solution into a new 1.5 ml eppendorf and centrifuged at 4000 rpm for 1 min. All the eluted supernatant was collected and

transferred to the eluted sample tube which contained first and second elution (approximately 500 µl of elution supernatant in total). Each 500 µl of eluted sample was mixed with 25 µl of 5 M NaCl, whereas for the 60 µl PCR input control samples, 2.5µl of 5 M NaCl was added and incubated at 65°C for 6 hours to reverse cross-link. Then 48 µl proteinase K buffer (prepared by mixing 14.76 µl of 0.5 M EDTA pH8, 29.5 µl of 1 M Tris-HCl pH 6.5 and 3.7 µl of 20 mg/ml) was added to each sample (except for PCR input control) and incubated at 55°C for overnight.

Chromatin DNA purification was performed by using phenol-chloroform ($CHCL_3$) precipitation method. Equal volume of phenol-$CHCL_3$ IAA (Invitrogen, Carlsbad, CA, USA) was added into the samples and centrifuged at maximum speed for 10 mins. Top layer of supernatant was collected and transferred into a new 1.5 ml eppendorf. Added equal volume of $CHCL_3$ to the eppendorf and centrifuged at maximum speed for 10 mins. Top layer of supernatant was collected and transferred into a new 2 ml eppendorf then 1/10 $NaAc_3$, 1 µl of glycogen and twice amounts of absolute ethanol were added to the tubes. The tubes were stored at -20°C for 3-4 hours and centrifuged at 13500 rpm for 10 mins. The supernatant was carefully removed without disturbing the white pellet and washed twice with 70% ethanol. The pellet was air-dried for 30 mins and resuspended with 30 µl of Milli-Q water. 4 µl of the samples was used to perform the SYBR Green QRT-PCR. Totally we had 4 set of primers to amplify different regions of TXNIP promoter, primers including:

-3200 regions (forward-5'-TCACCACTTGGTTTCCTGTT-3'),

(reverse-5'-AACAGCAAAGTAG GGCACAG-3');

-2500 regions (forward-5'-CAAGTGACACTGCATTCCAA-3'),

(reverse-5'-AGATGGTGGCTAGTGATGGA-3');

-180 regions (forward-5'-AGAG CGCAACAACCATTTT-3'),

(reverse-5'-GTGCACATCCCTCCCATT -3');

+1800 regions (forward-5'-TGCTAGGTGATGAGATTTCCA-3'),

(reverse-5'-CCATTGGC AAGGTAAGTGTG -3').

3.9 Vector construction and preparation

3.9.1 TXNIP recombinant vector

Amplification primers were design to amplify the intact cDNA of human TXNIP (NCBI reference sequence nos. NM_006472.1), using forward primer (5'-CTGACGGACAAGCTTGTGATGTTCAAGAAGA-3') and reverse primer (5'-CATTGACTCTAGATCACTGCACATTGTTGT-3'). A product of approximately 1400 bp was amplified with a NotI and a xhoI restriction enzyme site. The product was gel-purified using QuickClean DNA Gel Extraction kit (Genscript Corporation, Piscataway, NJ, USA) and subcloned into the multiple cloning site of pcDNA^{TM} 3.1 (+) Expression Vector (Invitrogen Corporation, Carlsbad, CA, USA) using NotI and a xhoI restriction enzyme (New England Biolabs, Ipswich, MA, USA). Sequencing was performed using 10 pmole of sequencing primer and 2 μl of BigDye® Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems Inc, Foster City, CA, USA) according to the manufacturer's protocol and sequenced by ABI 3130 Genetic Analyzer (Applied Biosystems Inc, Foster City, CA, USA). After verification by DNA sequencing, the DNA plasmid was purified using PureLink^{TM} HiPure Plasmid Midiprep Purification kit (Invitrogen Corporation, Carlsbad, CA , USA). 2 μg of the plasmid was transfected

into various gastric cancer cells using 5μl of lipofectamine™ 2000 transfection kit (Invitrogen Corporation, Carlsbad, CA, USA).

3.10 Immunostaining

3.10.1 Gastric cancer tissue array (TMA) block preparation

For the construction of TMA block, a hollow needle was used to punch the region of interest in each paraffin-embedded gastric cancer block and the tissue core was then inserted into a recipient paraffin block in a array pattern. For each block, it contained 100 cases of gastric cancer tissue samples. For each gastric cancer sample, 3 cores were taken and embedded in 3 different TMA blocks and which would improve the representative sampling for each case. In total, we included 150 cases of gastric cancer in the study. The clinical-pathological data of each case was recorded.

3.10.2 Immunohistochemical staining

0.4μm thick paraffin sections were prepared from the 10% buffered formalin fixed paraffin embedded tissue blocks. The sections were stained with UltraView™ Universal DAB Detection Kit (Ventana Medical System Inc., AZ, USA) and NexES IHC Staining Module Machine (Ventana Medical System Inc., AZ, USA) using procedure iVIEW DAB Paraffin and TXNIP antibody (1:500,MBL) was used with primary incubation time of 32 mins.

3.10.3 Scoring method

The cytoplasmic expression of TXNIP was scored and graded. The scoring was independently assessed by two pathologists, Prof. To Ka Fai (my supervisor) and Dr. Anthony W.H. Chan. The cytoplasmic expression of TXNIP was assessed by assigning a proportion score and an intensity score. The proportion score was scored according to the proportion of tumor cells with positive cytoplasmic staining (0, none; 1, <=10%; 2, 10 to <=25%; 3, >25 to 50%; 4, >50%). The intensity score was assigned for the average intensity of positive tumor cells (0, none; 1, weak; 2, intermediate; 3, strong). The cytoplasmic score of TXNIP was the product of proportion and intensity scores, ranging from 0 to 12. The cytoplasmic expression was categorized into different grading – grade 0 (score 0); grade 1 (score 1 to 3, low expression), grade 2 (score 4-6, intermediate expression), and grade 3 (score 7-12, strong expression).

3.10.4 Statistical analysis

Correlations between *TXNIP* cytoplasmic stain and clinicopathologic parameters were assessed by the non-parametric Spearman's rho rank test. The patient survival was measured from the date of histological diagnosis to death or last follow-up. The Kaplan-Meier method was used to estimate the survival rates for each variable. The equivalences of the survival curves were tested by log-rank statistics. For those variables being statistically significant found in the univariate survival analysis ($P < 0.05$), the Cox proportional hazards model with the likelihood ratio statistics was employed to further evaluate them for multivariate survival analysis. All statistical analyses were carried out by using statistical

program SPSS version 16.0. A two-tailed *P*-value of <0.05 was regarded as statistically significant.

3.11 Cell proliferation assay

Cell proliferation was measured by 3-(4,5-dimthylthiazol-2yl)-2,5- diphenyl-tetrazolium bromide (MTT) (Promega, Madison, WI, USA), which access the number of viable cells and determine the IC_{50}. For SAHA MTT assay, 50 µl of culture medium containing various concentrations of SAHA were added to 96-well plate and equilibrated at 37°C in a humidified, 5 % CO_2 atmosphere. Cell in log phase growth were seeded into the pre-equilibrated 96-well plates at a density of 1 x 10^4 cells in 50 µl to give a total volume of 100 µl/ml. For overexpressed TXNIP MTT assay, different recombinant vector-transfected cells at a density of 8 x 10^3 cells were seeded into the 96-well plate and equilibrated at 37°C in a humidified, 5 % CO_2 atmosphere. After different times of incubation, 15 µl of Dye solution was added to each well. The plate was then incubated in a humidified atmosphere of 5 % CO_2 at 37°C for 4 hours. After incubation, 100 µl of Solubilization/Stop solution was added to each well. One hour after the addition of the Solubilization/Stop solution, the contents of the wells were mixed to get a uniformly colored solution. Absorbance at 570 nm was measured using 96-well plate reader (Rainbow Spectra, Tecan, Switzerland).

3.12 Flow cytometry

3.12.1 Cell preparation

Cells were harvested by complete trypsinization to ensure single tumor cell suspension. Approximately 1 x 10^6 cells were collected and washed twice with cold PBS. Harvested cells were then resuspended in 70% ethanol and fixed overnight at -20°C for Propidium Iodide cell cycle assay. For Annexin V-FITC apoptosis assay, cells were directly stained with Annexin V-FITC probe.

3.12.2 Propidium Iodide staining

Propidium Iodide (PI) is a standard flow cytometric viability probe which used to distinguish viable and dead cells. The samples were vortex gently and centrifuged at 3,000 rpm for 8 minutes. The ethanol was discarded but less than 100 µl of ethanol was left behind in the samples. 400µl of PBS was added into the samples to resuspend the cell pellet. Then 12µl of PI staining solution (Sigma-Aldrich Co., St. Louis, MO, USA) was added to the samples and the samples were briefly vortex. The samples were incubated in dark at room temperature for 30 minutes. The samples were then transferred to a 5 ml round bottom tube before flow cytometer analysis.

3.12.3 Annexin V-FITC staining (Apoptosis assay)

For the detection and/or quantification of apoptosis, Annexin V-FITC binding method was used. Annexin V staining can identify apoptosis at earlier stage than

other assays that based on nuclear changes such as DNA fragmentation. Annexin V-FITC is a sensitive probe specific for identifying apoptotic cells at earlier stage with exposed phospholipids phosphatidylserine. Annexin V binding was determined using Annexin V-FITC apoptosis detection kit (BD Biosciences, San Jose, CA, USA) according to manufacturer's protocol and analyzed by flow cytometry as described below. Cells were washed twice with cold PBS and resuspended in 1 x binding buffer at a concentration of 1 x 10^6 cells/ml. 100 µl of the solution (1 x 10^5 cells) was transferred to a 5 ml round bottom tube and 5 µl of FITC-labeled Annexin V and 5 µl Propidium Iodide were added to the solution. The solution mixture was gently vortex and incubated for 15 minutes at room temperature in dark. 400 µl of 1 x binding buffer was added to the solution mixture. The samples were analyzed by flow cytometer immediately.

3.12.4 Flow cytometry analysis

After staining procedure, the samples were subjected to FACSCalibur multicolor flow cytometer (BD Biosciences, NJ, USA) and operated by CellQuest software (BD Biosciences, NJ, USA). CellQuest was also used to analyze Annexin V flow data and ModFit LT (Verity Software House) was used to analyze cell cycle flow data.

3.13 Intracellular ROS assay

Intracellular ROS was determined using the oxidant-sensitive fluorogenic probe CM-H_2DCFDA (5-(6)-chloromethyl-2'7'-dichlorodihydrofluorescein

diacetate, acetyl ester) from (Invitrogen; Carlsbad, California, USA). Cells were transiently transfected with TXNIP recombinant vector or empty vectors using Lipofectamine™ 2000 transfection kit (Invitrogen Corporation, Carlsbad, CA, USA).for 24 hrs. After 24 hrs, the medium was removed and 2 ml fresh medium (without phenol-red and serum) containing 5μM of DCF was added into the plate and incubated for 30 mins at 37°C incubator. The cells were washed once with PBS and fresh medium was added into the plate. The fluorescent intensity was visualized and captured under the fluorescent microscope.

3.14 Mitochondrial functional assay

The mitochondrial transmembrane potential value was determined using rhodamine 123 (Calbiochem, Darmstadt, Germany). Cells were transiently transfected with TXNIP recombinant vector or empty vectors using Lipofectamine™ 2000 transfection kit (Invitrogen Corporation, Carlsbad, CA, USA) for 24 hrs. After 24 hrs, the medium was removed and 2 ml fresh medium containing 2μl of rhodamine (1mg/ml) was added into the plate and incubated for 10 mins at 37°C incubator. The cells were washed once with PBS and fresh medium was added into the plate. The fluorescent intensity was visualized and captured under the fluorescent microscope.

3.15 Cell migration assay

Cell migration was assayed by using 24 well Costar® 8μM Transwell™ polycarbonate membrane permeable chamber (Corning Incorporated Life Sciences, Lowell, MA, USA) according to the manufacturer's instructions. AGS cells were

transiently transfected with indicated plasmids using Lipofectamine™ 2000 transfection kit (Invitrogen Corporation, Carlsbad, CA, USA). After 4 hours of transfection, cells were trypsinizaton and detached and plated at $3x10^4$ cells per insert chamber with plain medium. 600 µl of growth medium with 10% FBS was added onto the bottom of the chamber. Cells were then incubated in a 37°C incubator, supplied with humidified atmosphere containing 5 % CO_2 and 95% air. After 22 hours, cells were removed from the upper side of the membrane of the insert chamber with a cotton swab. The lower cells were washed with PBS, fixed with 100% methanol and stained with 2% crystal violet for 15 mins. The membranes were cut off and mounted in mounting medium on glass slides. Stained cells were counted under microscope and images were captured

3.16 *In vitro* cell invasion assay

Cell invasiveness was assayed by using 24 well BioCoat™ Matrigel™ invasion chamber with 8µM pore size (BD Biosciences, NJ, USA) according to the manufacturer's instructions. AGS cells were transiently transfected with indicated plasmids using Lipofectamine™ 2000 transfection kit (Invitrogen Corporation, Carlsbad, CA, USA). After 4 hours of transfection, cells were trypsinizaton and detached and plated at $3x10^4$ cells per insert chamber with plain medium. 600 µl of growth medium with 10% FBS was added onto the bottom of the chamber. Cells were then incubated in a 37°C incubator, supplied with humidified atmosphere containing 5 % CO_2 and 95% air. After 22 hours, cells were removed from the upper side of the membrane of the insert chamber with a cotton swab. The lower cells were washed with PBS, fixed with 100% methanol and stained with 2% crystal violet for 15 mins. The membrane were cut off and mounted in mounting

medium on glass slides. Stained cells were counted under microscope and images were captured

Chapter 4 Results

4.1 Identification of *TXNIP* as a potential target regulated by epigenetic histone acetylation

4.1.1 SAHA inhibited cell proliferation in gastric cancer cells

In our previous study, we had demonstrated the anticancer effects of SAHA in the gastric cancer cell model AGS. In this study, we included additional gastric cancer cell lines and examined their cell proliferation in response to SAHA treatment. We performed MTT proliferation assay in a panel of gastric cancer cell lines including MKN1, MKN7, MKN28, MKN45, SNU1, SNU16, AGS, N87 and KatoIII. The MTT results showed that SAHA exhibited cytotoxicity against these gastric cancer cell lines at both 48 and 72 hours after SAHA treatment (Figure 4.1). Among the panel of gastric cancer cell lines being investigated, AGS, N87 and SNU16 were more vulnerable to the cytotoxic effects induced by SAHA treatment at both time points. The cell growth of these three cell lines was dramatically decreased with increasing dosage of SAHA. Fifty-percent of growth inhibition (IC_{50}) of AGS was recorded at 4 μM and 2 μM SAHA at 48 hours and 72 hours respectively. For N87, the IC_{50} was 3 μM and 1.5 μM at 48 hours and 72 hours respectively. The IC_{50} of SNU16 was found to be at 4.5 μM at 48 and 72 hours and with almost the same numbers of viable cells at both time points. SNU1 had a very similar cell viability curve pattern at both time points and had the same IC_{50} (at 7

μM SAHA) at both 48 and 72 hours after SAHA treatment. In addition, same IC_{50} was also observed in Kato III (10 μM) at both 48 and 72 hours, although the cell viability curves were quite different at 48 and 72 hours.

The result from the MTT proliferation assay demonstrated that SAHA exhibited cytotoxicity against a panel of gastric cancer cell lines at both 48 hours and 72 hours time point. SAHA induced higher percentage of cell death at 72 hours than that of 48 hours at same concentration in several gastric cancer cell lines tested, including MKN1, MKN7, MKN28, MKN45, AGS and N87, suggesting that the response of these cells to the cytotoxic effects induced by SAHA may be time dependent.

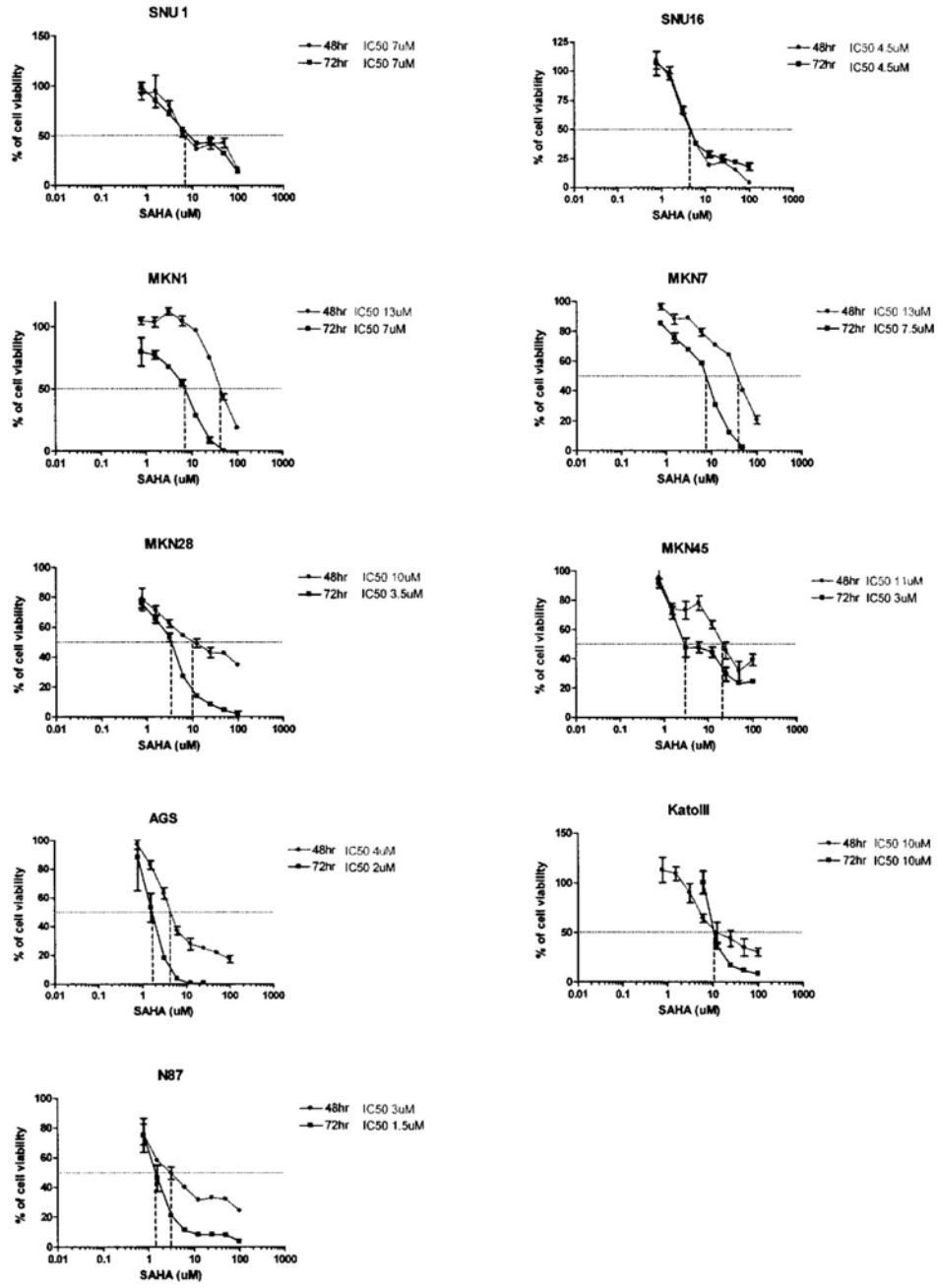


Figure 4.1 MTT proliferation assay in a panel of gastric cancer cell lines after 48 hrs or 72 hrs of SAHA treatment. Results were obtained from 3 independent experiments (means $\pm$ SD of triplicate experiments,* $P<0.05$).

