GENOMIC ANALYSIS OF CHEMO-RESISTANCE TO HDAC INHIBITORS IN GASTRIC CANCER CELLS

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2013

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(M.Sc. NUS)

A THESIS SUBMITTED FOR

THE DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF PHYSIOLOGY

YONG LOO LIN SCHOOL OF MEDICINE

NATIONAL UNIVERSITY OF SINGAPORE

2013

ACKNOWLEDGEMENT

I am very grateful for all I have received from many people for the past 4 years of PhD trainings. The training has shaped me up to be a better qualified person in both work and life.

I would like to convey my first thanks and my deepest gratitude to my supervisor, Prof. Patrick Tan for his encouragement, inspiration, patience, funding and also his continuous support. I am also thankful for the excellent example that he had provided as a successful scientist and also speaker. I also want to thank for his efforts and advices on my manuscripts and this thesis. His trust in me allowed me to grow and lead me to who I am today.

The supply of cell lines from other cancer types is important to this project. I want to thank Dr. Shang Li for kindly providing these important cell lines.

I would like to thank my graduate committee: Assoc. Prof. Reshma Taneja, Dr. Shang Li and Dr. Goh Liang Kee for all the constructive criticism and advice.

I also thank to Dr. Kakoli Das, Mrs. Jeanie Wu and Ms. Ming Hui Lee for their important technical support, advice and kind help.

I thank to my family as I got warmest support from them for the past few years while pursuing my personal interest. Although they cannot read English,

I sincerely thank to my parents for always giving me the best support and always being proud of me.

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Abstract

Histone deacetylase inhibitors (HDAC inhibitors) are regarded as very promising anti-cancer drugs for their high selectivity and relatively low effective concentrations in causing tumor growth inhibition. However, like other groups of anti-cancer drugs, HDAC inhibitors also are faced with the problem of chemo-resistance in some specific cancer types, especially solid tumors such as gastric cancer. This project aims to investigate possible mechanisms of HDAC inhibitor resistance in gastric cancer by a genomic screening method.

From 17 gastric cancer cell lines covering diverse origins and souces, we identified AGS, YCC11, Ist1, AZ521 and SCH cells as sensitive cell lines to HDAC inhibitor treatment, and YCC3, YCC7, MKN7 cells as the resistant cell line group. Our sensitivity indexes included cell proliferation assay (MTT assay), apoptotic assay (PARP cleavage by Western blot) and cell anchorage independent growth assay. The experimental drugs included Trichostatin A (TSA, class I, II HDAC inhibitor), SAHA (another hydroxamate HDAC inhibitor, which is similar to TSA but approved for clinical use) and MS275 (benzamide HDAC inhibitor, which can specifically inhibit class I HDACs).

Combining gene expression data from both the Affymerix U133 platform and the Illumina 6 platform, an integrated genomic analysis was performed using Partek software to investigate genes differentially expressed

between the sensitive and resistant gastric cancer cell lines group. Two gene candidates, STAT1 and RNH1, were nominated and subsequently validated at the protein level.

Of the two genes, STAT1 has been previously reported to contribute to HDAC inhibitor resistance in Kras-mutated colon cancer cells providing confidence in the robustness of our genomic analysis. We focused on investigating the effects of RNH1 on HDAC inhibitor-resistance in gastric cancer cells.

In order to investigate the importance of the RNH1 in gastric cancer HDAC inhibitor resistance, stable knock-down of RNH1 in YCC3 and YCC7 cell lines were established. Using cell proliferation, apoptosis and colony formation assays, we found that RNH1 knock-down in YCC3 and YCC7 cells reversed their HDAC inhibitors-resistance. These results were observed using two independent RNH1 shRNA sequences, demonstrating that this is not due to off-target effects.

The effect of RNH1 over-expression in sensitive cell lines was also tested. RNH1 overexpression in YCC11 and AZ521 cells caused higher resistance to HDAC inhibitors.

We hypothesized that the effects of RNH1 might be mediated through the production of reactive oxygen species (ROS) induced by HDAC inhibitors. Indeed, sensitive gastric cancer cell lines showed higher ROS production by

TSA treatment. Experimental deregulation of RNH1 in selected cell lines could also alter ROS production by TSA treatment Moreover, treating the cell lines with redox modulation molecules, such as GSH, could rescue sensitive cell lines from TSA induced growth inhibition, while PEITC treatment could enhance the growth inhibition of previously-resistant cell lines by TSA.

Finally, the effect of RNH1 on HDAC inhibitor sensitivity in normal gastric epithelial cell lines (GES1 and HFE145) and other types of cancer cell lines (Hela, MCF7, HepG2 and HCT116) were also tested. Similar to gastric cancer, cell lines with higher RNH1 expression level (GES1, HFE145 and HepG2) showed higher resistance to TSA treatment.

Taken collectively, our results demonstrate that RNH1 can contribute to HDAC inhibitor resistance in gastric cancer cells through regulating ROS production. These results improve our understanding the HDAC-related biology, and could prove useful in guiding the design of future clinical trials evaluating HDAC inhibitors.

List of Publications Related to This Study

Zhu Y, Das K, Wu J, Lee MH, Tan P. *RNH1* Regulation of Reactive Oxygen Species Contributes to Histone Deacetylase Inhibitor Resistance in Gastric Cancer Cells. *Oncogene*, 2013 Apr 15. doi: 10.1038/onc.2013.104. [Epub ahead of print]