4.1.2 SAHA induced gene expression profiles

To identify genes regulated by SAHA, AGS cells were cultured in two concentrations (2μM and 4μM) of SAHA for 24 hours and differentially gene expressions were analyzed by duplicate microarray experiments. The microarray data were normalized with median normalization which is the median that is used for scaling to obtain normalized values for each sample. The normalized data was analyzed by significance analysis of microarrays (SAM) method simultaneously in order to identify differential gene expressions with statistical significance. The SAM method automatically calculated the false discovery rate (FDR) using a permutation test. The FDR provides information of the expected proportion of genes that are false-positive. Thus, it helps to eliminate some falsely predicted genes according to the expected proportion of false discoveries is controlled. The principle of the FDR is based on the idea that one can tolerate more false discoveries if the number of the tests is large. For example, FDR of 5%, means that if 100 genes out of 10000 are predicted to be differentially expressed, then on average 5 genes would be predicted falsely. In the present study, gene was considered as significantly induced if the FDR was controlled to be less than 5% and was induced at least 2-fold upon SAHA treatment as compared to the control. In order to identify potential tumor suppressor genes that are regulated by histone acetylation, genes that were upregulated upon SAHA treatment were selected for further study. A total of 224 genes were found to be induced upon SAHA treatment (Table 4.1). The majority of the 224 genes were associated with cell proliferation and apoptosis.

Table 4.1 Differentially expressed genes that were induced upon SAHA treatment with a false discovery rate <5% and with a >2-fold upregulation in our microarray data. Thirty-two genes were downregulated (>1.5-fold) in nine gastric cancer cell (highlighted) in the gastric cancer expression array data.

	Accession	Gene.symbol	Fold.change
1	NM_006928	SILV	37.385
2	NM_005025	SERPINI1	23.389
3	U60873	TncRNA	29.126
4	NM_000729	CCK	32.657
5	231491.7	Data not found	14.485
6	NM_005794	DHRS2	17.426
7	NM_014400	LYPD3	10.345
8	NM_002933	RNASE1	9.098
9	NM_001785	CDA	9.154
10	NM_012240	SIRT4	8.608
11	NM_024028	PCYOX1L	7.638
12	NM_003518	HIST1H2BG	10.383
13	NM_000584	IL8	9.746
14	NM_006472	TXNIP	12.995
15	NM_004192	ASMTL	9.29
16	NM_000188	HK1	9.535
17	NM_004925	AQP3	6.807
18	NM_017786	FLJ20366	7.726
19	NM_002676	PMM1	6.66
20	NM_022661	SPANXC	7.507

	Accession	Gene.symbol	Fold.change
21	NM_022128	BRE	5.873
22	AF095719	CPA4	12.974
23	NM_015149	RGL1	7.118
24	NM_005619	RTN2	7.767
25	NM_005165	ALDOC	9.202
26	NM_006747	SIPA1	5.725
27	NM_001512	GSTA4	4.918
28	AK024443	PNPLA7	6.998
29	NM_003492	TMEM187	5.658
30	NM_002373	MAP1A	5.192
31	NM_000272	NPHP1	6.735
32	NM_031958	KRTAP3-1	5.873
33	NM_001145	RNASE4	6.092
34	BC013149	SLC27A1	5.076
35	NM_031301	APH1B	5.74
36	NM_032359	C3orf26	4.219
37	NM_006176	NRGN	5.228
38	NM_005410	SEPP1	5.277
39	NM_016487	C6orf203	4.612
40	NM_012446	SSBP2	7.674

	Accession	Gene.symbol	Fold.change
41	NM_012320	LYPLA3	7.262
42	NM_000904	NQO2	5.816
43	NM_001901	CTGF	4.539
44	NM_004726	REPS2	4.794
45	AK001460	Data not found	4.504
46	NM_000434	NEU1	6.298
47	NM_032898	C3orf34	5.092
48	NM_024507	KREMEN2	5.176
49	NM_001902	CTH	4.735
50	NM_018956	C9orf9	4.136
51	NM_006072	CCL26	4.565
52	NM_000700	ANXA1	4.747
53	NM_002274	KRT13	4.543
54	M97164	FTH1	4.076
55	NM_025047	ARL14	7.496
56	NM_017983	WIPI1	4.752
57	NM_017885	HCFC1R1	4.107
58	NM_032910	C21orf119	4.732
59	NM_014268	MAPRE2	3.883
60	NM_032933	C18orf45	4.042

	Accession	Gene.symbol	Fold.change
61	AL834175	FLJ36166	5.102
62	NM_004095	EIF4EBP1	3.177
63	NM_005252	FOS	4.671
64	NM_032705	C1orf97	4.781
65	NM_033292	CASP1	3.791
66	AB002360	BRSK2	4.097
67	NM_139025	ADAMTS13	3.978
68	NM_024306	FA2H	4.277
69	NM_030657	LIM2	4.518
70	NM_003582	DYRK3	3.867
71	NM_002923	RGS2	4.75
72	NM_032854	CORO6	4.056
73	NM_014040	Data not found	4.836
74	NM_000802	FOLR1	5.436
75	AK000028	FLJ20021	4.119
76	NM_003029	SHC1	4.95
77	NM_032251	CCDC88	4.716
78	NM_020524	PBXIP1	3.817
79	NM_019018	FAM105A	3.78
80	NM_017691	LRRC49	4.621

	Accession	Gene.symbol	Fold.change
81	AL365404	GPR108	4.644
82	NM_002206	ITGA7	3.784
83	201531.1	Data not found	6.63
84	NM_000062	SERPING1	4.042
85	NM_032751	Data not found	3.84
86	NM_001321	CSRP2	4.137
87	NM_145245	EVI5L	4.185
88	NM_000930	PLAT	3.414
89	AL122109	LOC90835	4.523
90	NM_015071	ARHGAP26	4.011
91	NM_002778	PSAP	4.715
92	NM_024681	KCTD17	4.067
93	NM_005345	HSPA1A	5.705
94	NM_002970	SAT1	3.868
95	NM_001165	BIRC3	6.112
96	NM_003101	SOAT1	5.897
97	NM_017805	RASIP1	4.519
98	NM_139205	Data not found	3.51
99	AK055031	LYPD5	4.476
100	BC008502	C4orf34	3.617

	Accession	Gene.symbol	Fold.change
101	NM_022172	PC	3.774
102	NM_016532	SKIP	3.489
103	BC012170	C3orf54	3.648
104	NM_005562	LAMC2	3.491
105	NM_032409	PINK1	4.335
106	NM_007088	CALB2	3.244
107	NM_014451	BBS9	3.786
108	NM_014718	CLSTN3	3.309
109	NM_007181	MAP4K1	3.702
110	NM_003516	HIST2H2AA3	5.02
111	NM_005135	SLC12A6	3.442
112	NM_019021	C11orf71	3.926
113	L14565	Data not found	4.265
114	NM_032039	ITFG3	3.684
115	NM_014585	SLC40A1	4.049
116	NM_004453	ETFDH	3.861
117	NM_138432	SDSL	3.958
118	406202.1	Data not found	3.696
119	NM_003979	GPRC5A	4.625
120	Z56123	Data not found	4.021

	Accession	Gene.symbol	Fold.change
121	NM_002661	PLCG2	3.544
122	NM_001928	CFD	4.44
123	NM_014471	SPINK4	3.619
124	Z58424	Data not found	3.304
125	NM_001089	ABCA3	3.875
126	NM_002254	KIF3C	3.074
127	NM_003273	TM7SF2	3.269
128	NM_002070	GNAI2	3.418
129	NM_006992	LRRC23	3.457
130	NM_003125	SPRR1B	3.434
131	NM_152544	C4orf23	1.917
132	AK094595	UNC5B	3.539
133	NM_024296	CCDC28B	2.928
134	NM_001731	BTG1	3.384
135	NM_000347	SPTB	2.99
136	BC000566	RABL4	3.725
137	NM_006270	RRAS	3.534
138	NM_012257	HBP1	3.501
139	AL049370	Data not found	2.861
140	BC004888	SUSD4	3.552

	Accession	Gene.symbol	Fold.change
141	NM_024927	PLEKHH3	4.239
142	NM_015896	ZMYND10	4.336
143	NM_020322	ACCN3	3.487
144	AF112213	C20orf24	3.677
145	NM_000235	LIPA	3.01
146	NM_058189	C21orf69	2.588
147	NM_021827	CCDC81	1.77
148	BM801869	Data not found	3.178
149	NM_014297	ETHE1	3.017
150	NM_005231	CTTN	1.808
151	NM_003897	IER3	3.123
152	NM_020448	NPAL3	3.022
153	NM_007209	RPL35	2.279
154	NM_031924	RSHL2	3.916
155	NM_000053	ATP7B	3.168
156	NM_004529	MLLT3	3.019
157	NM_001621	AHR	3.323
158	AF070641	ETV1	3.416
159	BQ316331	ETFDH	3.308
160	D01059	IGL@	3.328

	Accession	Gene.symbol	Fold.change
161	NM_005384	NFIL3	3.265
162	NM_005811	GDF11	3.142
163	AF258578	Data not found	3.377
164	NM_032118	WDR54	3.816
165	AB040965	SPPL2B	2.861
166	NM_014399	TSPAN13	3.097
167	NM_004647	DPF1	3.15
168	NM_022830	TUT1	2.91
169	NM_012268	PLD3	3.709
170	NM_004223	UBE2L6	2.762
171	NM_001062	TCN1	3.314
172	NM_003165	STXBP1	2.875
173	NM_024064	Data not found	2.718
174	NM_003275	TMOD1	3.065
175	NM_032903	Data not found	3.514
176	NM_018706	DHTKD1	4.129
177	NM_021979	HSPA2	4.401
178	BC009612	Data not found	3.729
179	NM_031437	Data not found	2.951
180	NM_032622	LNX1	2.905

	Accession	Gene.symbol	Fold.change
181	NM_003528	HIST2H2BE	4.131
182	NM_002284	KRT86	3.153
183	NM_004457	ACSL3	3.105
184	BC037223	MED19	3.394
185	AB037810	SIPA1L2	3.001
186	NM_025084	FLJ22795	2.76
187	NM_023948	MOSPD3	2.817
188	NM_012079	Data not found	2.88
189	NM_007236	CHP	2.565
190	NM_014905	GLS	3.83
191	NM_014962	BTBD3	3.799
192	NM_021195	CLDN6	3.355
193	NM_004252	SLC9A3R1	3.112
194	NM_025160	WDR26	2.907
195	AL137722	VWA1	2.516
196	NM_017893	SEMA4G	2.645
197	NM_022818	MAP1LC3B	3.085
198	NM_003107	SOX4	2.629
199	NM_007065	CDC37	2.671
200	AF052151	FAM89B	3.308

	Accession	Gene.symbol	Fold.change
201	NM_052936	ATG4A	2.689
202	NM_018070	SSBP3	2.662
203	NM_001628	AKR1B1	2.647
204	NM_018691	C5orf3	3.375
205	NM_001554	CYR61	3.153
206	NM_006332	IFI30	2.789
207	NM_004040	RHOB	3.275
208	NM_001175	ARHGDIB	2.869
209	AW955692	USP2	2.795
210	NM_004716	PCSK7	2.146
211	NM_002601	PDE6D	2.909
212	NM_022356	LEPRE1	2.664
213	NM_022460	HS1BP3	2.78
214	NM_005194	CEBPB	3.894
215	AY008445	Data not found	2.961
216	BC035778	NSUN6	3.017
217	AF170307	C9orf130	2.862
218	1513001.2	Data not found	2.701
219	D49387	LTB4DH	3.199
220	NM_000375	UROS	4.139

	Accession	Gene.symbol	Fold.change
221	NM_024578	OCEL1	2.744
222	NM_006931	SLC2A3	2.899
223	AL833056	ZNF438	2.7
224	NM_006688	C1QL1	2.663

4.1.3 The comparison of SAHA-induced gene expression profiles and gastric cancer cells gene expression profiles

In addition, we compared the SAHA-induced gene expression profiles with our in-house gastric cancer cells gene expression profiles. In our in-house gastric cancer cells gene expression profiles, the expression profiles of 9 gastric cancer cell lines verus normal gastric tissue were obtained by microarray gene expression analysis and the data collected were first normalized and then compared with the normal gastric control total RNA that purchased commercially from Ambion. Those genes that were downregulated with a 1.5 fold cutoff among the 9 gastric cancer cells were selected. Totally, there were 2770 genes (2770 genes out of 27750 probes) selected according to above criteria. These 2770 downregulated genes were crosschecked with the 224 SAHA-induced target genes. Thirty-two genes (highlighted in Table 4.1) were found to be overlapped across both gene sets. These 32 genes were commonly downregulated in the gastric cancer cell lines and were significantly induced by SAHA treatment.

4.1.4 Identification of target gene with the highest gene expression induced by SAHA

We compared the induction fold change of these 32 genes selected from gastric cancer gene expression profiles vs SAHA-induced expression profiles. Thioredoxin Interacting Protein (also known as *VDUP-1/TBP2/TXNIP*) had the highest induction levels (13 folds) upon SAHA treatment. Thus, we further selected and focused on *TXNIP* as it could be the potential candidates of tumor suppressor

gene in gastric cancer.

4.1.5 Cross checked with in house array-CGH data

We then cross checked *TXNIP* to our in-house whole genome array comparative genomic hybridization (array CGH) data. *TXNIP* was found to be genetically stable among all cell lines except for MKN45. In our array-CGH data (Figure 4.2), the results revealed that *TXNIP* showed genomic gain in MKN45 with a log 2 ratio of 1.29. Other cell lines have a log 2 ratio range from 0.018 to 0.83 which showed neither amplification nor LOH in these gastric cancer cells.

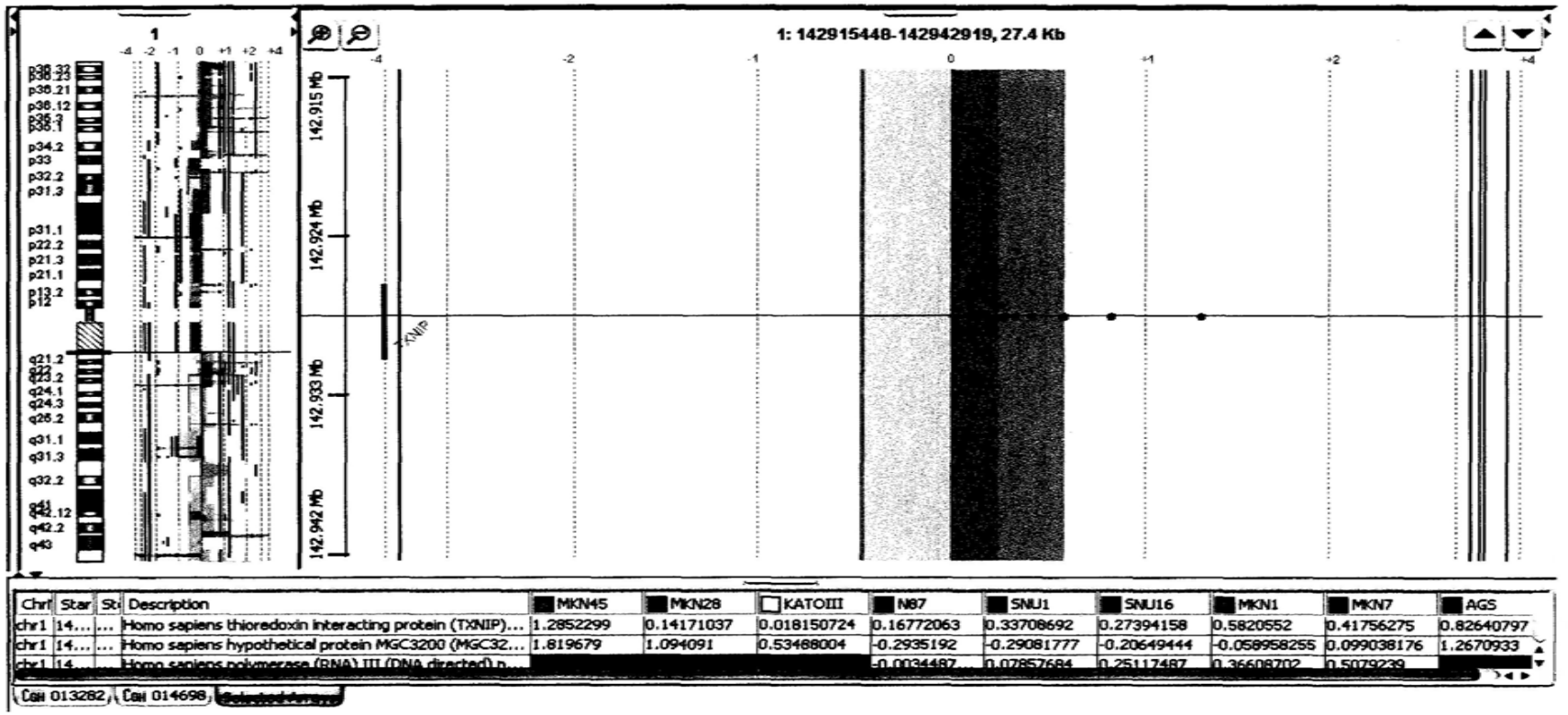


Figure 4.2 Whole genome array comparative genomic hybridization (array-CGH) of nine gastric cancer cell lines on chrosomre 1q21.1.

4.2 *TXNIP* is regulated by histone acetylation

4.2.1 Effect of SAHA and 5aza treatments in the expression of *TXNIP* in gastric cancer cells

Gene promoter hypermethylation is another major epigenetic mechanism which regulated the silencing of tumor suppressor genes. We investigated whether promoter methylation may also involve in the silencing of *TXNIP*. If *TXNIP* silencing is regulated by histone acetylation, SAHA treatment would induce the expression of *TXNIP*. Whereas, if *TXNIP* is regulated under gene promoter methylation, 5aza-dR treatment would induce the expression of *TXNIP*. As mentioned in the literature review section, previous studies have demonstrated an interaction between DNA methylation and histone modification. Thus, we would also like to investigate whether combined treatment may synergistically induced *TXNIP* expression in gastric cancer cells.

mRNA expression levels of *TXNIP* were analyzed by quantitative real-time PCR. Taqman® assay was performed in nine gastric cancer cell lines which cultured with histone deacetylase inhibitor SAHA alone, demethylating agent 5aza-dR alone or synergistically to see whether the expression of *TXNIP* was altered by the treatments. For combined treatment (SAHA and 5aza-dR), the gastric cancer cells were first subjected to 5aza-dR demethylating agent treatment for 3 days before subjected to SAHA treatment for another 24 hours (Figure 4.3).