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Abbreviations

Ac	Acetylation of histone tail
AML	Acute myeloid leukemia
APS	Ammonium persultate
CBP/p300	CREB-binding protein/ E1A binding protein p300
CDH1	Cadherin-1
CRA	13-cis-retinoic acid
CTCL	Cutaneous T-cell lymphoma
DMEM	Dulbecco's modified eagle medium
DNMT	DNA methyltransferase
EGFR	Epidermal growth factor receptor
FBS	Fetal Bovine Serum
GCL	Cysteine ligase
GFP	Green fluorescence protein
GI50	Growth inhibition by 50%
GSR	Glutathione reductase
GST	Glutathione S-transferase
H. Pylori	Helicobacter Pylori
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
HRP	Horseradish peroxidase
IARC	International Agency for Research on Cancer
LC20	Lethal concentration by 20%
MALT	Mucosa-associated lymphoid tissue

mCRC	Metastatic colorectal cancer
MeCP	Methyl-CpG binding proteins
Met	Methylation of CpG island
MEM	Minimum essential medium of eagle
METH	5-methylenetetrahydrofolate-homocysteine S-methyltransferase
MLH1	MutL homolog 1
MORF	Morpholino oligomer
MS275	entinostat
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
Nrf2	E2-related factor 2
NSCLC	Non–small cell lung cancer
PARP	Poly ADP-ribose polymerase
PBS	Phosphate buffered saline
PCAF	P300/CBP-associated factor
PEITC	Beta-phenylethyl isothiocyanate
PI	Propidium iodide
RNH1	Ribonuclease inhibitor
ROS	Reactive oxygen species
RUNX3	Runt-related transcription factor 3
SAHA	Vorinostat
SDS-PAGE	SDS-Polyacrylamide gel electrophoresis
SOD	superoxide dismutase
STAT1	signal transducer and activator of transcription 1
TBP2	Thioredoxin-binding protein 2
Trx	Thioredoxin

TSA	Trichostatin A
TSG	Tumor suppressor gene
TF	Transcription factor;

Chapter One. Introduction

1.1 Gastric Cancer

Gastric cancer refers to cancer originating from any part of the stomach and mainly includes four histological types: adenocarcinoma, lymphoma , carcinoid tumor and gastrointestinal stromal tumor. Adenocarcinomas originating in mucosa (inner lining of the stomach) possess 95% of the gastric cancer cases. (1) 4% of gastric cancer is attributed by slow-glowing mucosa-associated lymphoid tissue (MALT) lymphoma, and 3% of gastric cancer is carcinoid tumor arising from hormone-making cells of stomach in neuroendocrine system. Gastrointestinal stromal tumor originated in interstitial cell of Cajal in the stomach wall possesses the rarest portion. Gastric cancer is defined into proximal and distal according to the site of cancer origin. Cancer develops near the gastro-esophageal junction is defined as proximal while cancer develops in the lower part of stomach is defined as distal gastric cancer. (1)

1.1.1 Epidemiology of Gastric Cancer

There is up to 10-fold difference in gastric cancer incidence rate throughout the world, and most gastric cancers (two-third) occur in developing countries (2, 3).(Figure 1.1) the highest gastric cancer rates are reported in Japan and Korea (4, 5). Other countries with high-incidence for gastric cancer include East Asia, Eastern Europe, and Central and South America, while relative low rates are found in South Asia, North and East Africa, North America, Australia, and New Zealand. (6) Gastric cancer is a late-onset disease with a peak incidence at the age of 50-70 years, and the incidence rated in males are as twice as the one in females (7, 8). So the estrogen could be considered as a important protection factor. Blacks and lower socio-economic groups in developing countries also possess significantly higher gastric cancer incidence rates (8).

Figure 1.1. Global variation in cancer incidence for gastric cancer.

The incidence of gastric cancer for men of all ages is highest in developing countries (orange and red) such as Asia and South America and lowest in developed countries (green) such as in North America. Graphic adopted from reference (3)

1.1.2 Classification of Gastric Cancer

There are mainly two gastric cancer classification including the Ming classification which is based on growth pattern, and Lauren's classification which is based on various predominant histological pattern (9). Lauren's classification is the most widely-used and accepted approach to classify gastric cancer as it has proven useful in evaluating the natural history of gastric cancer (9). In Lauren's classification, gastric cancer is classified into two subtypes: the intestinal-type, a well differentiated tumor characterized by cohesive neoplastic cells forming gland-like tubular structures and the diffuse-type, a poorly differentiated tumor resulting in individual cells infiltrating and thickening the stomach wall. (10) These two types have distinct morphologic appearance, pathogenesis, and genetic profiles. There are still gastric cancer cases which do not fit into either histological type and present a mixed pattern (intestinal and diffuse) (10). The intestinal gastric adenocarcinomas have a better prognosis than the diffuse ones. (9) Gastric adenocarcinoma of intestinal type is causally related to *Helicobacter pylori* (*H. pylori*). In the past decades, steady and slow fall could be seen in the incidence of intestinal gastric adenocarcinoma, which may due to an improvement in socioeconomic situation, sanitation, food preservation and declining *H. pylori* incidence (11, 12).

1.1.3 Prognosis of Gastric Cancer

Gastric cancer incidence rate ranks the fourth among all type of cancers and it is the second common reason of cancer-related death worldwide.(13, 14) 5-year survival depends on tumor stage when the diagnosis is confirmed. The survival rate is fairly high for patients with localized disease (62%), but dramatically decreases when the tumor has already spread to regional lymph nodes (22%) or distant organ sites (3%). (15) the survival rates also vary among the different countries. In US from 1995 to 2001 it was only 23% (16) and in Europe 21% in 1991–1994 (17), while the corresponding survival rate in Japan is reported to be approximately 60% (18). The reasons contributing to survival differences may involve better disease screening program and treatment experiences. (19) In general, developing countries with higher incidence rates of gastric cancer show better survival rates than developed countries with lower incidence due to the difference in the tumor location in stomach (9). It is reported that proximal cancers are predominant in developed countries and are associated with higher socio-economic class, poor prognosis compared with distal cancers which are common in developing countries (19).

1.1.4 Risk Factors of Gastric Cancer

Gastric cancer is a disease affected by multi-factors. The environmental or lifestyle factors are major contributors to the etiology of this disease.