The mRNA expressions of *TXNIP* were found to be significantly upregulated upon the treatment of SAHA in AGS, MKN1, MKN28, MKN45, SNU16, N87 and Kato III ($p<0.05$). Upon SAHA treatment, *TXNIP* expression was significantly induced by 3-folds in MKN1, 6-folds in MKN45, 24-folds in AGS, 6-folds in Kato III, 5-folds in N87, 2-folds in SNU16, and 2-folds in MKN28, respectively. Whereas upon 5aza-dR treatment, MKN7, MKN28, MKN45, AGS and N87 *TXNIP* expression were found to be significantly upregulated ($p<0.05$), although to a lesser extend in terms of fold change and number of cell lines when compared with the treatment of SAHA. Combined treatment of 5'aza and SAHA synergistically resulted in marked upregulation of expression of *TXNIP* in MKN28 (317-folds), MKN45 (18-folds), N87 (10-folds) cells, but not the other gastric cancer cell lines. These data suggested that apart from histone acethylation, DNA methylation may also contribute to the regulation of *TXNIP* expression in a subset of gastric cancer cells.

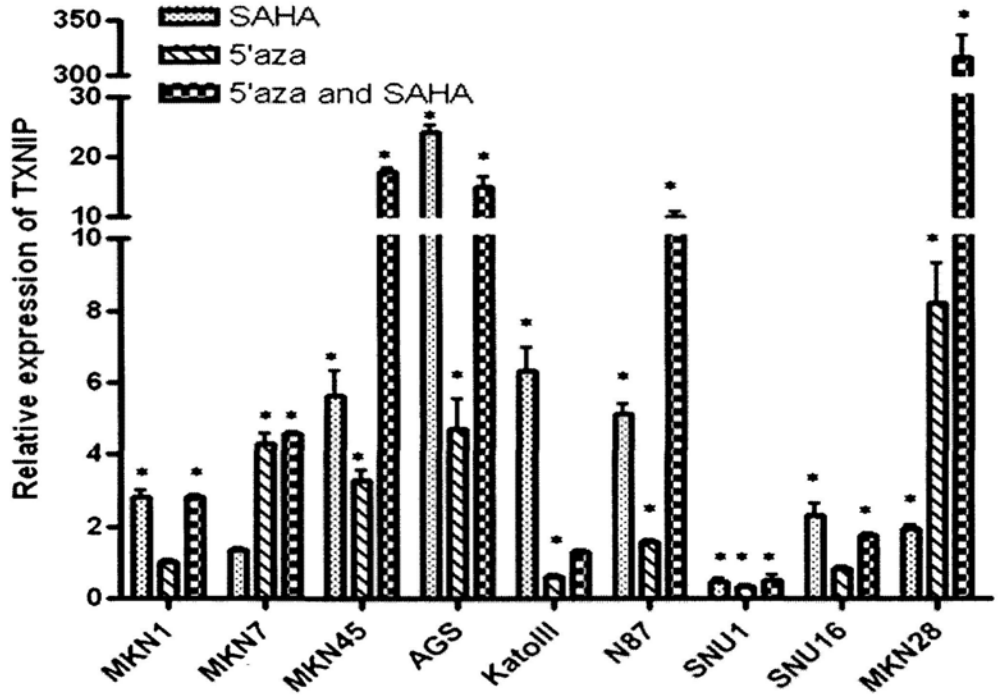


Figure 4.3 *TXNIP* mRNA expression induced in gastric cancer cell lines upon SAHA, 5aza-dR or combine treatments. *TXNIP* mRNA expression was significantly up-regulated in various gastric cancer cell lines upon the treatments (t-test; * $p<0.05$). Relative expression of *TXNIP* in terms of fold change was plotted as compared to control. Data was shown as mean $\pm$ SD (n=3). The mRNA expression levels were quantified by using Taqman QRT-PCR.

We have also examined the protein levels of *TXNIP* upon various treatments in the nine gastric cancer cell lines by a series of immunobloting experiments (Figure 4.4). Without any treatment, *TXNIP* was normally expressed in Kato III, N87, SNU1, SNU16, MKN1, MKN7 and MKN45. However, expression of *TXNIP* could not be detected in AGS and MKN28 cells. Given that various concentrations of monoclonal anti-*TXNIP*, as well as prolonged incubation and exposure time were tested, *TXNIP* endogenous expression in AGS and MKN28 cells remained undetectable. The results may be attributed to the unusually low basal expression of *TXNIP* in AGS and MKN28 cells.

Upon treatment of SAHA, the protein levels of *TXNIP* of most of the gastric cancer cell lines increased dramatically. *TXNIP* expression in KatoIII, MKN45 and N87 were significantly induced at least 2-fold by SAHA treatment. SAHA treated AGS cells showed even much higher protein expression of *TXNIP* as compared to untreated cells and in fact *TXNIP* expression was not observable in AGS untreated cells. For MKN1, SAHA also capable to induce *TXNIP* expression but the induction was less compare to other SAHA inducible gastric cancer cell lines. No difference could be observed in MKN7 upon SAHA treatment. Interestingly, for gastric cancer cells SNU1 and SNU16, *TXNIP* expression was downregulated upon treatment of SAHA. For MKN28, SAHA treatment was unable to induce the expression of *TXNIP* to a detectable level. The concordant increase of both mRNA and protein levels of *TXNIP* upon SAHA treatment in most gastric cancer cells supported that histone deacetylation plays an important role in the gene silencing of *TXNIP*. For combine treatment, none of the cell lines showed synergestic effect upon treatment of both 5aza-dR and SAHA at the protein levels. Interestingly, in the

immunoblotting results of the protein expression of *TXNIP* of combine treatment of AGS (Figure 4.4, lower panel), the protein expression of AGS was detected in the mock of combine treatment. Normally, the protein expression of *TXNIP* was undetectable in mock. We think that the protein expression of *TXNIP* was increased to a detectable level in the mock was due to the increased density of AGS cells in the culture plate or caused by serum deprivation in the growth medium which resulted in increased *TXNIP* protein expression.

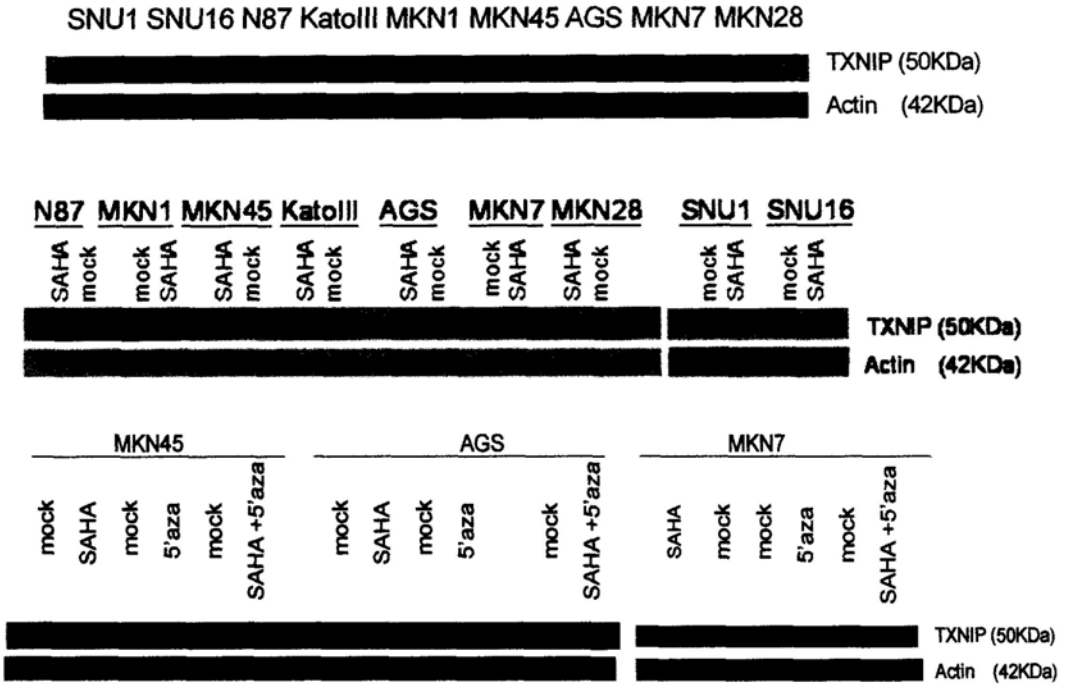


Figure 4.4 Western blot analysis of *TXNIP* protein expression in baseline and upon SAHA or 5aza-dR treatment. *TXNIP* protein expression was induced in a panel of gastric cancer cell lines except for MKN7, MKN28, SNU1 and SNU16 upon SAHA treatment. 5aza-dR treatment mildly up-regulated *TXNIP* protein expression in AGS cells. Upper panel; basal protein expression of *TXNIP*; middle panel; *TXNIP* protein expression after SAHA treatment; lower panel; *TXNIP* protein expression upon treatment of SAHA or 5aza-dR or combine treatment.

4.2.2 Investigation of gene promoter methylation in epigenetic silencing of *TXNIP* in gastric cancer cells

4.2.2.1 Promoter methylation

By using the Methylprimer Express software from Applied Biosystems, we found that the 5' region of *TXNIP* (AB051901) contains a CpG island that spans approximately 1000bp (GC content >50%; CpG observed/ CpG expected ratio >0.6), located on +1 of the transcriptional start site (2281bp to 3231bp of nucleotide sequence of AB051901), The CpG island is located on approximately 1kb upstream of the first exon. Since hypermethylation of promoter CpG-dinucleotides often associated with inactivation of gene transcription, we first used bisulfate sequencing method with primers that amplify the regions around the TATA-box and another region around the first exon to access the methylation status of *TXNIP* in a panel of gastric cancer cell lines. We directly sequenced the genomic DNA after sodium bisulfate modification. Sodium bisulfate converts DNA base C to T, but not methylated C of the CpG-dinucleotide. The bisulfate sequencing primers were designated according to the published journal papers (Figure 4.5) (Ahsan et al., 2006). Totally there were 21 CpG sites and 17 CpG sites located within the primers flanking the TATA-box region and exon one region respectively. As shown in figure 4.6, the CpGs in both promoter region and exon 1 region of *TXNIP* were heavily methylated in MKN28 cells, more that 90% of the CpG sites were methylated. CpGs were also heavily methylated in exon one region in SNU16 cells (Figure 4.7). Some of the cell lines including SNU16 and KatoIII showed methylation in some CpG sites, the percentage of the methylation status of those CpG sites was less than

or approximately 30%. Althought some of the cell lines showed increased RNA levels of *TXNIP* after treatment of 5aza-dR, the induction could be a result of indirect or secondary effects. Another possible explanation is that, 5aza-dR may alter TXNIP expression via alteration of the regulation of histone methylation, which in turn influences the transcriptional activity of TXNIP. Based on the bisulfate sequencing results, DNA methylation may not be a frequent or important mechanism of silencing *TXNIP* expression in gastric cancer cells.

4.2.2.2 Methylation may not be involved in the silencing of *TXNIP* in gastric cancer cells

As mentioned in the previous section, we used the DNA demethylating agent 5aza-dR to access the mRNA and protein expression of *TXNIP* in gastric cancer cells (Figure 4.3 and 4.4). The expression of *TXNIP* was significantly induced by 4-folds in MKN7 cells, 8-folds in MKN28 cells, 3-folds in MKN45 cells, 5-folds in AGS cells and 1.6-folds in N87 cells at the mRNA levels after the demethylating treatment. We then investigated the protein levels of MKN7, MKN28, MKN45 and AGS cells upon the treatment of 5aza-dR. However, only AGS cells showed a mild increase of *TXNIP* protein level after the treatment. MKN45 and MKN7 were not unregulated upon treatment.

Theoretically, MKN28 could be a potential cell line model in investigation of *TXNIP* regulation by gene promoter methylation since the promoter region of *TXNIP* in MKN28 was densely methylated. Indeed, MKN28 showed markedly increase in the relative fold change of mRNA expression upon the treatment of 5aza-dR. However, the protein level was remained undetectable by western blot

analysis. As we mentioned in the previous section, most of the cell lines including MKN28 showed no genomic gain or loss in *TXNIP* by the array CGH result. In order to exclude gene mutation in *TXNIP* in MKN28 cells, we sequenced all the exons of *TXNIP* gene in MKN28 cells. Totally, there are 8 exons in *TXNIP* gene and there was no mutation detected by direct sequencing analysis (Appendix I). The basal mRNA expression of MKN28 is extremely low (CT value was undetermined) as compared to other gastric cancer cell lines. Even after the treatment of 5aza-dR or combined treatment (5aza-dR and SAHA), the CT values were approximately 34 and was low as compared to that of other gastric cancer cell lines. A likely possible explanation for the absence of detectable protein expression of *TXNIP* is the extremely low level of TXNIP that is beyond the detection limit of the anti-TXNIP antibody. In summary, gene promoter methylation may not be an important epigenetic mechanism but may play a role in regulating the expression of *TXNIP* in gastric cancer cells.

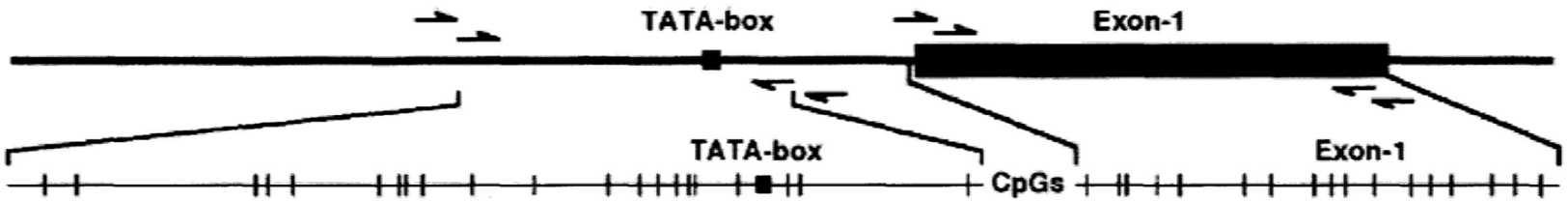


Figure 4.5 Schematic diagram of the location of the primers for sodium bisulfate sequencing of genomic DNA in gastric cancer cells [adopted from (Ahsan et al., 2006)].

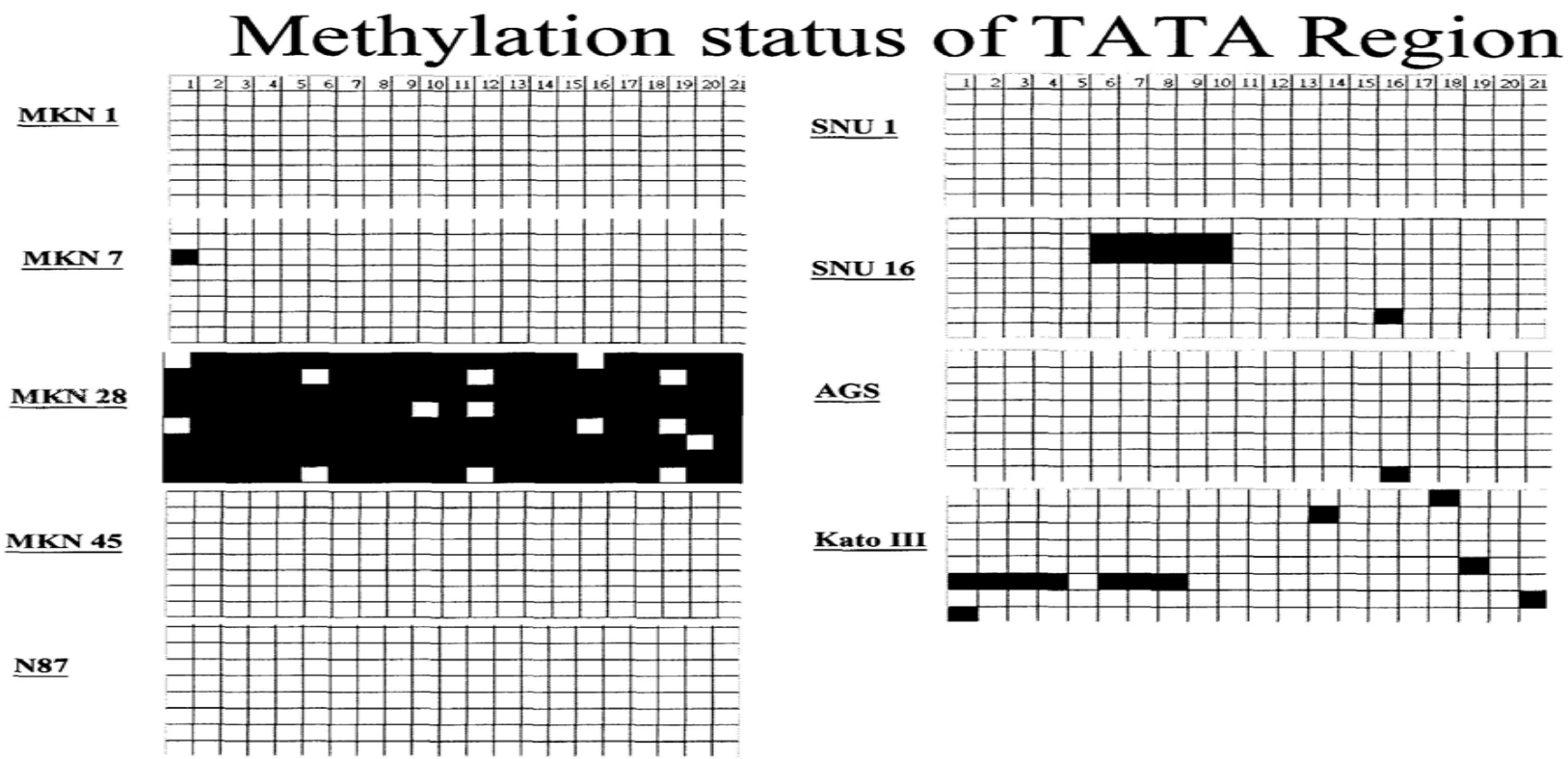


Figure 4.6 Bisulfate sequencing analysis of methylation statuses at the TATA-box region on the promoter of *TXNIP* in gastric cancer cell lines.