1.1.4.1 Helicobacter Pylori infection

Helicobacter Pylori (*H. Pylori*) infection was regarded as a group I carcinogen by the World Health Organization's International Agency for Research on Cancer (IARC). (20) A study supports the concept, in which a cohort of 4.655 healthy people was monitored for 7.7 years by measuring blood pepsinogen levels (markers of atrophy) and anti-*H. Pylori* antibodies. (21) *H. Pylori* is a gram-negative bacterium and is associated to the development of chronic gastritis, peptic ulceration, gastric carcinoma and MALT lymphoma. (22) Countries with high gastric cancer incidence rates always have a high prevalence of *H. Pylori* infection. (7) *H. Pylori* Infection is usually acquired during childhood by oral ingestion and is highly associated with low socioeconomic status (23, 24). *H. Pylori* may promote gastric carcinogenesis through the stage of chronic gastritis and gastric atrophy due to higher gastric pH which permit the proliferation of nitrate-reducing anaerobic bacteria, resulting in the production of N-nitroso compounds.(25)

H. Pylori infection has been also reported to inhibit ascorbic acid secretion in stomach, and ascorbic acid is the strong scavenger of *N*-nitroso compounds and oxygen free radicals (26).

1.1.4.2 Dietary factors

Consumption of salty foods and *N*-nitroso compounds and low intake of fresh fruits and vegetables have been reported to increase the risk of gastric cancer incidence. (27) A high intake of salty food and N-nitroso compounds and low intake of fresh fruits and vegetables increase the risk of *H. Pylori* infection, gastritis and then gastric carcinogenesis by providing ideal conditions for the growth of *H. Pylori*, which in turn facilitates the growth of N-nitrosating bacteria such as *Escherichia coli* and also reduces the resistance to carcinogenic N-nitroso compound in the stomach (28, 29). There are prospective studies reporting the negative relationship between gastric cancer risk and fruit and vegetable consumption (30-32).

Polyphenols in green tea have shown antitumor and anti-inflammatory effects in animal studies through the antioxidant activities and the ability to inhibit nitrosation, which have been implicated as anti-risk factor of gastric cancer (33, 34).

1.1.4.3 Smoking

Prospective studies have proved a significant dose dependent relationship between smoking and gastric cancer risk (35, 36). The prolonged consumption of tobacco products is highly related to the increased gastric cancer mortality in both male and female (37). All this evidence supports the considering of smoking as an important risk factor for gastric cancer.

1.1.4.4 Other Factors

The association between alcohol and gastric cancer seems little supported (38). Other common risk factors with less effects on gastric cancer include radiation (39), Epstein-Barr (EB) virus (40, 41), blood type A (42), pernicious anemia (43) and prior gastric surgery for benign conditions (44). In addition, a positive family history is also regarded as a significant risk factor, especially with genetic syndromes such as hereditary non-polyposis colon cancer and Li-Fraumeni syndrome (45-47).

1.2 Epigenetics and Gastric Cancer

Epigenetics refers to heritable changes of gene expression which is not due to the alterations of the nucleotide sequence of DNA, and DNA methylation and histone post-translational modifications are regarded as the most important two aspects among widely characterized epigenetic modifications in mammals (48) (Figure1.2) Epigenetic alterations in cancer affect a wide range of genes involved in different and fundamental cellular pathways including apoptosis, angiogenesis, cell cycle control, immune recognition and tumor cell invasion and metastasis. Epigenetic abnormalities in cancer cells can be completely or partially rescued through the effects of pharmacologic inhibitors of the enzymes responsible for building and maintaining the balanced epigenetic status. (49) Gastric cancer is a genetic disease, and both multiple genetic and epigenetic alterations play equally important roles in the process. Genetic alterations such as p53 (50), ErbB2/HER2 (51, 52)and FGFR2 (53, 54) etc have been reported to be closely associated with gastric cancer carcinogenesis and prognosis. This thesis will focus on the discussion of epigenetic alterations of gastric cancer, especially in histone modification.

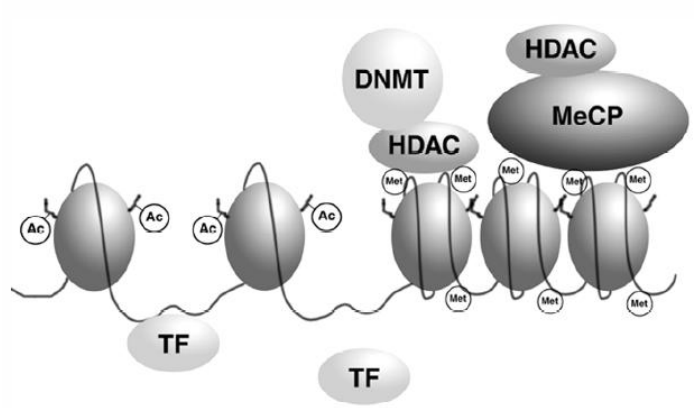


Figure 1.2 Schematic illustration of the regulation of gene expression by histone acetylation and CpG methylation.

Ac, acetylation of histone tail; Met, methylation of CpG island; TF, transcription factor; DNMT, DNA methyltransferase; HDAC, histone deacetylase; MeCP, methyl-CpG binding proteins. Figure obtained from reference (48)

1.2.1 DNA methylation and Gastric Cancer

DNA methylation refers to the process where methyl groups are added to the 5' position of the nucleotides, which is usually the base cytosine right after guanine and leads to gene silencing. Only about 1% sequence In human genome is CpG rich, called CpG islands (55). Around 50% of CpG islands are associated with gene promoter regions (56). DNA methylation can occur at both CpG islands and also non-CpG rich region (57-59). There are mainly two types of DNA methylation: global methylation and promoter-specific DNA methylation (60, 61).

Aberrant methylation of some tumor suppressor genes (TSGs) is a fundamental abnormality in many cancers including gastric cancer by silencing TSGs or promoting inactivating mutations of TSGs (58). For instance, MLH1 is a DNA repair gene, responsible for the repairing of mistakes in replication error (RER) in the tandem repeats of the short sequences. Hypermethylation of MLH1 is almost exclusively found in microsatellite instability-high tumors representing the RER phenotype. (62) This suggests the significance of MLH1 hypermethylation in the RER phenotype in gastric cancer. Surprisingly, MLH1 in surrounding normal mucosa is also similarly hypermethylated, which suggests this biomarker could indicate an early stage of carcinogenesis.(63, 64) in addition, E-cadherin, one of the members of the trasmembrane glycoprotein family, is a cell adhesion molecule and plays an

important role in growth development and carcinogenesis. (65) It was reported that E-cadherin promoter hypermethylation was seen in primary gastric carcinomas, especially in diffuse type, and E-cadherin promoter hypermethylation was observed at similar frequencies in both early and advanced gastric cancer cases (66). Unfortunately, E-cadherin gene methylation can also be observed in non-neoplastic gastric mucosa, which may provide obstacles to determining its role in gastric cancer development. Environmental impact could be considered as another possible contributing factor for hypermethylation. For example, *H. Pylori* infection is associated with promoter hypermethylation of TSGs such as *RUNX3*, *CDH1* (67, 68) probably through nitric oxide production of microphages in gastric cancer.