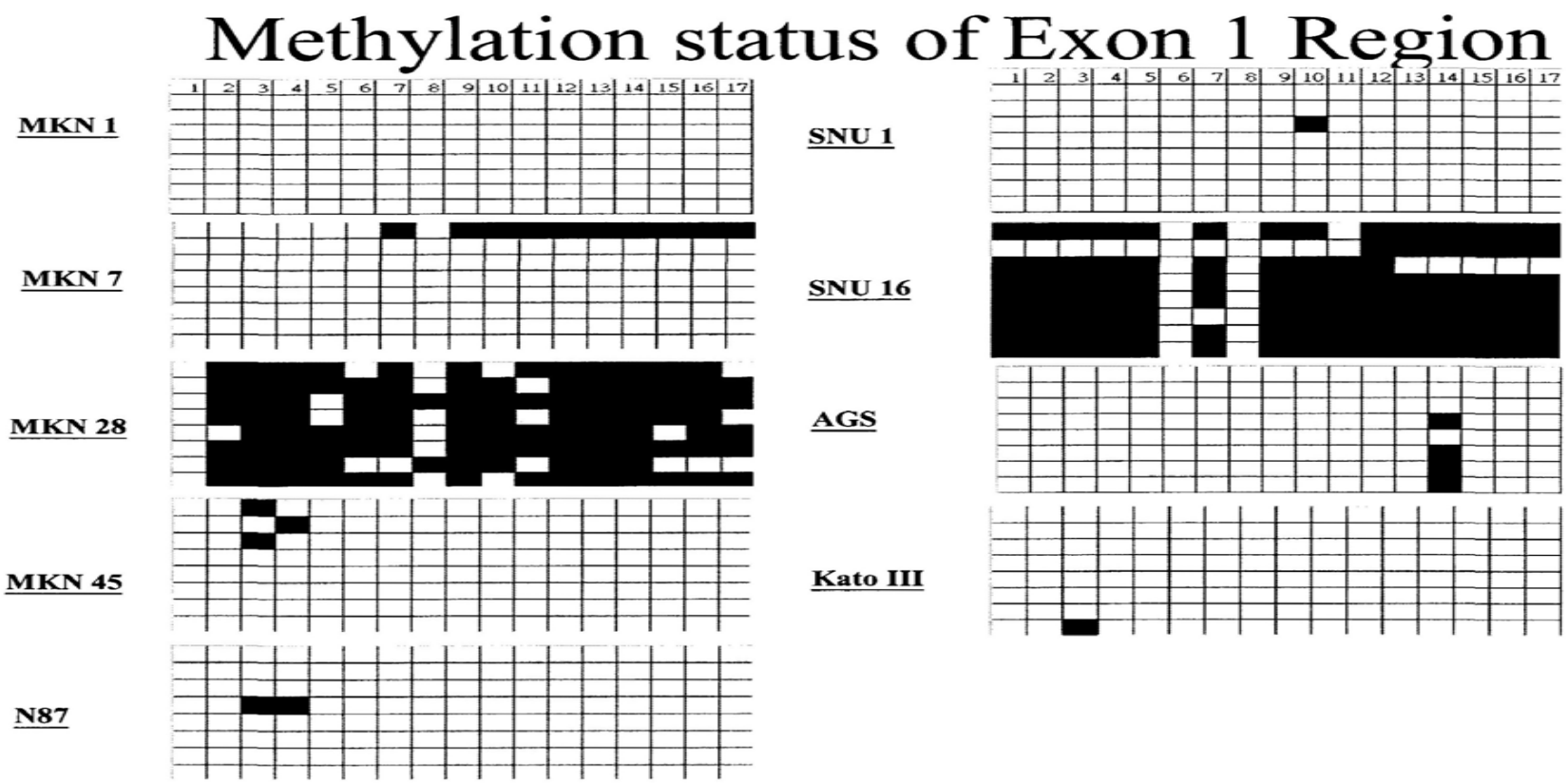
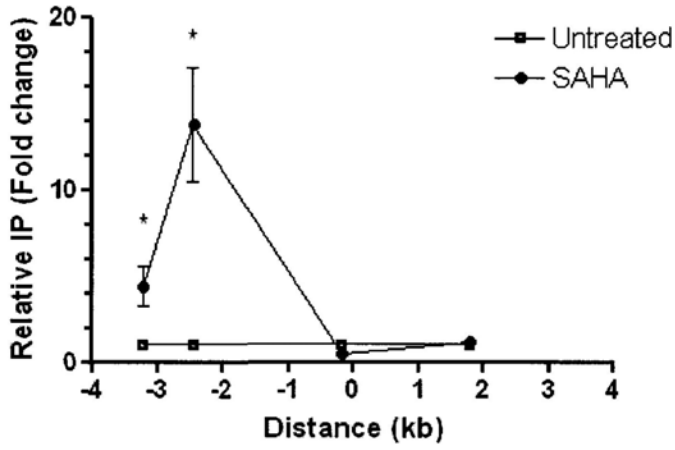


Figure 4.7 Bisulfate sequencing analysis Methylation statuses at first exon of *TXNIP* in gastric cancer cell lines.

4.2.3 SAHA enhances the transcriptional activity of *TXNIP*

We further investigated whether the expression of *TXNIP* is under the regulation of histone acetylation. To examine the histone acetylation status in the region around the promoter of *TXNIP*, chromatin immunoprecipitation assays were carried out using antibodies against acetyl-histone H3 and acetyl-histone H4 respectively. As shown in figure 4.8, SAHA treatment increased both acetylated H3 and H4 at the *TXNIP* promoter region. Compared to the input control, promoter acetylation of H3 during the SAHA treatment was about 4-fold higher at -2.5kb and 14-fold higher at -3kb upstream of the transcriptional start site of *TXNIP* than that of the untreated samples. SAHA treatment also increased the binding of acetylated H4 by approximately 1.5-fold at -3kb and 3-fold at -2.5kb upstream and 5-fold at 2kb downstream of the transcriptional start site of *TXNIP* as compared to the control. Since histone acetylation correlated with active gene transcriptions and such effect would in turns increased acetylated histones. In our data, SAHA inhibit HDAC activity and induced *TXNIP* expression via increasing histone acetylation at the *TXNIP* promoter region which leads to enrichment of acetylated histone H3 and H4. Thus, enrichment of acetylated histone H3 and H4 on the *TXNIP* promoter and increased transcription activity upon SAHA treatment supported that the expression of *TXNIP* is regulated by histone acetylation.

A



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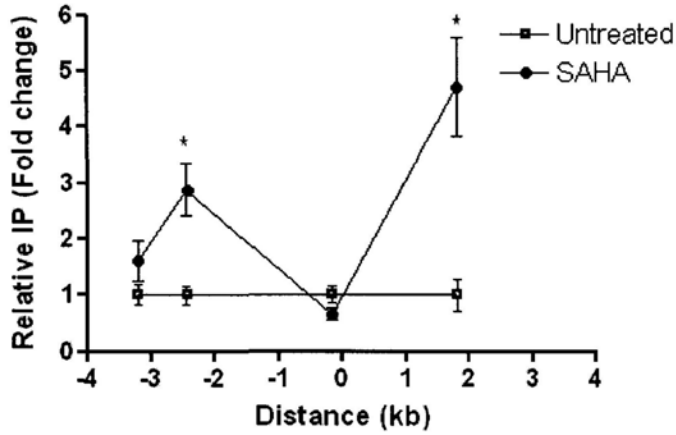


Figure 4.8 Increased histone acetylation by SAHA treatment at the *TXNIP* promoter region by using antibodies against (A) acetylated histone H3 and (B) acetylated histone H4 near the promoter region. All tests were performed in 3 independent experiments. Data was shown as mean $\pm$ SD (n=3). 0kb represented the transcriptional start site of *TXNIP*.

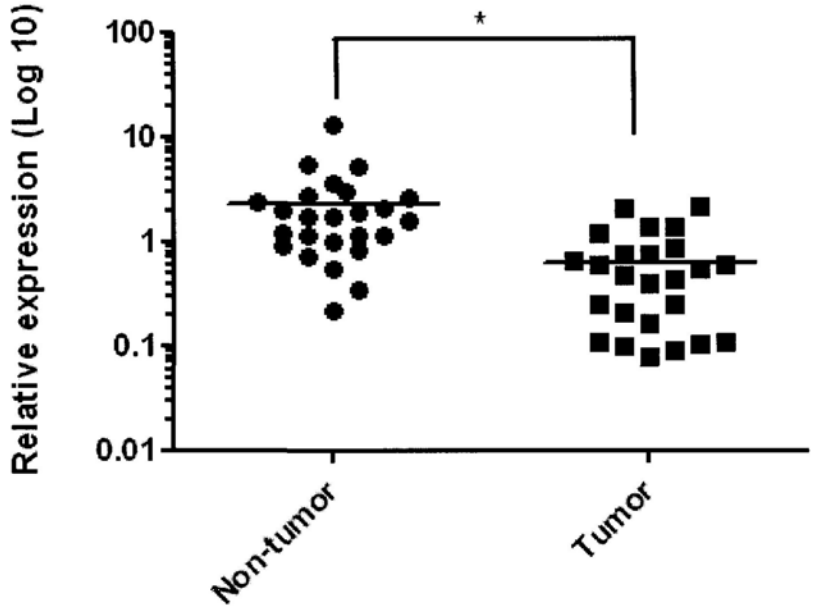
4.3 Expression of *TXNIP* in primary gastric cancer samples

4.3.1 *TXNIP* expression level in paired primary gastric cancer tissues

In order to assess whether down-regulation of *TXNIP* occurs in primary gastric cancer, mRNA and protein expression were investigated by quantitative RT-PCR and western blotting analysis respectively. *TXNIP* mRNA and protein levels of each primary gastric cancer sample were compared to its paired-up non-cancer gastric mucosal tissue sample. All primary samples were obtained from Prince of Wales Hospital, Hong Kong between the years of 1998 to 2000. In total, 25 paired primary samples were investigated, expressions of *TXNIP* in 21 of them (84%) were found to be down-regulated when compared with their paired non-cancer gastric tissues (Figure 4.9 A). The mRNA expression level of *TXNIP* in gastric cancer tissues were significantly lower than that of the pair-up non-cancer gastric tissues ($p=0.002$, pair t-test).

We managed to recruit 5 cases of pair-up gastric cancer and non-cancer gastric mucosal tissues samples for western blotting analysis (Figure 4.9 B). Three out of five cases (60% of cases) showed significant reduction of protein expression level of *TXNIP* in gastric cancer tissues (T) as compared to the pair-up non-cancer gastric tissues (N) (cases number 1-3). Taken together, the results indicated that expression of *TXNIP* was frequently down-regulated in primary gastric cancer.

A



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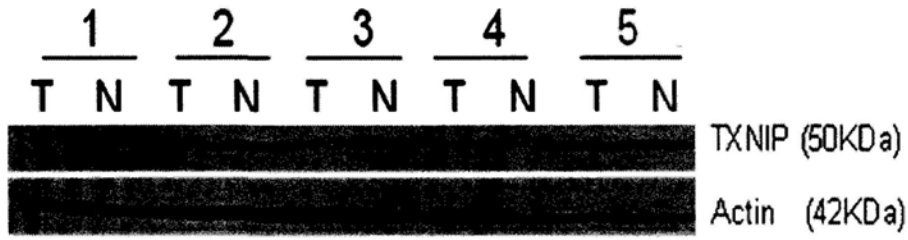


Figure 4.9 *TXNIP* expression in primary gastric cancer samples. (A) The relative expression of *TXNIP* mRNA was significantly lower ($p<0.002$) in cancer tissues as compared to paired non-cancer gastric mucosal tissues in 25 cases of pair-up primary gastric samples. The expressions were normalized with *GAPDH*. (B) The protein expressions of *TXNIP* (T, Tumor; N, Non-tumor).

4.3.2 *TXNIP* expression and correlation with clinicopathological characteristics

4.3.2.1 Clinicopathological characteristics of gastric carcinoma samples

Tissue microarray (TMA) blocks including 150 cases of primary gastric adenocarcinoma paraffin tissue samples were constructed. The samples were obtained from Prince of Wales Hospital, Hong Kong between the years of 1998-2002 and the basic clinicopathological characteristics were summarized in table 4.2. The staging classification was according to the American Joint Commette on Cancer (AJCC) Staging Manual (6th edition) as mentioned in the "Literature Review" section. In the 150 of gastric carcinoma patients, 63% were male and 37% were female (male to female ratio of 1.7:1). The median age was 69 years, mean age was 66 years (+/-12 years, range 35 to 88). The median follow up time was 16.2 months, mean follow up times was 36.2 months (+/- 38 months, range 0.3 to 143.4). Histology of the gastric cancer included intestinal-type (59%), diffuse-type (28%) and mixed-type (13%). Thus, 41% of cases contained diffuse component. For the histology grading of gastric cancer, 5% were well differentiated, 40% were moderately differentiated and 55% were poorly differentiated adenocarcinoma. For the overall staging, 13% were stage I, 13% were stage II, 37% were stage III and 37% were stage IV. The presence of helicobacter pylori was also observed in 63% of gastric samples at the time of diagnosis.

As mentioned in the "Materials and Methods" section, the cytoplasmic expression of *TXNIP* immunohistochemical staining was scored and graded. The

cytoplasmic score of *TXNIP* was the product of proportion and intensity scores, ranging from 0 to 12. Then the scoring was categorized into grade 0 (score=0, complete loss of staining), grade 1 (score 1 to 3, weak staining), grade 2 (score 4-6, intermediate staining), and grade 3 (score 7-12, strong staining). The non-cancer gastric mucosa normally expressed *TXNIP* with weak staining in foveolar epithelium and intermediate to strong staining in the gastric glands. The representative images of *TXNIP* staining are illustrated in fig 4.10 and 4.11. Overall, strong *TXNIP* staining was observed in 7.3% (11/150), intermediate staining 26% (39/150), weak staining 40.7% (61/150) and complete loss of staining in 26% (39/150) of gastric cancer samples.

Table 4.2 Clinicopathological characteristics of 150 cases of gastric carcinoma.

Characteristic	Number	Percentage
Sex		
Male:Female	95:55	63.3:36.7
Age (year)		
Mean (±SD), Range	66 (±12), 35-88	
Median	69	
Follow updated (months)		
Mean (±SD), Range	36.2(±38), 0.3-143.4	
Median	16.2	
Type		
Intestinal	89	59.3
Diffuse	42	28
Mixed	19	12.7
Diffuse component		
Absence	89	59.3
Presence	61	40.7
Grade		
Well differentiated	7	4.7
Moderate differentiated	60	40
Poorly differentiated	83	55.3
Stage		
I	20	13.3
II	19	12.7
III	55	36.7
IV	56	37.3
Helicobacter pylori		
Negative	56	37.3
Positive	94	62.7
TXNIP expression		
0	39	26
1	61	40.7
2	39	26
3	11	7.3

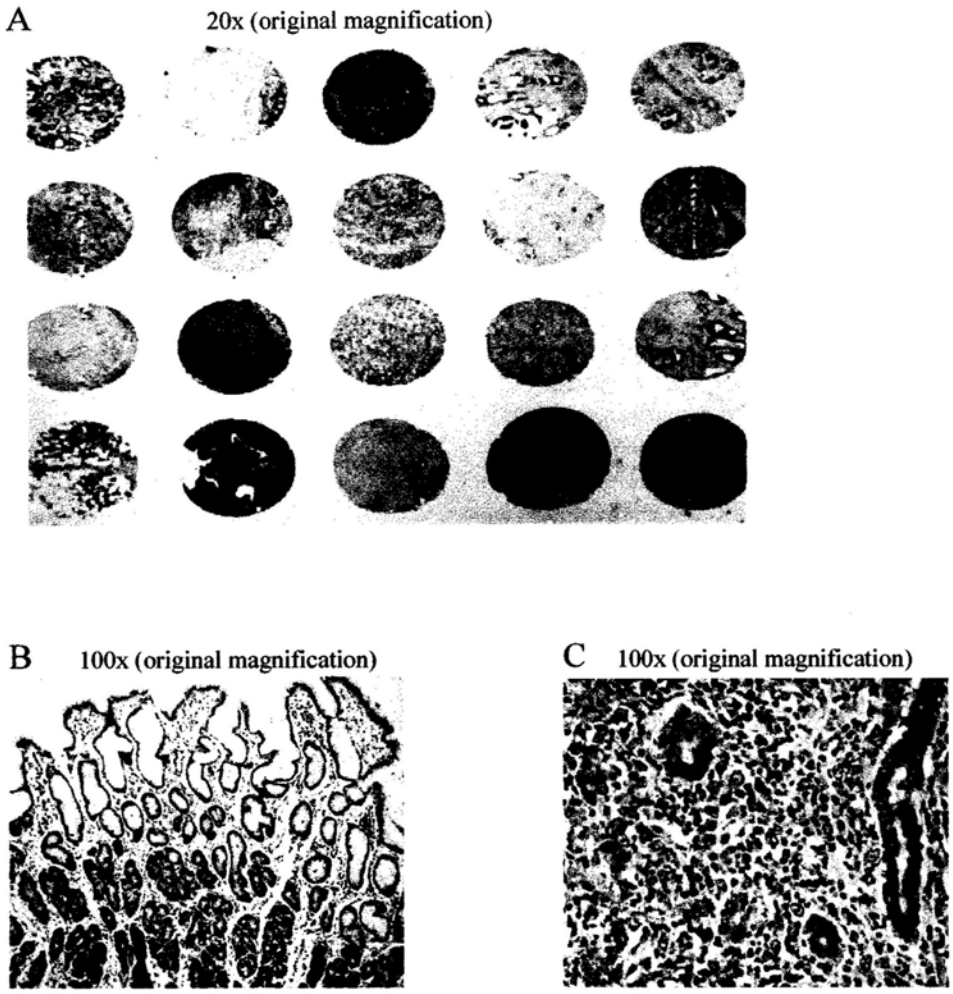


Figure 4.10 Microscopic imaging of the *TXNIP* cytoplasmic expression in the gastric carcinoma samples in the tissue microarray (TMA), A) partial region of the TMA; B) paired non-cancer gastric mucosa; C) paired diffuse-type gastric adenocarcinoma.

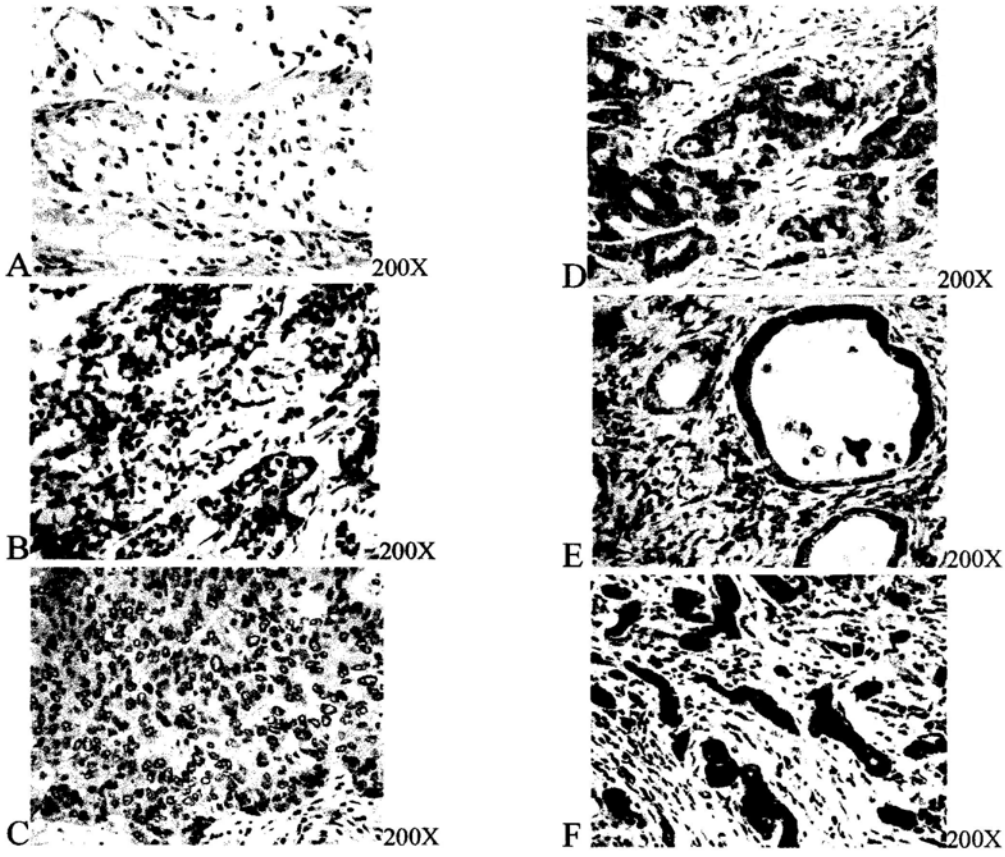


Figure 4.11 Representative microscopic imaging of the of the *TXNIP* cytoplasmic expression in representative gastric carcinoma samples within the tissue microarray (TMA), A) diffuse-type grade 0; B) diffuse-type grade 1; C) intestinal-type grade 0; D) intestinal-type grade 1; E) intestinal-type grade 2; and F) intestinal-type grade 3), with 200x original magnification.

4.3.2.2 Association of *TXNIP* expression with clinicopathological characteristics in gastric carcinoma

Correlations between TXNIP cytoplasmic stain and clinicopathological parameters were assessed by non-parametric Spearman's rho rank test. Loss or low *TXNIP* expression was significantly associated with several clinicopathological features, including the presence of diffuse histology, more advance overall staging, tumor (T) stage, lymph node (N) stage and metastasis (M) stage. Interestingly, it also associated with the presence of helicobacter pylori detected at the time of diagnosis. *TXNIP* expression was not associated with gender, age or histology grading of cancer. The data was summarized in table 4.3.

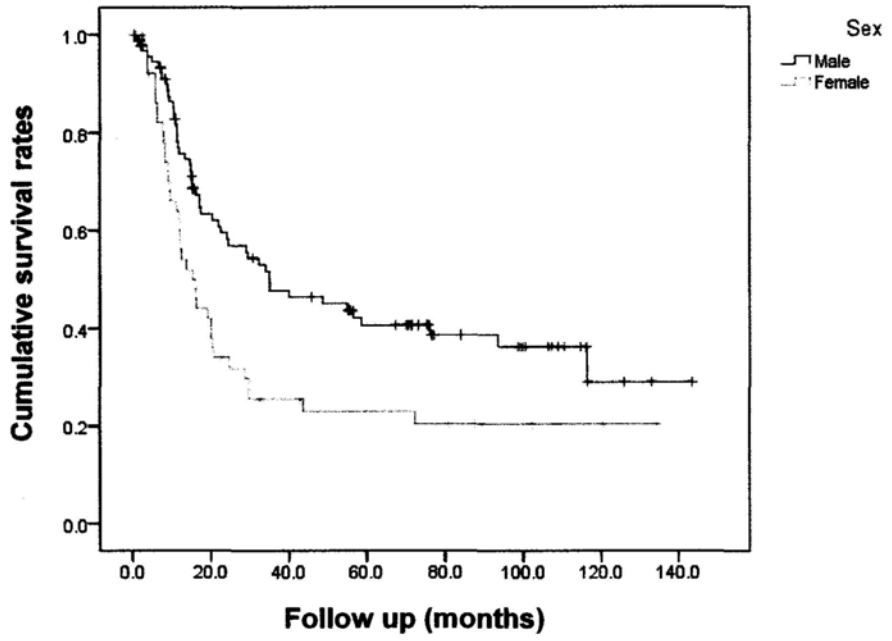
Table 4.3 Correlation of TXNIP expression with clinicopathological characteristics in gastric carcinoma.

Characteristic	Number	Number of cases with TXNIP expression				P-value
		0	1	2	3	
Sex						
Male	95	22	39	27	7	N.S
Female	55	17	22	12	4	
Type						
Intestinal	89	14	36	28	11	0.000
Diffuse	42	19	16	7	0	
Mixed	19	6	9	4	0	
Diffuse component						
Absence	89	14	36	28	11	0.000
Presence	61	25	25	11	0	
Grade						
Well differentiated	7	1	1	5	0	N.S
Moderate differentiated	60	11	28	15	6	
Poorly differentiated	83	27	32	19	5	
Stage						
I	20	0	6	13	1	0.000
II	19	1	10	2	6	
III	55	20	17	14	4	
IV	56	18	28	10	0	
Stage (T)						
1	10	0	2	7	1	0.001
2	38	6	17	10	5	
3	93	31	27	20	5	
4	9	2	5	2	0	
Stage (N)						
0	22	0	8	11	3	0.000
1	40	11	13	11	5	
2	50	15	22	10	3	
3	38	13	18	7	0	
Stage (M)						
0	127	31	49	36	11	0.038
1	23	8	12	3	0	
Helicobacter pylori						
Negative	56	22	20	11	3	0.009
Positive	94	17	41	28	8	

4.3.2.3 Survival analysis of clinicopathologic characteristics and *TXNIP* expression in gastric carcinoma patients.

By univariate analysis, female gender (P=0 .006), diffuse histology type (P =0 .000), presence of diffuse component (P =0 .006), poorer histology grade (P = 0.025) and more advanced stage (P = 0.000) were correlated with poor disease specific survival (Table 4.4 and Figure 4.12-14). Loss or low *TXNIP* expression was also significantly correlated with poor disease specific survival (Table 4.4 and Figure 4.14B). The median disease survival time for *TXNIP*-negative (grade 0, n=39) subgroup was 16.1 months (95% CI, 10-22.2 months), and for low TXNIP expression (grade 1, n=61) subgroup was 20.4 months (95% CI, 9.7-31.1 months). In contrast, patients with immediate and strong expression of TXNIP (grade 2 and grade 3, n=50) are still alive during the follow-up period. By Cox multivariate analysis, only gender and stage remained independently associated with disease specific survival ($P < 0.005$).

A



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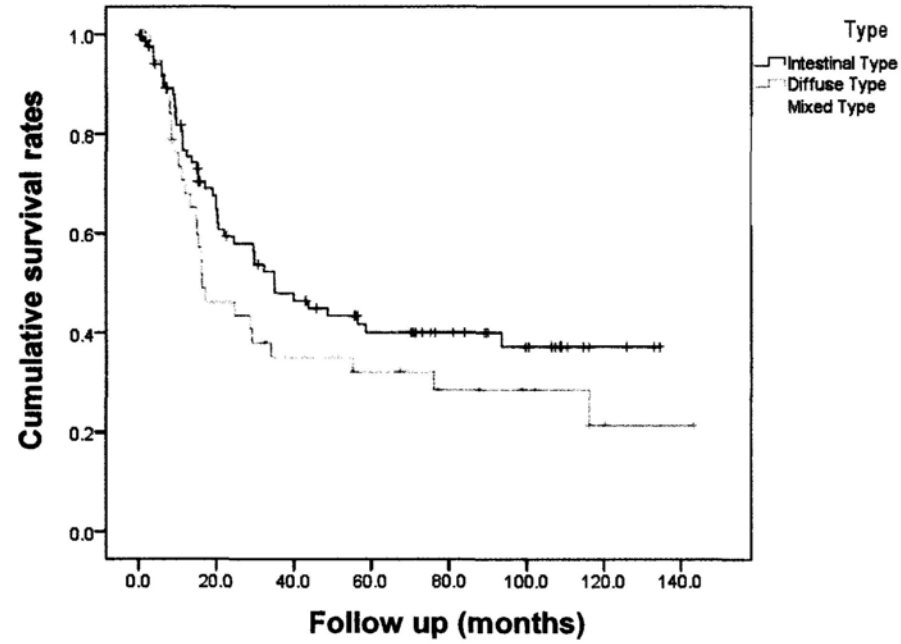
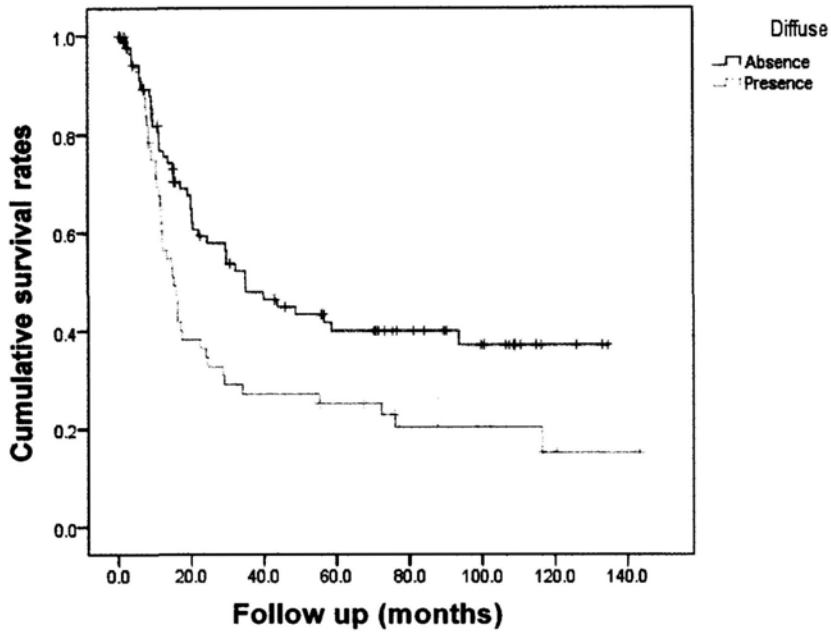


Figure 4.12 Disease specific survival curves of patients with gastric cancer based on (A) gender, (B) histology type.

A



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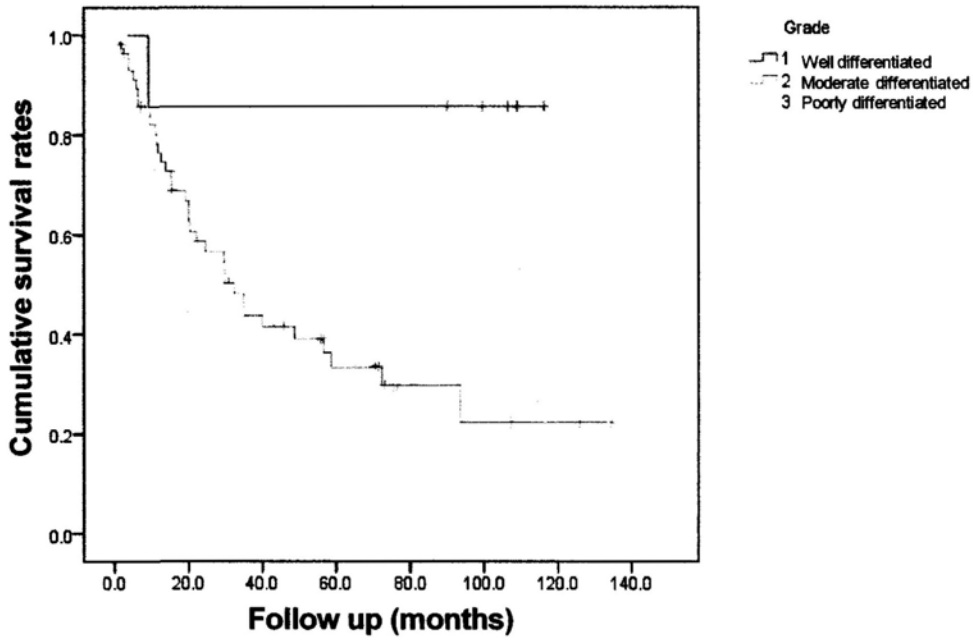
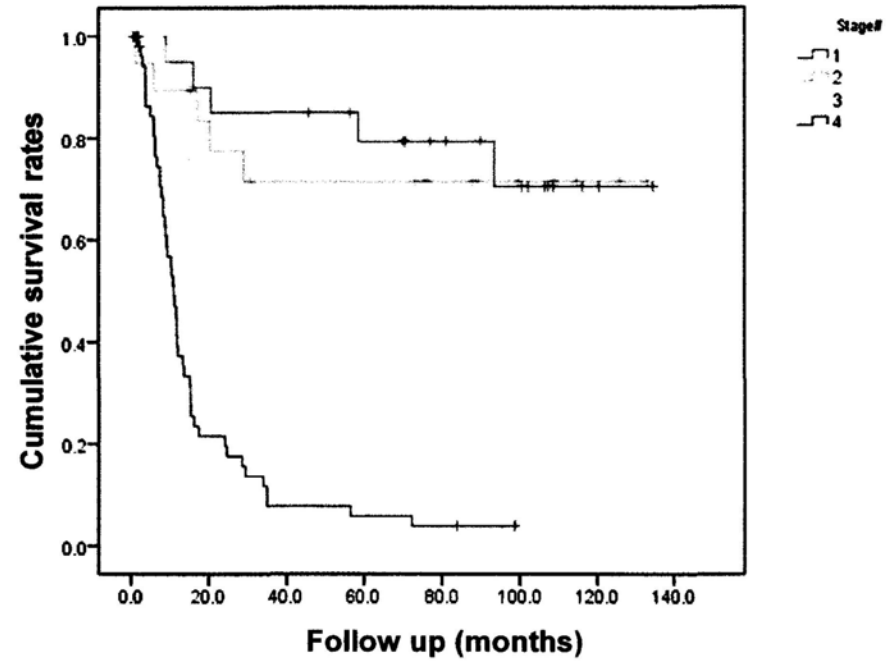


Figure 4.13 Disease specific survival curves of patients with gastric cancer based on (A) presence of diffuse components, (B) histology grade.

A



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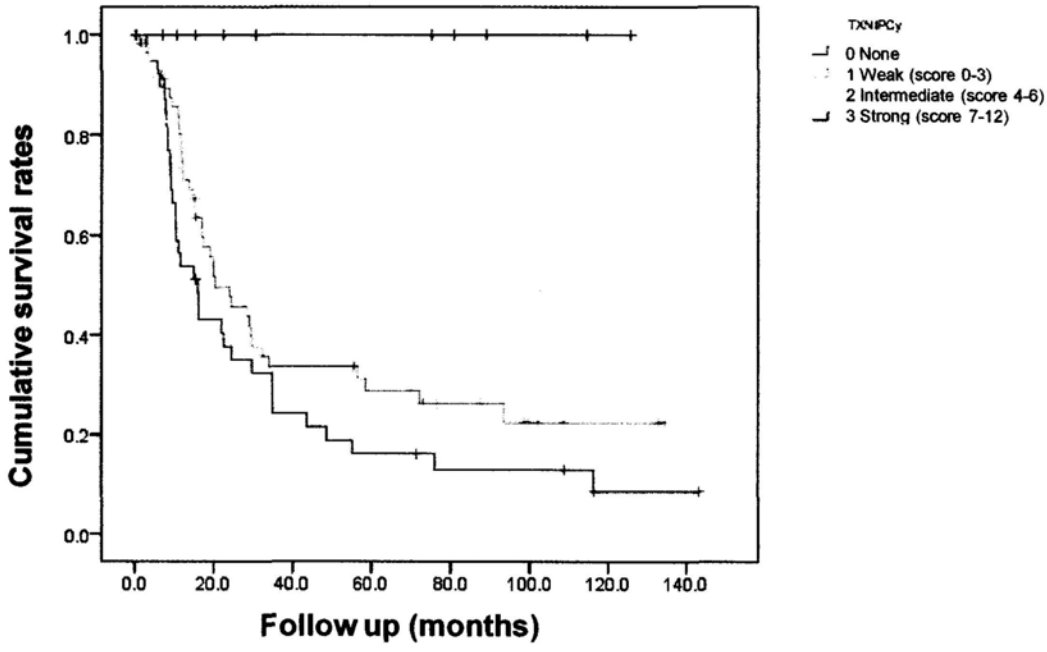


Figure 4.14 Disease specific survival curves of patients with gastric cancer based on (A) stage, (B) *TXNIP* expression.

Table 4.4 Univariate analysis of the correlation of clinicopathological characteristics and disease specific survival in gastric carcinoma.

Characteristic	Number	Median survival time (95% CI) months	Univariate analysis (Kaplan Meier) P-value
Sex			0.006
Male	95	35.1 (11.4-58.8)	
Female	55	16.1 (11.5-20.7)	
Type			0.000
Intestinal	89	35.1 (21.2-49.0)	
Diffuse	42	16.2 (5.2-27.2)	
Mixed	19	11.9 (11.5-12.3)	
Diffuse component			0.006
Absence	89	35.1 (21.2-49.0)	
Presence	61	15.4 (11.3-19.4)	
Grade			0.025
Well differentiated	7	-	
Moderate differentiated	60	32.3 (20.9-43.7)	
Poorly differentiated	83	16.2 (14.0-18.5)	
Stage			0.000
I	20	-	
II	19	-	
III	55	29.8 (17.4-42.2)	
IV	56	11.1 (9.0-13.3)	
TXNIP expression			0.001
0	39	16.1 (10.0-22.2)	
1	61	20.4 (9.7-31.1)	
2	39	-	
3	11	-	

4.4 Functional characterization of *TXNIP* in gastric cancer cells

We demonstrated that *TXNIP* is frequently down-regulated in gastric cancer cell lines and histone acetylation represents an important epigenetic mechanism in regulating the expression of *TXNIP*. *TXNIP* not only down-regulated in gastric cancer cell lines, but also frequently down-regulated in primary gastric cancer samples. Thus, we would like to explore the possible tumor suppressor functions of *TXNIP*. We explored the possible functional roles of *TXNIP* through a series of *in-vitro* experiments including the effects of *TXNIP* in cell proliferation, colony formation, cell cycle, apoptosis, reactive oxygen species production, transmembrane potential, cell migration and cell invasion.