DNA methyltransferases (DNMT) are enzymes responsible for DNA methylation. There are four types of DNMTs: DNMT1, for methylation maintenance following DNA replication; DNMT2, for some de novo CpG methylating capacity (69); DNMT3A and 3B, for de novo methylation on unmethylated sites (70). Aberrantly high expression of DNMTs could be a potential mechanism of DNA hypermethylation in cancer. It is reported that higher DNMT1 protein expression level is significantly related to DNA methylation of multiple CpG islands in gastric cancer with poor differentiation, which suggests an important role of DNMT1 through frequent DNA methylation of multiple CpG islands in the poorly differentiated gastric cancer development (71). Although the role of altered expressions of DNMTs

in human cancer is still not fully understood, people are still interested in applying DNMT inhibitors to cancer therapy.

Two DNMT inhibitors, 5-azacitidine and decitabine were approved by the FDA for clinical use in myelodysplastic syndrome (72, 73). As cytidine analogs, both drugs are phosphorylated by uridine/cytidine kinase of cells, and then can incorporate into DNA strands like natural cytosines during DNA synthesis. (74) It is reported that the 5-azacitidine efficacy in myelodysplastic syndromes and leukemia is due to the reactivation of cyclin dependent kinase inhibitor, p15, which is normally silenced by promoter methylation (75). Despite the promising activity in myelodysplastic syndrome, early clinical trials showed that DNMT inhibitors have low anticancer activity and significant toxicity as single agent in solid tumors. (76) Recent studies suggest that low concentrations of DNMT inhibitors may synergistically promote other chemotherapies and contribute to overcoming intrinsic or acquired chemoresistance (77, 78). The possibility of using 5-azacitidine and decitabine as a single or combined treatment for solid tumor is still under investigation by many research groups. (79) So far, there is no report about DNMT inhibitor treatment in gastric cancer.

1.2.2 Histone Modification and Gastric Cancer

The importance of histone modifications in the pathogenesis of gastric cancer has been underscored by recent studies. (80) Histones are the basic unit of the nucleosome, consisting of the core histones, H2A, H2B, H3 and H4, each contains two copies (81, 82). With long tails protruding from nucleosome H3 and H4 histones can be covalently modified by other molecules. The modification allows regulatory proteins to access DNA and regulate the transcription process. Modifications of histones include methylation, acetylation, phosphorylation, ubiquitination, SUMOylation, citrullination and ADP-ribosylation. Histone modifications play a role in different aspects of biology, such as DNA repair, gene regulation, and cell proliferation. (80) The patterns of histone H3 and H4 acetylation in gastric cancer have been evaluated, as well as the expression of acetylated H3K9, acetylated H4K16, H3K9triMe and H4K20triMe. The results suggest that global histone modification patterns could serve as an independent predictor for gastric cancer recurrence and survival. (83) in this thesis, we focus on the histone acetylation modification.

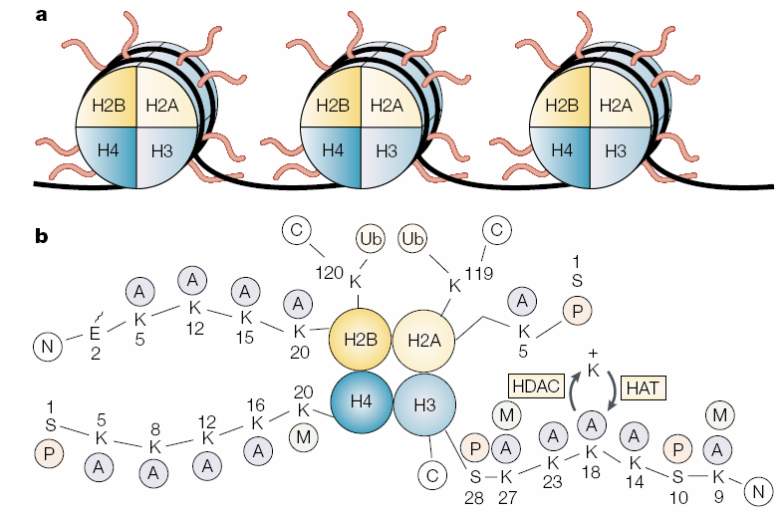


Figure 1.3 Schematic diagram of A. histone structure in nucleosomes; B N-terminal tails protruding from core histones

K, Lysines are potential acetylated/deacetylated sites for histone etyltransferase (HAT) and histone deacetylase (HDAC); A, Acetyl; C, Carboxy-terminus; N, Amino-terminus; E, glutamic acid; M, methyl; P, Phosphate; S, Serine; Ub, Ubiquitin. Figure obtained from reference (84)

1.2.2.1 Histone Acetylation and Deacetylation

The acetylation and deacetylation of key lysine residues of histones are controlled by Histone acetyltransferase (HAT) and histone deacetylase (HDAC). (84)

HATs transfer the acetyl groups from acetyl coenzyme A to e-NH3+ groups of lysine residues within histones, or by adding an acetyl group to the a-amino group of the first residue of the polypeptide.(85) There are two classes of HAT based upon their sub-cellular location, and acetylation activities, which are the cytosolic (type-B) HATs and the Nuclear (type-A) HATs (86). Acetylations on histone lysine residues by HAT result in transcriptional activation (87). The acetyl groups are added to and neutralize the positive charge of lysine of histone, which can influence the interaction between the histone tails and DNA, as well as RNA and other proteins. The acetyl group also provides a specific binding site for certain proteins via their bromodomain. (88) Histone acetylation, subsequently, results in opening up of a specific DNA region allowing the access of transcription factors to promoters for transcription. (89)