4.4.1 Cell variability and cell growth

The endogenous *TXNIP* expression levels in AGS is the lowest among all nine gastric cancer cell lines and was selected as the cell line model to investigate the functional phenotype of *TXNIP* by transfection study. MTT proliferation assay was performed to determine whether the inducible expression of *TXNIP* causes growth inhibition in gastric cancer cells. In addition to AGS cells, MKN7 cells were also included in the experiments. Cell lines were transfected with the *TXNIP* recombinant vector and over-expression of *TXNIP* was confirmed with RT-PCR or western blot analysis (Figure 4.15). Over-expression of *TXNIP* resulted in a significant decrease ($p<0.05$) in cellular proliferation between 48 hrs and 96 hrs as compared with the empty vector transfected cells (Figure 4.16). In AGS cells, the over-expression of *TXNIP* greatly inhibited cell proliferation approximately 2-fold

in different time points, from 24 hrs to 96 hrs, as compared with that of the cells transfected with empty vector control. Similar results were obtained in the over-expression of *TXNIP* in MKN7 which inhibit around 1.3 fold of cell growth as compared to that of empty vector control from 24 hrs to 96 hrs. This study suggested that *TXNIP* plays a role in cell growth regulation.

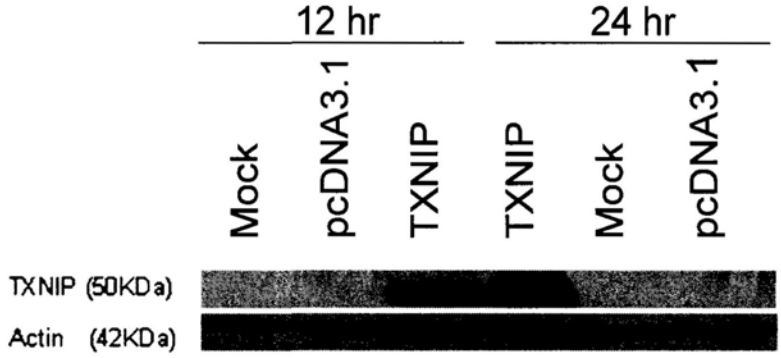
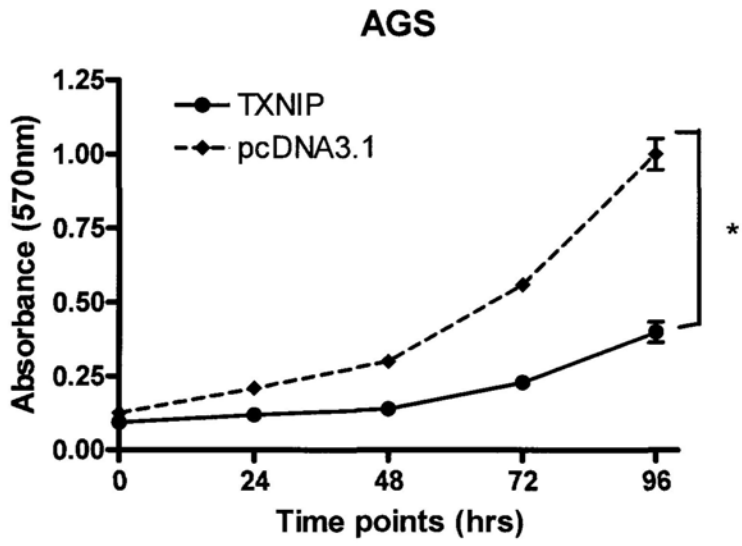


Figure 4.15 Western blot analysis of gastric cancer AGS cells transfected with empty vector (pcDNA3.1) or *TXNIP* for 12 and 24 hrs.

A



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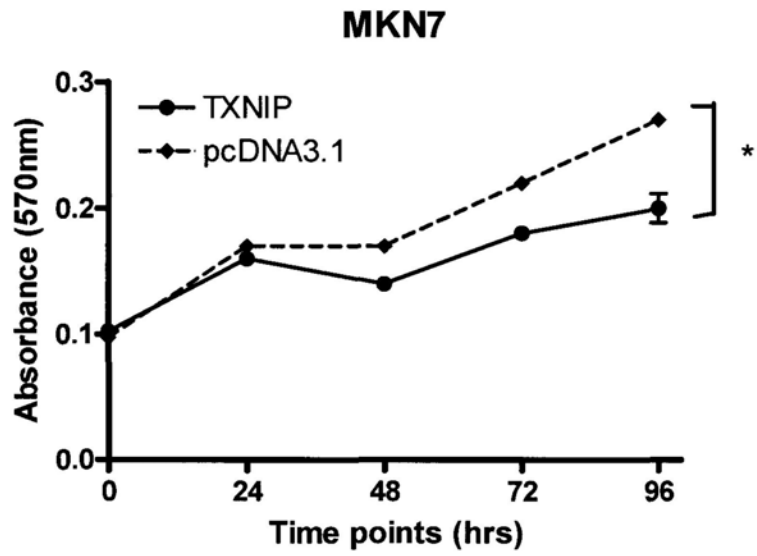
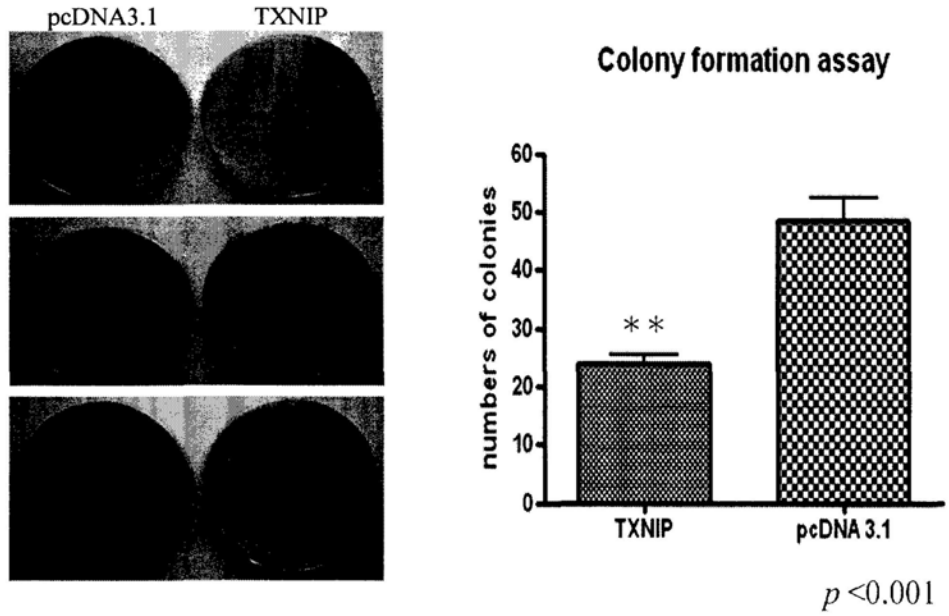


Figure 4.16 Over-expression of *TXNIP* inhibited the cell growth in AGS and MKN7 cell lines. All experiments were performed in 3 independent experiments. (means $\pm$ SD of triplicate experiments) and data in each time point represented the mean of a triplicate samples.

4.4.2 Colony formation

We further characterized the functional changes upon *TXNIP* transfection by colony formation assay. In addition to AGS cells, MKN28 cells were also included in the experiment. Over-expression of *TXNIP* in AGS and MKN28 cells, inhibited the colony formation as compared with the empty vector control under Geneticin (G418) selection conditions (Figure 4.17). Geneticin is used to select genetically engineered cells. Under the selection conditions, the numbers of clones were significantly reduced more than 50% in the presence of over-expressed *TXNIP* in both gastric cancer cell lines. This finding suggested that ectopic expression of *TXNIP* abrogates colony formation of gastric cancer cells.

A



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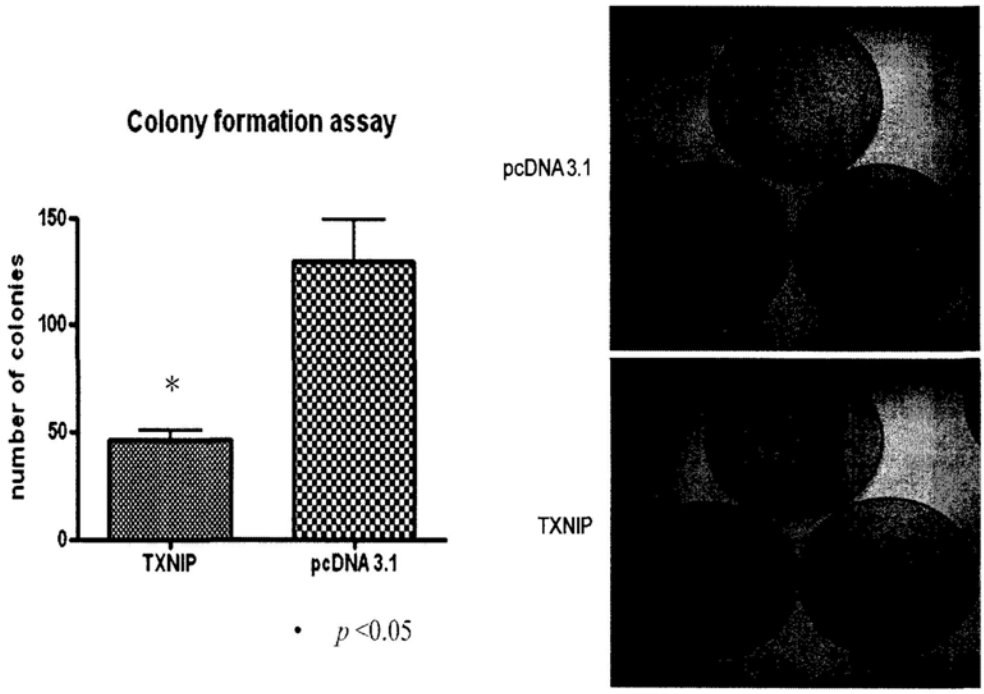


Figure 4.17 Over-expression of *TXNIP* inhibited colony formation ability in (A) MKN28 and (B) AGS. All experiments were performed in 3 independent experiments (means ± SD of triplicate experiments).

4.4.3 Cell cycle regulation

The MTT assay and colony formation assay indicated that over-expression of *TXNIP* in gastric cancer cells would lead to cell growth inhibition. We further explored the possible roles of *TXNIP* expression in cell cycle regulation. In this study, we examined the effects of the *TXNIP* over-expression in AGS and MKN7 cells on the cell cycle regulation by using propidium iodide flow cytometry analysis. Three independent experiments were performed and representative result was shown. No significant alteration in cell cycle was observed in both cell lines with over-expression of *TXNIP* at 24 and 48 hours (Figure 4.18).

4.4.4 Annexin V assay for apoptosis

We used annexin V flow cytometric analysis to evaluate the numbers of apoptotic cells in AGS cells (Figure 4.19). The results demonstrated that ectopic expressed *TXNIP* in AGS cells would increase the numbers of apoptotic cells as compared to the empty vector control (pcDNA3.1). As shown in the upper panel of figure 4.19 A, both of the numbers of early and late apoptotic cells were increased in *TXNIP*-over-expressing AGS cells as compared to the vector control. As shown in the lower panel of figure 4.19B, the first peak (M1) represented the numbers of viable cells and the second peak (M2) represented the numbers of apoptotic cells. The percentage of apoptotic cells in *TXNIP* transfected AGS cells was ~43% and for pcDNA3.1 vector control was ~32%. Therefore *TXNIP* increased the percentage apoptotic cells by approximately 11%.

A

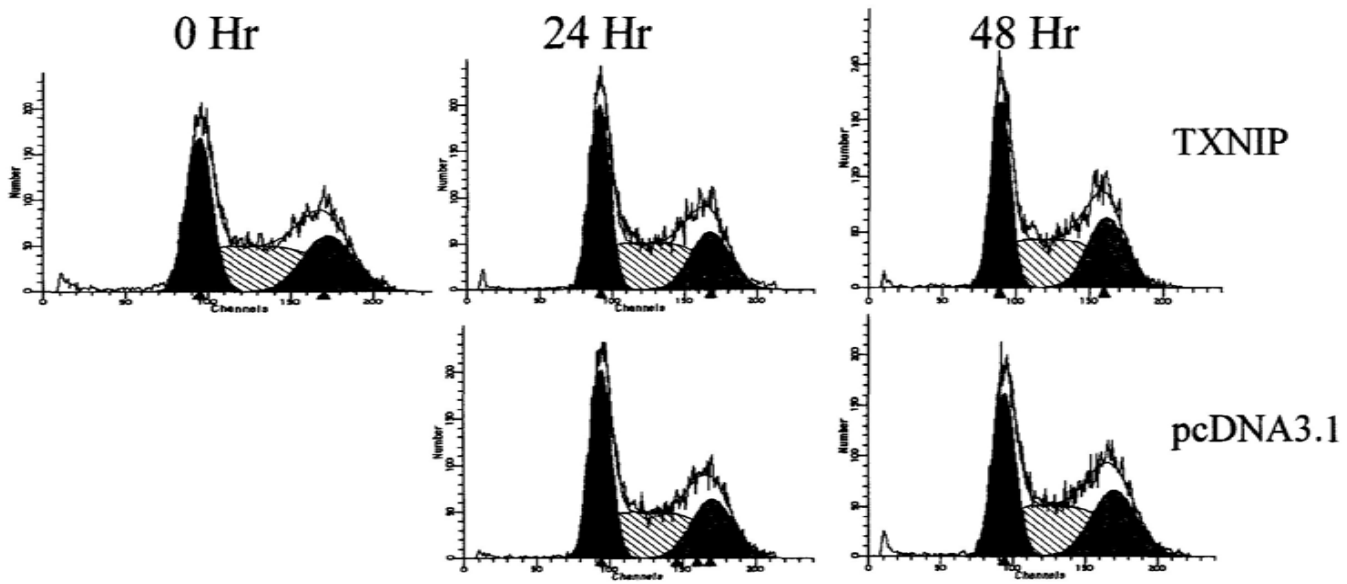


Figure 4.18A Representative cell cycle in *TXNIP*-over-expressed AGS cells. No significant effect was observed in the cell cycle progression in *TXNIP*-over-expressed cells in AGS cells at 24 or 48 hours.

B

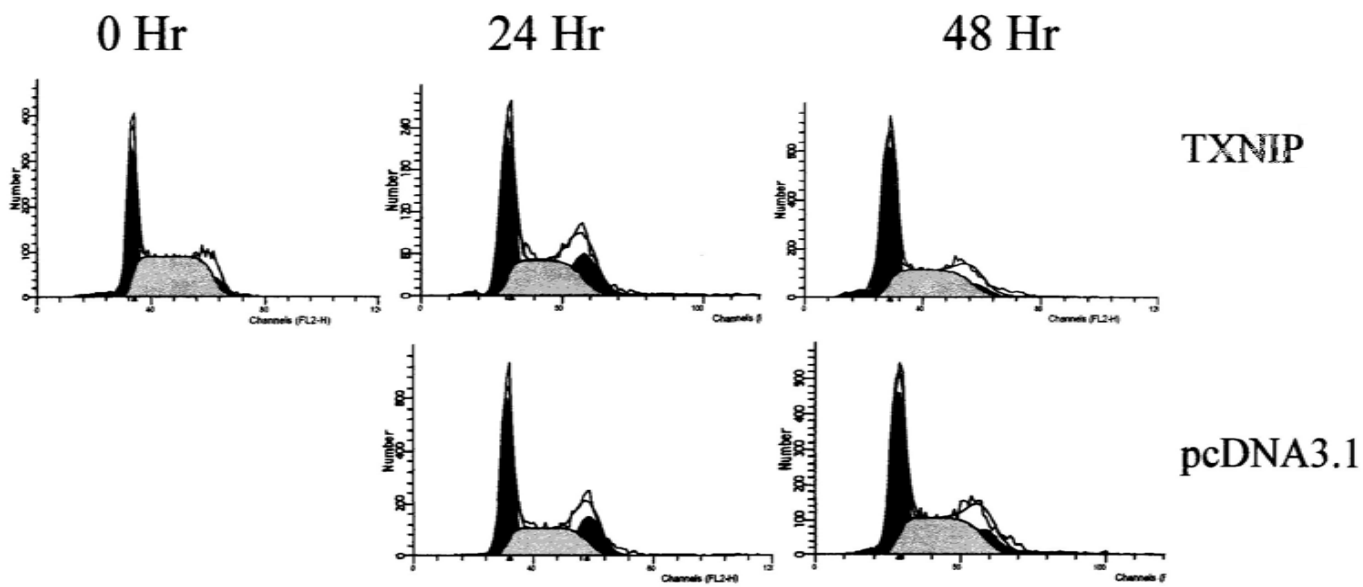
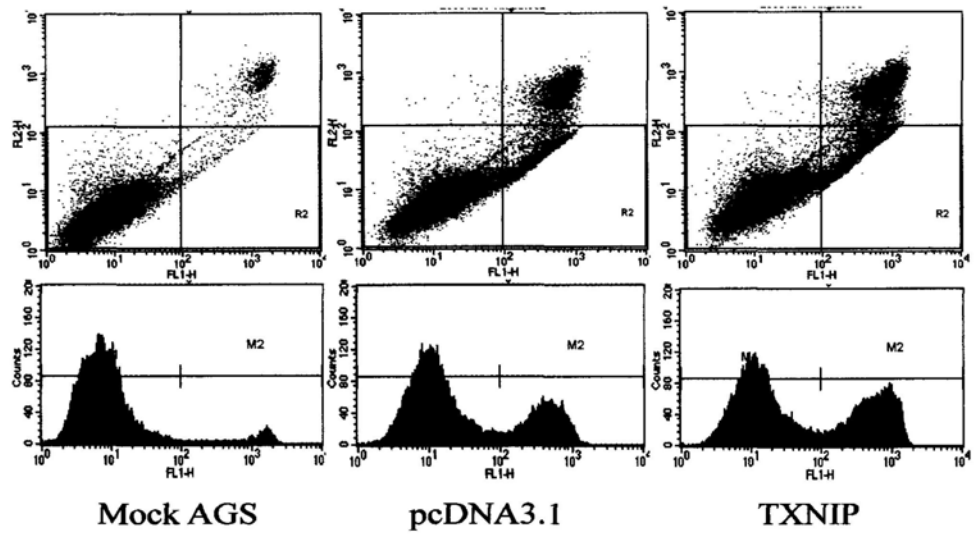


Figure 4.18B Representative cell cycle in *TXNIP*-over-expressed MKN7 cells. No significant effect was observed in the cell cycle progression in *TXNIP*-over-expressed cells in MKN7cells at 24 or 48 hours.