Histone acetylation status is balanced by highly dynamic interactions between HAT and HDAC. HDAC removes the acetyl group, reverses the charge neutralization effect, promotes the deacetylation and thus tightens the chromatin structure inhibiting genetic transcription (90). In

humans, 18 HDAC enzymes have been identified and classified, based on homology to yeast HDACs. (91) Class I HDACs include HDAC1, 2, 3 and 8, which are related to yeast RPD3 deacetylase(92); Class II HDACs include HDAC4, -5, -6, -7, -9 and -10, which are related to yeast Hda1.(93) All class I and II HDACs are zinc-dependent enzymes. Class III HDACs, sirtuins, require NAD+ for their enzymatic activity. (94) Class IV HDACs, HDAC11, like yeast Hda 1 similar 3, have conserved residues in the catalytic core region shared by both class I and II enzymes. (95) HDACs can target both histone and non-histone proteins. (91)

1.2.2.2 Histone Acetylation Status and Gastric Cancer

The balance between histone acetylation and deacetylation mediated by HATs and HDACs is impaired in cancer cells. Accumulated evidence from past few years suggests that the modifications of acetylation status play a central role in gastric cancer development. (96, 97) The global acetylation status of histones during carcinogenesis was studied by examining the expression of acetylated histone H4 by Western blotting in samples of non-neoplastic gastric mucosa and different stages of gastric cancer tissues (98). The level of acetylated histone H4 expression was shown to be reduced in 70% of gastric cancer tissues compared to non-neoplastic mucosa samples, while the total amount of histone did not differ significantly between tumor and

normal tissues. The results suggest that global hypoacetylation could be observed in gastric cancer. Reduced histone H4 acetylation was also found in some gastric lesions exhibiting intestinal metaplasia which is usually regarded as the predisposing condition to gastric cancer. Thus, hypoacetylation could be closely associated with tumorigenesis as well as invasion and metastasis of gastric cancer.

1.2.2.3 Histone Acetyltransferase (HAT) and Gastric Cancer

Previous studies showed the aberrations of HATs have both tumor suppressor and oncogene functions in gastric cancer. For example, the histone acetyltransferase gene EP300 may function as a tumor suppressor gene because it is reported somatically mutated in breast, colorectal, gastric and pancreatic cancers, and is located on a region of chromosome 22 that has been reported with loss of heterozygosity in many cancer types. (99) While, another member of the HAT family, Hbo1, which is unique among HAT enzymes in that it serves as a positive regulator of DNA replication, shows strong protein expression in carcinomas of the testis, ovary, breast, stomach/esophagus, and bladder detected by immunohistochemistry. (100)

1.2.2.4 Histone Deacetylase (HDAC) and Gastric Cancer

Most of the histone deacetylase (HDAC) family proteins are over-expressed in cancers compared with normal tissues except HDAC10 and HDAC11. (91, 101-104) Class I HDAC isoforms HDAC1, HDAC2, and HDAC3 has been reported to be highly expressed in primary tissue in the patients with gastric cancer, and the high expression of all three of these isoforms together was significantly associated with nodal tumor spread and decreased overall patient survival. (105) HDAC1 is over-expressed in 60% gastric cancer compared with normal tissue. Over-expression or hyper-activation of histone deacetylases in gastric cancer cells could induce transcriptional repression of various sets of genes in regulating proliferation, migration, angiogenesis, differentiation, invasion, and metastasis.(98, 106) Knockdown of HDACs inhibited cancer features by inducing autophagy, apoptosis, senescence, growth inhibition and sensitized cells for chemotherapy as reviewed by Witt O. *et al.* (107, 108). It should be noted that the knockdown-induced phenotypic effects could be dependent on the cell context.

1.2.2.5 Histone Deacetylase Inhibitors and Gastric Cancer

Because histone or non-histone acetylation is reversible, histone deacetylases have been regarded as an attractive target for cancer therapy by epigenetic inhibitors. So far, many types of HDAC inhibitors have been purified from natural sources or artificially synthesized.(109) HDAC inhibitors can be structurally classified as four groups, which are hydroxamate, cyclic peptide, aliphatic acids and benzamide. (91, 110) HDAC inhibitors inhibit HDAC by binding into the active site pocket and chelating the catalytic zinc-ion located at its base, hence, preventing HDAC from being bound by its natural substrates which is demonstrated by Figure 1.4 using vorinostat (SAHA) as an example. Since the enzymatic pocket is highly conserved among HDACs, most HDAC inhibitors inhibit at least several or even all HDACs, such as SAHA and trichostatin A (TSA), which are named pan-HDAC inhibitors. (111) Some other HDAC inhibitors, such as entinostat (MS275), depsipeptide and MGCD0103 specifically inhibit class I HDACs (102, 112). Currently, at least 16 HDAC inhibitors have been developed and entered into phase I and II clinical trials, with different efficacy and specificity (91, 110, 112, 113). (Table 1.1) Two of them, Vorinostat (SAHA) and Romidepsin were approved by the US FDA in 2006 and 2009 respectively for the treatment of advanced and refractory primary cutaneous T-cell lymphoma (CTCL) (114, 115). Despite the higher selectivity of HDAC inhibitor in inducing transformed cell death than

normal cells (116), it is not surprising that HDAC inhibitors induce various side-effects including bone marrow depression, diarrhea, weight loss, taste disturbances, electrolyte changes, disordered clotting, fatigue and cardiac arrhythmias in phase I and II clinical trials (117). The non-specific toxicity of pan HDAC inhibitors leads to the intense interest of exploiting the application of specific HDAC inhibitors. Three class I HDAC inhibitors, MS275, depsipeptide and MGCD0103, are currently being evaluated in Phase I/II trials in patients with solid or hematological malignancies. (118) Class I HDAC inhibitors displayed similar clinical efficacy as pan HDAC inhibitors, however, the toxicity profiles induced were also similar (118). In this case, it will be rational to evaluate the efficacy and toxicity of class II HDAC inhibitors in clinical trials.

So far, there is no report about clinical trials of HDAC inhibitors as single agents to treat gastric cancer patients. An analysis of global human gastric cancer gene signatures and connectivity maps suggest that HDAC inhibitors such as SAHA and TSA have an inversely correlated gene signature compared to the gastric cancer specific gene signature, which suggests HDAC inhibitors as lead therapeutic candidates for gastric cancer (119).

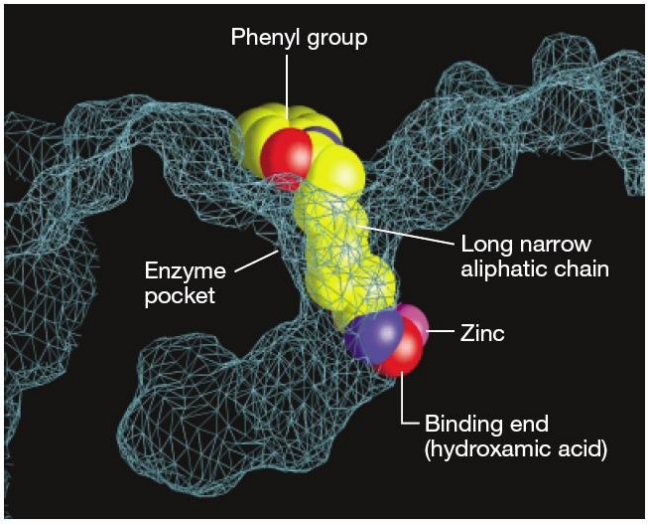


Figure 1.4 Structure of SAHA bound to an HDAC-like protein.

HDAC-like protein is based on the crystal structure of HDAC homologue from the hyperthemophilic bacterioum, *Aquifex aeolicus* (108) and its active site structure was represented by netting. The hydroxamic acid moiety of SAHA binds to the zinc at the bottom of the catalytic pocket. The burying of most of the structure of SAHA into the active site of HDAC prevents HDAC from binding to its natural substrate, thus, blocking its enzymatic deacetylation. Figure obtained from reference (111).

Class	Compound	Specificity	Dose range	Development phase	Adverse effects
Hydroxamate	Vorinostat (SAHA)[24]	Class I/II	µmol/L	US FDA approved	Fatigue, nausea, vomiting, diarrhoea, anaemia, anorexia, thrombocytopenia, QTc prolongation[32-37]
	Belinostat (PXD 101)[25,31]	Class I/II	µmol/L	II	Fatigue, nausea, vomiting, diarrhoea, constipation, flushing, QTc prolongation[38]
	LAQ 824[27]	Class I/II	nmol/L	I	Fatigue, nausea, vomiting, diarrhoea, anorexia, constipation, thrombocytopenia, neutropenia, lymphopenia, anaemia, QTc prolongation, ST segment/T-wave changes, headache[39]
	Panobinostat (LBH 589)[24,31]	Class I/II	nmol/L	I/II	Nausea, vomiting, diarrhoea, anorexia, thrombocytopenia, hypokalaemia, QTc prolongation, ST segment/T-wave changes, pericardial effusion[40]
	Pyroxamide[27]	Class I	µmol/L	I	NA
	Givinostat (ITF 2357)[24,26]	Class I/II	nmol/L	I	Fatigue, diarrhoea, thrombocytopenia, leukopenia, neutropenia, QTc prolongation[41,42]
	PCI 24781[24]	Class I/II	nmol/L	I	NA
Cyclic peptide	Romidepsin (depsipeptide, FK 228)[24]	HDAC1, 2	nmol/L	II	Fatigue, nausea, vomiting, anorexia, thrombocytopenia, lymphopenia, leukopenia, neutropenia, anaemia, QTc prolongation, ST segment/T-wave changes, sinus or ventricular tachycardia[43-45]
Aliphatic acid	AN 9 (pivaloyloxymethyl butyrate)[24,25]	NA	µmol/L	II	Fatigue, nausea, vomiting, diarrhoea, anorexia, dysgeusia, fever, hyperglycaemia, hypokalaemia, hepatic transaminase elevation, anaemia[46,47]
	Sodium Phenylbutyrate[24]	Class I/IIα	mmol/L	II	Fatigue, nausea, vomiting, dyspepsia, neutropenia, anaemia, somnolence, confusion, light-headedness[48-51]
	Valproic acid[26]	Class I/IIα	mmol/L	II	Fatigue, nausea, vomiting, leukopenia, thrombocytopenia, neurological toxicities: neurosensory, neurocortical, vertigo, somnolence[52]
	Valproic acid, topical (Baceca®)[24]	Class I	NA	II	NA
	Valproic acid, oral (Savicol™)[24]	Class I	NA	II	NA
Benzamide	Entinostat (MS 275)[24,26]	HDAC1, 2, 3	µmol/L	II	Fatigue, nausea, asthenia, anorexia, anaemia, thrombocytopenia, hypoalbuminaemia, hypophosphataemia, hyponatraemia, headache[53,54]
	Tacedinaline (CI 994)[27]	NA	µmol/L	I/II	Fatigue, nausea, vomiting, diarrhoea, constipation, mucositis, thrombocytopenia[55]
	MGCD 0103[24,25]	Class I	µmol/L	II	Fatigue, nausea, vomiting, anorexia, diarrhoea, dehydration, constipation, abdominal pain, dyspnoea[56,57]

NA = not available; **QTc** = corrected QT interval; **SAHA** = suberoylanilide hydroxamic acid.

Table 1.1 Classification of histone deacetylase inhibitors in clinical trials

Table obtained from reference (109)

1.2.2.6 Histone Deacetylase Inhibitors Resistance in Cancer

In hematological malignancies most single-agent HDAC inhibitor trials showed complete or partial responses in up to a third of patients enrolled. (109, 120) However, the response rates in solid tumors were relatively much lower, which was mostly less than 10%. (109) As an exciting new group of agents for the treatment of cancer, HDAC inhibitors will be applied into more and more clinical trial of different type of cancers. Thus it is necessary to understand the resistance mechanism of HDAC inhibitor in cancers, especially solid tumor treatment, which will provide useful information in cancer type and drug selection for clinical application. Some genes, such as STAT1 in colon cancer cells harbored with active RAS (121) and HR23B in cutaneous T-cell lymphoma (CTCL) cells (122) were reported to be responsible for HDAC inhibitor resistance. So far there is no publication about potential genes related to HDAC inhibitors resistance in gastric cancer cells.