A



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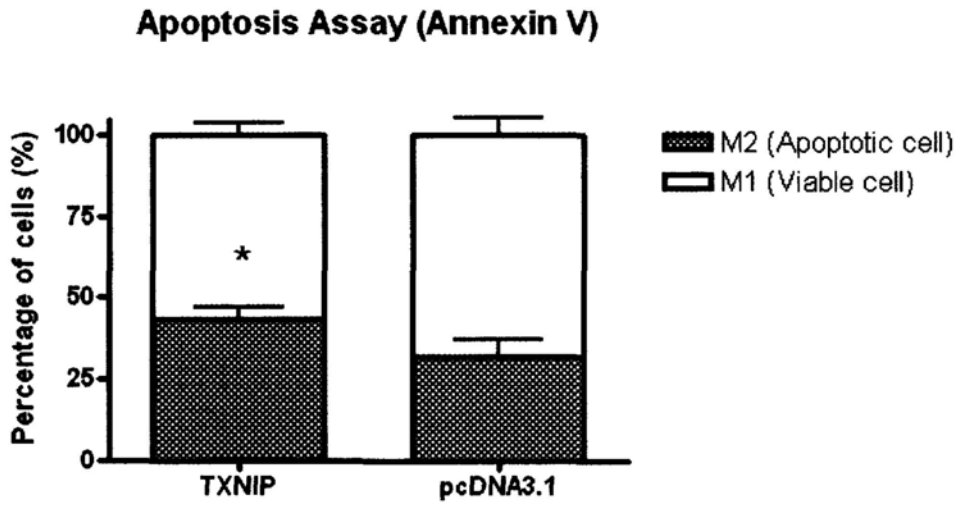

Figure 4.19 *TXNIP* significantly induced apoptosis in AGS gastric cancer cells. (A) Representative histograms and (B) the percentages of apoptotic and viable cells were shown (means ± SD of triplicate experiments;* $P<0.02$).

4.4.5 ROS production

Cellular oxidative stress is controlled by redox regulation and it involves reduction and oxidation. It is well know that *TXNIP* is a negative regulator of thioredoxin (*TRX*) and one of the essential functions of *TRX* is to regulate the reactive oxygen species (ROS) (Yamanaka et al., 2000, Junn et al., 2000, Nishiyama et al., 2001). ROS is often recognized as a cellular toxic stress and ROS levels could alter other cellular processes including cell cycle, apoptosis and proliferation. Previous studies have demonstrated that transfection of *TXNIP* antisence RNA reduced intracellular ROS level in melanoma cells (Song et al., 2003). Another study also revealed that over-expression of *TXNIP* increased the levels of ROS in fibroblast (Yoshioka et al., 2004). In addition, *TXNIP* knock-out mice have reduced levels of ROS (Lee et al., 2005). Thus, we also investigated whether *TXNIP* might regulate ROS level in gastric cancer cells. The production of intracellular ROS in AGS cells were measured by DCF fluorescent oxidation assay. This assay used the redox-sensitive fluorogenic probe CM-H_2DCFDA to treat the cells and then visualized under fluorescent microscope. There was no significant difference in fluorescent signals between *TXNIP*-over-expressing cells and control cells at 24 hours in 3 independent experiments (Figure 4.20). Thus, based on the DCF fluorescent oxidation assay, *TXNIP* has no observable effects on the production of ROS in gastric cancer cells.

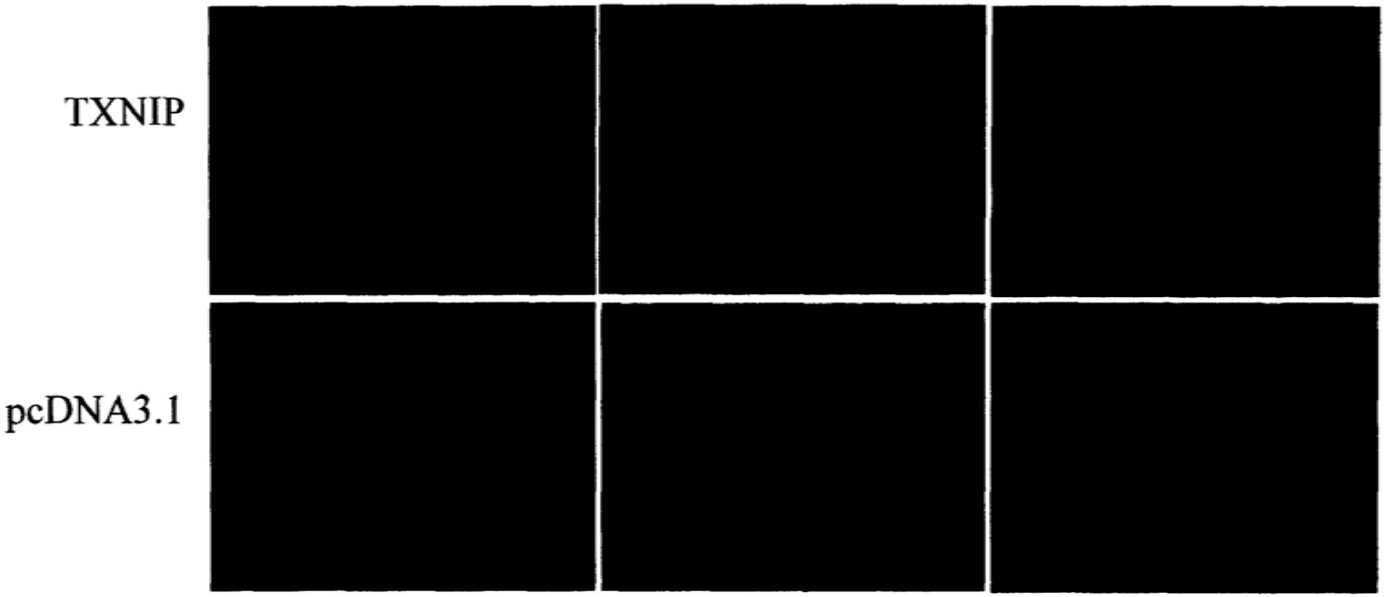

Figure 4.20 Representative fluorescent signal images of reactive oxygen species of AGS cells transfected with *TXNIP* and vector control. No significant difference of the signals was noted between *TXNIP* transfected AGS cells and the vector control cells.

4.4.6 Transmembrane potential

Recent studies have shown that lack of *TXNIP* could protect against mitochondria-mediated apoptosis in pancreatic beta cells (Chen et al., 2009). Thus, it prompts us to evaluate whether *TXNIP* would induce apoptosis through mitochondrial death pathway. Transmembrane potential is the voltage across the mitochondrial membrane of the cell (interior and exterior). The membrane potential is regulate by the interaction of ion channels and ion pumps embedded in the membrane to maintain ion concentrations across the membrane. Thus, if *TXNIP* mediated apoptosis via mitochondrial pathway, the mitochondrial transmembrane potential would decrease. The mitochondrial transmembrane potential value was determined using rhodamine-123 followed by microscopic visualization. No significant difference was observed on the intensity of fluorescent signals in AGS cells transfected with *TXNIP* as compared with vector control (Figure 4.21). The results suggested that over-expression of *TXNIP* has no observable effects on the mitochondria transmembrane potential.

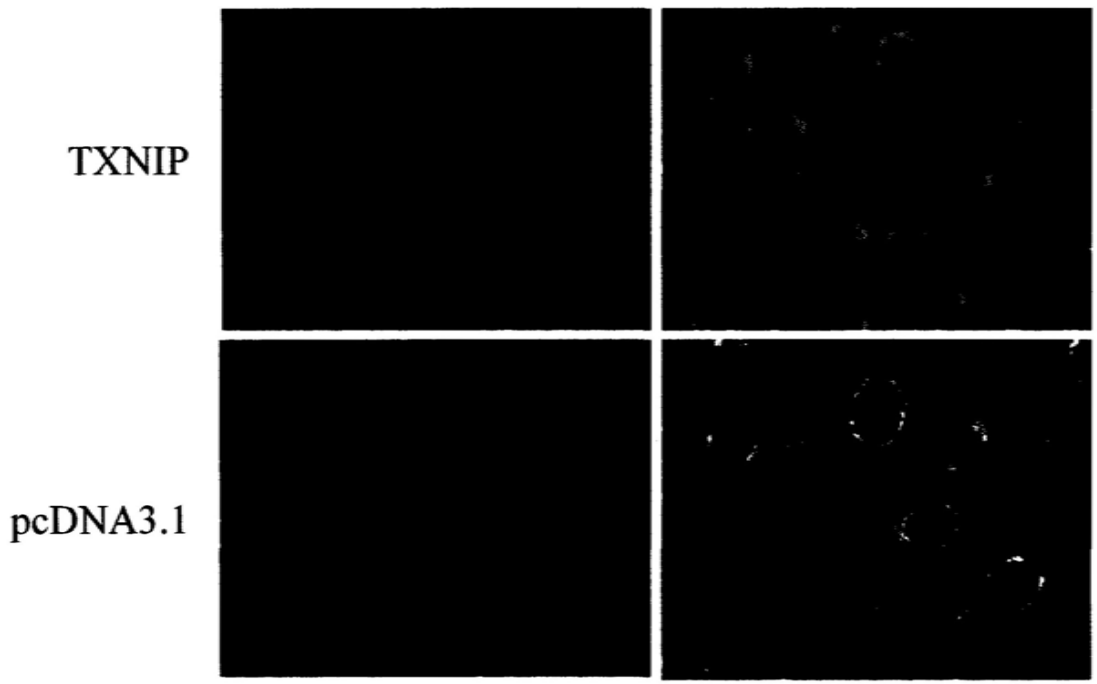

Figure 4.21 The mitochondrial transmembrane potential signal of AGS cells transfected with *TXNIP* and vector control. No significant difference of the signals was noted between *TXNIP* transfected AGS cells and the vector control cells.

4.4.7 *In vitro* cell migration study

Tumor cells have the property to increase migratory ability. Therefore, we also examined the effect of *TXNIP* on cell migration. The *TXNIP* over-expressing AGS cells and vector control cells were assayed for cell migration ability in response to serum using Transwell assay system (Figure 4.22). Cells that travelled through the membrane were visualized by crystal violet stain and counted. Ectopic expression of *TXNIP* in AGS cells markedly inhibited the migration ability of the cancer cells through the membrane. The mean of the total number of *TXNIP* over-expressing AGS cells that migrated through the membrane was 82 and for vector control cell was 862. There was approximately more that 80% reduction in the number of cells migrated through the membrane. The results indicated that *TXNIP* may act as a potential suppressor gene by inhibiting migration ability in gastric cancer cells.

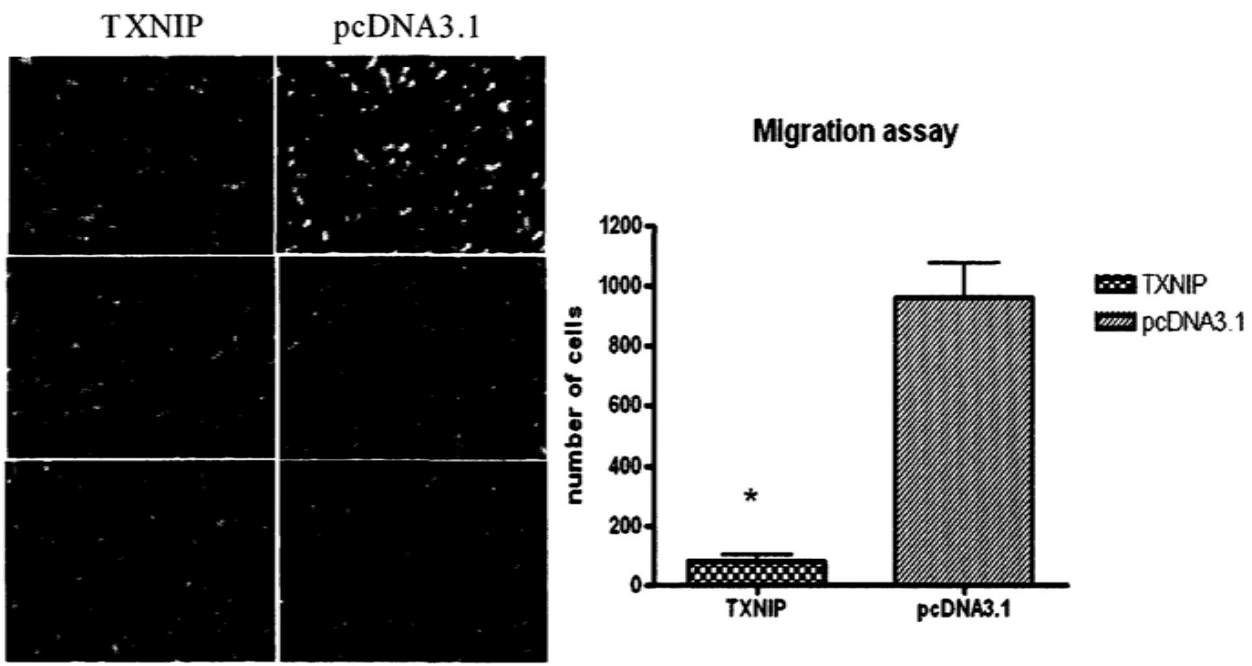

Figure 4.22 Effects of *TXNIP* on the migration of AGS cells in response to growth serum as analyzed by Transwell assay system. Forced expression of *TXNIP* in AGS cells significantly suppressed the migration ability through the membrane as compared with vector control cells. All experiments were performed in 3 independent experiments (means $\pm$ SD of triplicate experiments, *$p<0.05$.).

4.4.8 Cell invasiveness assay

We further investigated the effects of cell invasion in gastric cancer cells over-expressing *TXNIP*. We used the BD BioCoat™ Matrigel Invasion Chambers to study the cell invasion of gastric cancer cells over-expression of *TXNIP* (Figure 4.23). The chambers were coated with an extracellular matrix which served as a barrier to cancer cells. Over-expression of *TXNIP* significantly suppressed the invasive ability of AGS cells. The mean of the total number of *TXNIP* over-expressing AGS cells that invaded through the matrix membrane was 317 and for vector control cell was 826. Transfection of cells with *TXNIP* remarkably reduced the numbers of invasive cells to more than 60%. Thus, *TXNIP*-expressing cells significantly decrease the invasiveness in gastric cancer cells.

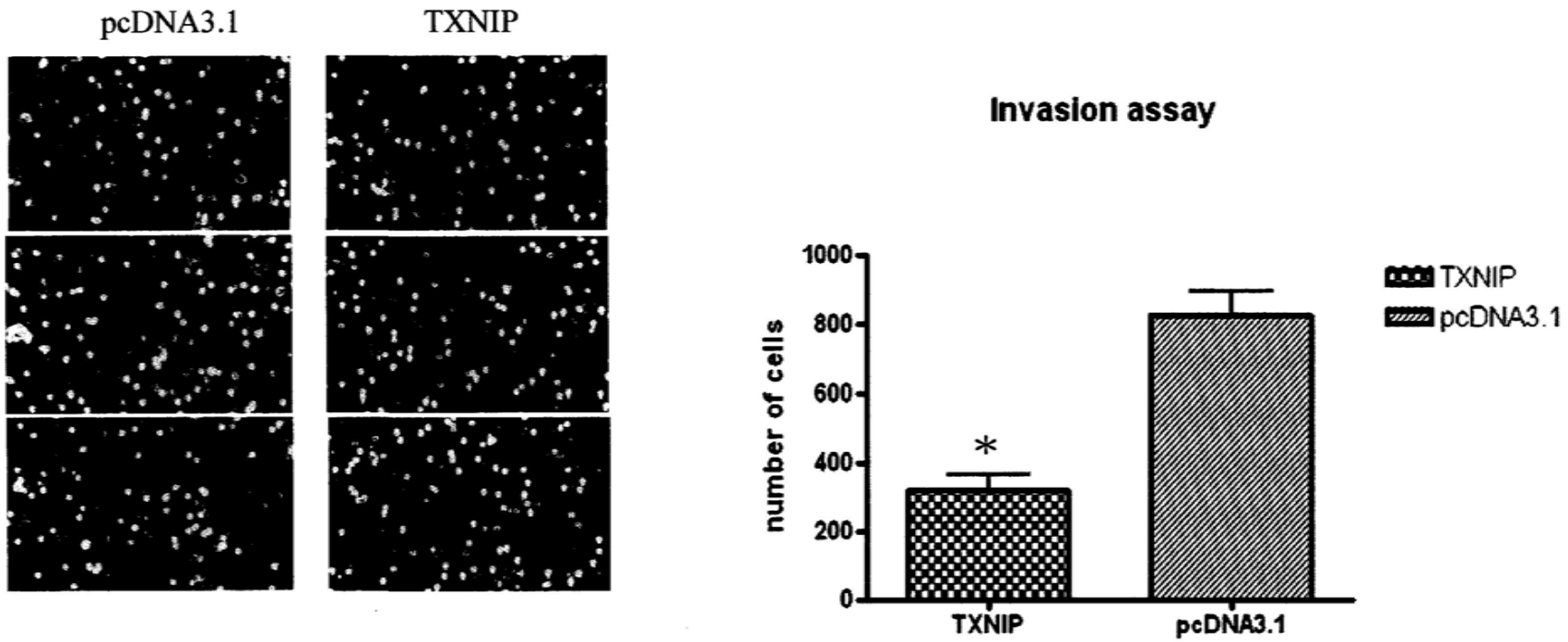

Figure 4.23 Effects of *TXNIP* on the invasiveness of AGS cells in response to growth serum as analyzed by BD Biocoat™ matrigel invasion chamber assay. Forced expression of *TXNIP* in AGS cells significantly suppressed the number of cells invaded through the matrix gel membrane as compared to vector control cells. All experiments were performed in 3 independent experiments (means ± SD of triplicate experiments, *$p<0.05$.

Chapter 5 Discussion

In the present study, we aimed to identify and characterize a potential target gene, which is silenced in gastric cancer and regulated by the epigenetic mechanism, histone acetylation. Thus, the potential target gene should be 1) commonly downregulated/silenced in gastric cancer cells 2) the expression of the gene could be induced by SAHA treatment, 3) the gene expression is regulated by histone acetylation in gastric cancer cell.

SAHA exhibit anti-proliferative effect in a panel of gastric cancer cell lines

We first showed that suberoylanilide hydroxamic acid (SAHA), a well known histone deacetylase inhibitor, has anti-proliferative effect towards a panel of gastric cancer cell lines. SAHA is a well known HDAC inhibitor and is a potential therapeutic drug to combat cancer. It has been already approved by the United States Food and Drug Administration (FDA) Department as a new drug application for the treatment of cutaneous T-Cell lymphoma (CTCL). Currently, there are more than 50 clinical trials on SAHA and it has been administrated intravenously or orally in cancer patient. It is at phase II clinical trials for the treatment of hematological malignancy including Hodgkin's disease, non-Hodgkin's

lymphomas and in some solid malignancy including thyroid, renal cell, mesothelioma, laryngeal and urothelial carcinomas (Kelly et al., 2005). In pre-clinical studies, SAHA has shown to inhibit proliferation, induce differentiation and apoptosis in animal models of solid tumors and hematological malignancies with little or no toxicity (Marks et al., 2004, Dokmanovic and Marks, 2005). The clinical trial data also indicated that SAHA has significant anticancer activity and with well toleration (Kelly et al., 2003). Although there are studies of SAHA in various cancers, data of the effect of SAHA in gastric cancer is very limited. To our knowledge, only one study has published so far which demonstrated that SAHA has the ability to reactivate the tumor suppressor gene, RUNX3 in gastric cancer cells (Huang et al., 2007). Huang et al reported that the treatment of SAHA induced the accumulation of acetylated histones and upregulated the expression of RUNX3. SAHA treatment also inhibited cell growth, induced cell cycle arrest and apoptosis in various gastric cancer cells. In addition, chromatin immunoprecipitation was performed and found that SAHA treatment remarkably increased RUNX3 transcriptional activity at the downstream of transcriptional start site (Huang et al., 2007). In our previous studies, we demonstrated that SAHA could induce apoptosis, inhibit cell proliferation and induced G1 arrest in AGS gastric cancer cells. In the present study, we began the investigation by examining whether SAHA would

induce cytotoxicity in nine gastric cancer cell lines, including MKN1, MKN7, MKN28, MKN45, SNU1, SNU16, AGS, N87 and KatoIII. We found that SAHA could induce remarkable cytotoxic effects in the panel of gastric cancer cell lines at both 48 and 72 hours of treatments. Cell growth in those gastric cancer cell lines was inhibited at a micromolar concentration which suggested that SAHA is a potential effective anticancer drug for gastric cancer.