1.3 Reactive Oxygen Species (ROS) and Gastric Cancer

Despite being essential for life, oxygen can cause various cellular stresses called oxidative stress by generating Reactive oxygen species (123). Reactive oxygen species (ROS) refer to oxygen-containing breakdown products of molecular oxygen such as hydrogen peroxide, hydroxyl radicals, and superoxide anion, which are highly reactive and are able to damage lipid membranes, proteins, and DNA when present in high amounts. (124) Cellular defences to ROS include antioxidant enzymes, such as superoxide dismutase, catalase, glutathione peroxidase and thioredoxin reductase, and antioxidant scavengers, such as ascorbate, glutathione and thioredoxin. (124)

There are many reports investigating or suggesting the mediation of ROS in gastric cancer cells apoptosis induced through ROS-mitochondrial pathway.(125, 126) However, ROS can also play an important role in gastric carcinogenesis. The relationship between ROS and gastric carcinogenesis could be mediated through *H Pylori*. A positive association between ROS production and *H Pylori* infection has been established for decades (127). (Figure 1.5) *H. Pylori* induces infiltration of the gastric mucosa by immune or inflammatory cells. If this immune or inflammatory response could not clear the infection, the host will be left prone to chronic inflammation. One adverse consequence of this inflammatory response may be gastric cancer. (128) It has also been shown that ROS production is enhanced by infection

with *cag*A-positive *H Pylori* strains (129). The most probable initiator of gastric carcinogenesis is oxidative or nitrosative DNA damage, and oxidative stress induced by *H. Pylori* can also modify epithelial cell turnover in stomach.(130) This idea is supported by studies describing an increase in both epithelial cell proliferation and cell apoptosis under *H pylori* infection. (131) Recent publications show that some genes, such as prostaglandin reductase 2 may act as a switch in modulating ROS-mediated cell death and tumor transformation of gastric cancer cells, although the gene itself is associated with higher mortality in gastric cancer patients. (132)

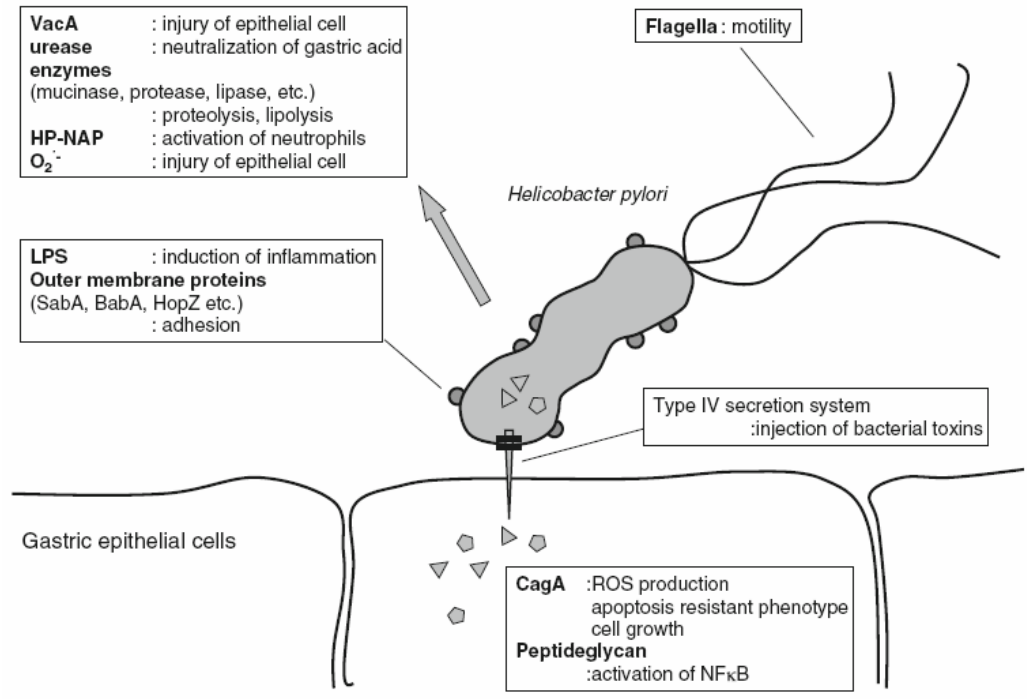


Figure 1.5 ROS production in *H. pylori*-infected gastric mucosa
Figure obtained from reference (127)

1.4 Histone Deacetylase Inhibitors and Reactive Oxygen Species (ROS)

The generation of ROS can be regarded as a potential method in the treatment of cancer. Interestingly, the ability of a cell to defend itself against ROS has been reported to be associated with resistance to chemo-therapies including HDAC inhibitors treatment. (91)

1.4.1 The Role of Reactive Oxygen Species (ROS) in Cancer Treatment by Histone Deacetylase Inhibitors

ROS production may play an important role in HDAC inhibitor-induced cell death. SAHA was reported to induce ROS production and then apoptosis in cancer cells. The pro-apoptotic Bcl-2 family member, Bid was up-regulated under the SAHA treatment. Bid translocation to mitochondria and subsequent disruption of mitochondria was identified as the source of ROS by SAHA (133). LAQ824, another hydroxamate, can induce apoptosis by inducing ROS increase and SM22 gene expression in 13-cis-retinoic acid (CRA)-resistant human melanoma cells (134). In addition, SAHA failed to increase the level of ROS in pan-HDAC inhibitors-resistant human acute myeloid leukemia (AML) HL-60 (HL-60/LR) cells (135). All these publications suggest the involvement of ROS production in HDAC inhibitor mediated

cancer cell apoptosis. HDAC inhibitor has been reported to up-regulate the expression of thioredoxin-binding protein 2 (TBP2) which can bind to and inhibit reduced thioredoxin (Trx) activity. Trx deactivation could induce ROS production in transformed but not in normal cells (116). Trx can also inhibit apoptosis signal-regulating kinase 1 (ASK1). ASK1 promotes SAHA induced apoptosis by enhancing the expression of pro-apoptotic protein Bim through a positive feedback on E2F1 activity (136).