Identification of putative tumor suppressor gene by expression profile approach

The expressions of suppressed or silenced target genes were found to be re-activated by SAHA treatments. In the current study, we are of interest to identify putative tumor suppressor gene which was regulated through the histone acetylation in gastric cancer. We compared gene expression profiles of SAHA treated vs control AGS cells to identify a set of genes that were differentially up-regulated by SAHA treatment. With the analysis based on SAM method, 224 genes that were significantly upregulated upon treatment of SAHA were identified.

Based on our microarray analysis in nine gastric cancer cell lines, a set of commonly down-regulated genes in gastric cancer cells was identified. We found that totally there were 2770 genes out of 27750 probes (approximately 10%) were

commonly downregulated with a cutoff fold change 1.5-fold (log2 ratio) in all 9 gastric cancer cell lines. By comparing these data sets, 32 genes that were down-regulated in gastric cancer cells and unregulated upon SAHA treatment were identified. Among these selected genes, *TXNIP* represents the one with the highest up-regulation (a fold change of 13-folds) upon SAHA treatment. *TXNIP* was also subsequently confirmed to be induced by SAHA treatment and regulated by histone acetylation in most gastric cancer cells. Thus, with this approach, we were able to identify the potential tumor suppressor gene that was regulated by histone acetylation in human cancer. In previous studies, similar microarray analyses were performed on prostate cancer cells using cells cultured with 7.5 μM SAHA vs control cells at different time points and then subjected to microarray. By comparing the microarray profiles using 2-fold as a cutoff value, they found that less than 2% of genes were induced by SAHA treatment and they also showed that *TXNIP* was upregulated by SAHA treatment (Butler et al., 2002).

TXNIP

Thioredoxin-interacting protein (*TXNIP*), also known as thioredoxin binding protein 2 (*TBP-2*) and vitamin D_3 upregulated protein 1 (*VDUP-1*). It is located on chromosome 1q21.1. *TXNIP* was originally identified in the human HL-60

promyelocyctic cells and was found to be induced after the treatment of 1,25-dihydroxyvitamin D-3 and therefore termed as vitamin D_3 upregulated protein 1 (*VDUP-1*) (Chen and DeLuca, 1994). The names *TXNIP* and *TBP-2* were termed because it was shown to be a negative regulator of thioredoxin (*TRX*) which binded to and inhibited thioredoxin function in cytoplasm (Yamanaka et al., 2000, Junn et al., 2000, Nishiyama et al., 2001). Thioredoxin is a small multifunctional protein consisting of 105 amino acids and plays a key role in cellular redox regulation. Thioredoxin take part in numerous of biological process including scavenging reactive oxygen species (ROS), activating ribonucleotide reductase required for DNA synthesis (Arner and Holmgren, 2000). It is a thiol-oxido reductase that acts through the dithiol/disulfide exchange reaction using the two cysteine residues in the active site. Reduced thioredoxin transfers a hydrogen ion from the protein dithiol (SH-SH) to the disulfide (S-S) of the target oxidized protein and then thioredoxin oxidized itself (Kaimul et al., 2007, Nakamura et al., 2006). In normal condition, thioredoxin interacts with apoptosis signal kinase 1 (ASK1) and inhibits the activity of ASK1 or promotes ASK1 ubiquitination and degradation to block the ASK1-mediated apoptosis (Liu and Min, 2002). *TXNIP* expression could be induced by various of chemicals and stress conditions including vitamin D3, SAHA, 5-fluorouracil, serum deprivation culture, H_2O_2, TGF-β, glucose, UV and heat

shock (Kaimul et al., 2007, Pang et al., 2009, Kim et al., 2004). As *TXNIP* is the endogenous inhibitor of thioredoxin, it thereby also prevents the inhibitory effect mediated by thioredoxin on AKS1 (Junn et al., 2000, Saitoh et al., 1998). Thus, *TXNIP* can act as a proapoptotic member to abolish the inhibition of thioredoxin on ASK1 and activates the ASK1-mediated apoptosis.

Studies have found that cancer cells that were resistant to therapy may have relatively high levels of thioredoxin but low levels of *TXNIP* (Arner and Holmgren, 2000, Butler et al., 2002, Ungerstedt et al., 2005). In previous studies, SAHA has been reported to directly act on the promoter region and induce the expression of vitamin D upregulated protein 1/thioredoxin-binding protein-2 (*TBP-2/VDUP-1/TXNI*P) and down-regulate the expression of thioredoxin (Butler et al., 2002). In the study, SAHA demonstrated the anti-cancer effects in various cancers including colon, bldder, prostate, kidney, breast, myeloma, murine erythroleukemia and arrests cancer cell growth (Butler et al., 2002). The induction of *TXNIP* in transformed cells by SAHA is associated with the decrease in thioredoxin which may in-turn facilitate ROS-related cell death while the normal cells are resistance to the HDAC inhibition. Several anticancer drugs including SAHA can increase ROS accumulation in transformed cells but not in normal cells

(Ungerstedt et al., 2005). In addition, ROS accumulation can lead to the transcriptional induction and activation of Bim, which is a member of BH3-only proapoptotic protein that involved in apoptosis pathway (Sade and Sarin, 2004).

TXNIP is then also linked to cancer development. The expression of *TXNIP* was reported to be downregulated in several types of cancers including breast cancer, colon cancer, prostate cancer, bladder cancer, myeloma, kidney cancer, murine erythroleukemia (Butler et al., 2002) gastrointestinal cancer (Ikarashi et al., 2002), pheochromocytomas (Ohta et al., 2005), B-cell lymphomas (de Vos et al., 2003) and adult T-cell leukemia (Ahsan et al., 2006). In a mouse model study, point mutation or knockout of the *TXNIP* was associated with high incidence of hepatocellular carcinoma (Sheth et al., 2006). *TXNIP* was shown to suppress the metastasis of melanoma in mice (Goldberg et al., 2003). Thus, *TXNIP* was also suggested to be a metastasis suppressor gene (Shin et al., 2008). However, the mechanisms or functional roles of *TXNIP* is still largely unclear. Data regarding the potential role of *TXNIP* in gastric cancer development is limited. The implication of *TXNIP* in gastric cancer development will be discussed in later part of the discussion.

Epigenetic regulation of TXNIP in gastric cancer

Gene promoter methylation and histone acetylation play a major role in epigenetic regulation of gene expression. Epigenetic alternations are implicated in various human cancers including gastric cancer. The down-regulation of gene expression for tumor suppressors such as *CDKN2A, p53, E-cad and VHL*, have been reported to be associated with DNA methylation and histone deacetylation (Ushijima and Okochi-Takada, 2005). However, little is known about the regulation of *TXNIP* in gastric cancer cells. Other studies had shown that *TXNIP* expression is inactivated in certain cancers via promoter hypermethylation (Ahsan et al., 2006, Dutta et al., 2005). A few gastric cancer cell lines showed upregulation of *TXNIP* mRNA level with treatment of demethylating agent, however AGS cells showed increased protein expression. For SNU16 gastric cancer cells, despite there was densely methylation at the *TXNIP* promoter region, *TXNIP* mRNA level was not up-regulated upon 5aza-dR treatment. *TXNIP* promoter regions in MKN28 gastric cancer cells were densely methylated and the mRNA level was also upregulated upon 5aza-dR treatment. However, we were unable to restore the protein expression of MKN28 after 5aza-dR treatment. The results suggested that gene promoter methylation may not be an important mechanism lead to silencing of *TXNIP* in gastric cancer cells.

As SAHA is a well known histone deacetylase inhibitor which could increase the protein levels of acetyl-H3 and acetyl-H4, it prompted us to investigate the histone acetylation status of *TXNIP* in gastric cancer cells. In this experiment, acetyl-H3 and acetyl-H4 were used to test whether SAHA can induce the promoter transcriptional activity of *TXNIP* in gastric cancer cell line MKN45. The enrichment of *TXNIP* transcriptional activity near the *TXNIP* promoter region after treatment would supported that *TXNIP* is regulated by histone acetylation. Previous studies shown that by using chromatin immunoprecipitation assay, 5aza-dR or sequential (SAHA and 5aza-dR) treatment would increase the PCR signal of *TXNIP* obtained using anti-acetylated-H3 or H4 antibody in leukemia cells (Ahsan et al., 2006). A recent study has also demonstrated that HDAC inhibitor TSA and glucose induced both histone H3 and H4 acetylation of *TXNIP* gene (Cha-Molstad et al., 2009). In addition, the study also revealed that glucose induced the expression of *TXNIP* via chREBP, p300, and histone H4 acetylation in pancreatic beta cells (Cha-Molstad et al., 2009). In our data, SAHA treatment could significantly increase both acetylated histone H3 and H4 to the *TXNIP* promoter region and resulted in increased *TXNIP* transcription. *TXNIP* transcription activity was enriched at -2.5kb to -3kb upstream of the transcriptional start site when using

acetylated histone H3 and at -2.5kb to-3 kb upstream and +2kb downstream regions of the transcriptional start site when using acetylated histone H4. Thus, we have demonstrated that the *TXNIP* promoter transcriptional activity was correlated with the acetylation of histone H3 and H4. To our knowledge, this is the first study to demonstrate *TXNIP* transcriptional activity could be induced specifically by SAHA through the acetylation of histone H3 and H4 in gastric cancer. We are also the first group to show the exact regions where the acetylated histone H3 and H4 recruited at and may promote the *TXNIP* transcription.

TXNIP as a tumor suppressor in gastric cancer

Studies on the functional roles of *TXNIP* in gastric cancer is limited. To our knowledge, only two research groups (Ikarashi et al., 2002, Shin et al., 2008) have published data regarding *TXNIP* in gastric cancer. Loss of expression in gastric cancer has been reported in 21% (60 out of 286) gastric cancer by an immunohistochemical study and suggested that the loss of expression was associated with cancer stage (Shin et al., 2008). However, the functional role of *TXNIP* in gastric cancer cell is largely unknown. Ikarashi and his team had demonstrated that *TXNIP* gene might act as an important stress response gene in gastric cancer (Ikarashi et al., 2002). They showed that *TXNIP* mRNA expression

level in gastric cancer was significantly lower than that in their adjacent non-cancer tissues in 12 samples by using QRT-PCR (Ikarashi et al., 2002). They found that *TXNIP* expression was correlated to the stage of cancer. The expression of *TXNIP* in stage II patients was significantly higher than that of stage III and IV patients. In these studies, the mechanism of regulation of *TXNIP* expression was not explored. Furthermore, the functional roles in gastric cancer cells have not been extensively investigated.

a. Downregulation of TXNIP in gastric cancer

Our data indicated that expression of *TXNIP* in all nine gastric cancer cell lines (MKN1, MKN7, MKN28, MKN45, AGS, KatoIII, SNU1, SNU16, N87) were down-regulated in gastric cancer. By RT-PCR, majority of the primary gastric cancer samples also showed down-regulation of *TXNIP* as compared to paired non-cancer gastric mucosal tissues. Down-regulation of *TXNIP* in protein level by western blot was also shown. In our immunochemistry staining study in tissue microarray containing 150 cancer tissues, 26% of gastric cancer tissues showed complete loss (grade 0) and 41% showed low expression (grade 1) of *TXNIP*. Thus, we demonstrated the frequent down-regulation of *TXNIP* in gastric cancer.

b. Functional analysis of TXNIP in gastric cancer cells

Previous studies suggested that *TXNIP* expression would result in growth suppression in several cancer cell types including gastric cancer cells, leukemia cells and breast cancer cells (Han et al., 2003, Nishinaka et al., 2004b, Nishinaka et al., 2004a). A Korean group has reported that *TXNIP* inhibited cell growth by blocking cell cycle progression in human kidney 293 cells (Han et al., 2003). Further study from this group showed that *TXNIP* suppressed cell invasiveness and tumor metastasis in melanoma cells (Shin et al., 2008). However, the functional role of *TXNIP* in gastric cancer is still remaining unknown. In order to investigate the functional role of *TXNIP* in gastric cancer, a series of functional studies were performed.

In our data, both AGS and MKN7 cell lines were subsequently transfected with the *TXNIP* and overexpression of *TXNIP* in both cell lines resulted in a significant reduction in cellular proliferation between 48hrs and 96 hrs by MTT assay. Similar results obtained from the study conducted by Song *et al.*, revealed that transfecting *TXNIP* antisense cDNA would reduce 50% of cell growth in melanoma cells (Song et al., 2003). In addition, our study also demonstrated *TXNIP* over-expressed gastric cancer cells could resulted in inhibition of colony formation.

The observations suggested that *TXNIP* play a role in cell growth regulation.

Previous studies demonstrated that *TXNIP* is involved in cell cycle regulation by inhibiting cyclin A at transcriptional level thereby leading to cell cycle arrest at the G0/G1 phase in synchronized cells (Han et al., 2003). The cell-cycle progression of TXNIP expressing cells was delayed at G0/G1 phase when compared with the control cells (Han et al., 2003). Other studies also found that TXNIP-mediated cell cycle arrest was associated with the regulation of p16 and $p27^{kip1}$ (Yamaguchi et al., 2008, Nishinaka et al., 2004b). However, in our transfection study, no prominent alteration was demonstrated in the cell cycle regulation in gastric cancer cells. Nevertheless, over-expression of *TXNIP* would induce apoptosis in the gastric cancer cells and this may explain for the aberrant regulation of cell growth. However, previous studies have reported that over-expression of *TXNIP* alone in gastric cancer cells was not sufficient to induce apoptosis (Han et al., 2003). Some investigators suggested that additional stimuli such as H_2O_2 and heat shock may be required to induce the cell apoptosis (Junn et al., 2000). Another study also revealed that over-expression of *TXNIP* was unable to induce apoptosis in MCF-7 breast cancer cells (Nishinaka et al., 2004b). In contrast, over-expression of *TXNIP* could induce apoptosis in T cell lymphoma and

primary rat cardiomycyte (Wang et al., 2006, Wang et al., 2002). Thus, the role of *TXNIP* in apoptosis may depend on the presence of additional stress and may be cell type dependent.

In our study, over-expression of *TXNIP* in gastric cancer cells was able to reduce cell migration as well as inhibited cell invasiveness. A previous study had demonstrated that transfection of cells with *TXNIP* resulted in a significant decrease in invasiveness in Hela cells (Shin et al., 2008). Furthermore, they also found that *TXNIP*-expressed mice suppressed melanoma metastases dramatically *in vivo* (Shin et al., 2008). To our knowledge, we are the first group to demonstrate the effect of *TXNIP* on cell migration and invasiveness in gastric cancer cells. Taken together, the experimental data not only provide important insight of the functional roles of *TXNIP*, but also supported that it is a tumor suppressor gene in gastric cancer.

c. TXNIP and it's correlation with clinicalpathology parameters

In our data, loss or low expression of *TXNIP* was significantly associated with several clinicopathological features, including the presence of diffuse histology, histology grade stage, overall clinical stage, T-stage, LN (lymph node) stage, M

(metastasis) stage, and the presence of *helicobacter pylori* at the time of diagnosis. Loss or low expression of *TXNIP* was also associated with a poorer disease specific survival in univariate analysis. The median survival in gastric cancer patients who had a negative *TXNIP* expression was 15.5 months as in contrast to that in patients with strong *TXNIP* expression had a median survival of 30.7 months. The results are consistent with a previous study (Shin et al., 2008). Their findings indicated that loss or low *TXNIP* expression is associated with more aggressive clinical behavior and which is also in keeping with our gastric cancer cell lines experiments. Our studies also suggested that *TXNIP* expression may be a potential biomarker in gastric cancer.

In summary, we have identified a tumor suppressor gene, *TXNIP*, which was regulated by histone acetylation in gastric cancer. Histone acetylation is likely the dominant epigenetic mechanism that regulated *TXNIP* expression. Functional studies supported its tumor suppressor functions in gastric cancer cells. *TXNIP* is frequently down-regulated in primary gastric cancer. The loss and low expression of *TXNIP* is associated with aggressive clinical behavior in gastric cancer and may be a potential biomarker. Our study enhanced the insights of the epigenetic

regulation and functional roles of *TXNIP* in human cancer and provided supporting data for future clinical trail of HDAC inhibitors in gastric cancer. .

Chapter 6 Further studies

Our study has identified the *TXNIP* is regulated under epigenetic control, histone acetylation in gastric cancer. Experimental data supported that *TXNIP* is functioned as tumor suppressor in gastric cancer cell lines. The loss or low expression is associated with more aggressive behavior of gastric adenocarcinoma and predict a poorer disease specific survival. In the further studies, we will like to investigate the possible underlying biological pathways that associated with the downregulation of *TXNIP* in gastric cancer cells. Our study also identified a list of potential candidate genes that down-regulated in gastric cancer cells but induced upon SAHA treatment. We will characterized each of this gene and see whether they are also regulated by histone acetylation and function as tumor suppressor gene in gastric cancer.

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Appendix I

dbj|AK226090.1| Homo sapiens mRNA for thioredoxin interacting protein variant, clone: bm05546

Length=1988

GENE ID: 10628 TXNIP | thioredoxin interacting protein [Homo sapiens]

Score = 2606 bits (1411), Expect = 0.0

Identities = 1411/1411 (100%), Gaps = 0/1411 (0%)

Strand=Plus/Plus

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Query  1    CCTAGTAGTTAATATTCATTTGTTTAAATCTTATTTTATTTTTAAGCTCAAACTGCTTAA  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  104  CCTAGTAGTTAATATTCATTTGTTTAAATCTTATTTTATTTTTAAGCTCAAACTGCTTAA  163

Query  61   GAATACCTTAATTCCTTAAAGTGAAATAATTTTTTGCAAAGGGGTTTCCTCGATTTGGAG  120
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Query  121  Ctttttttttcttccaccgtcatttctaactcttaaaaccaactcagttccatcatggtg  180
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Sbjct  944   GCCAATGGCCAGACCAAGGTGCTGACTCAGAAGTTGTCATCAGTCAGAGGCAATCATATT  1003


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