Although most observations support that ROS accumulation could be the mediator between HDAC inhibitors treatment and cancer cells death, the situation could be different in liver cancer cells. In response to the oxidative stress, hepatoblastoma cell Huh6 could be induced to undergo apoptosis through the nuclear translocation of p53, and the translocated p53 activated target genes which are essential in cell apotosis. Interestingly, HDAC inhibitor (nicotinamide) treatment strongly inhibited the nuclear translocation of p53 induced by ROS stress and the subsequent p53-dependent apoptosis in Huh6 cells, (137) which suggests that the relationship between ROS and HDAC inhibitors could be complicated according to different types of cancer.

1.4.2 The Role of Reactive Oxygen Species (ROS) on Cancer Chemo-sensitivity to Histone Deacetylase Inhibitors

A cDNA microarray analysis performed in a clinical trial suggested that a gene signature composed mainly of antioxidants was associated with clinical resistance to SAHA. (138) Thus, increased antioxidant expression may contribute to HDAC inhibitor resistance. In early stage, studies focused on the role of thioredoxin (Trx) in regulating sensitivity to HDAC inhibitors. Trx function as a ubiquitous antioxidant and electron donor for ribonucleotide reductase and the thioredoxin reductase keeps Trx in a reduced state by using NADPH as a cofactor. (139) SAHA can up-regulate the expression of Trx-binding protein 2 (TBP2) which can bind and inactivate Trx. This suggests that HDAC inhibitors could inactivate antioxidants and facilitate oxidative stress. (140) Some other study further enhances the idea of inactivation of Trx sensitizing certain cancer cells to HDAC inhibitor treatment with siRNA for Trx down-regulating Trx levels and increasing significantly the sensitivity of the cells to SAHA. (116) Another attractive point for Trx is that SAHA can surprisingly increase Trx levels in normal cells compared to different effects in transformed cells. (141) The interesting difference may provide a potential explanation for the higher resistance of normal cells to HDAC inhibitor induced ROS production and cell apoptosis, although the mechanism is not fully understood.

Recently, it is reported that other antioxidants could also be up-regulated in a pan-HDAC inhibitor-resistant leukemia cell line HL60/LR, while Trx was decreased in the same cell model under SAHA treatment. SAHA induces increased ROS and translocation of nuclear factor E2-related factor 2 (Nrf2) from cytoplasm to nucleus in HL60/LR cells. Nrf2 translocation up-regulates antioxidant genes including glutamate cysteine ligase (GCL), glutathione reductase (GSR), glutathione S-transferase (GST), and superoxide dismutase (SOD) as a cellular protective mechanism. Addition of beta-phenylethyl isothiocyanate (PEITC), which can deplete cellular glutathione, significantly sensitizes leukemia cell lines and primary leukemia cells to SAHA treatment. (142) The publication suggests other potential mechanisms for HDAC inhibitor resistance related to ROS pathway in cancer cells.

1.5 Ribonuclease Inhibitor (RNH1)

The molecular weight of Ribonuclease inhibitor (RNH1) is about 50-kDa, and the protein is found in the cytoplasm (143, 144) , nuclei and mitochondria (145) of mammalian cells. RNH1s are a family of highly conserved proteins. The conservation of the amino acid sequence between different hosts, such as pig, cow, rat, mouse, sheep and human is as high as nearly 70%. (144) All eukaryotic RNH1s share two characteristics: the high

percentage content of reduced cysteine amino acids (30-32 residues, 7% of total amino acids), and a central portion composed of 15-16 repeated hydrophobic leucine rich motives (144, 146). The high percentage content of reduced cysteines is observed in its cytoplasmic localization, which is required for its function as an ribonuclease inhibitor.(147) The role of leucine-rich repeats, like the ones in other family of proteins, is to provide surface areas to promote protein-protein interactions (148, 149).Previous publications suggest biological roles of RNH1 as a modulator of the biological functions of various ribonucleases or as an oxidative sensor to monitor the redox status in normal or aged cells.(150-152) In this study, RNH1 was identified through genomic analysis and functionally proved as a contributor to the HDAC inhibitor resistance of gastric cancer cells

1.6 Aims of This Study

In this project, we will examine the phenotypic and molecular effects of HDAC inhibitors, such as TSA, SAHA and MS275, on gastric cancer cells. With the genomic analysis of differently expressed genes between HDAC inhibitors sensitive and resistant gastric cancer cells, we tried to investigate the possible candidate genes or signatures for HDAC inhibitors response in gastric cancer cells.

Chapter Two: Materials and Methods

2.1 Cell Culture

2.1.1 Cell lines and Drug Treatments

Gastric cancer cell lines AGS, N87, and Hs746T were purchased from the American Type Culture Collection. AZ521, Ist1, TMK1, MKN1, MKN7, MKN28, HFE145, GES1 and IM95 cells were obtained from the Japan Health Science Research Resource Bank. SCH cells were provided by Yoshiaki Ito (Cancer Sciences Institute of Singapore). YCC cells (YCC3, YCC6, YCC7, YCC11 and YCC16) were a gift from Sun-Young Rha (Yonsei Cancer Center, South Korea). HepG2, MCF7, HCT116 and Hela cells were provided by Dr. Tingdong Yan (Duke-NUS Graduate Medical School). AGS, IST1, AZ521, SCH, GES1 and MKN7 cells were maintained in RPMI 1640 medium (Sigma, St Louis MO) with 10% FBS (Gemini Bio-Products, West Sacremento, CA). YCC cells were maintained in MEM medium with 10% FBS. IM95, HS746T, HFE145, HepG2 and HCT116 cells were maintained in DMEM medium with 10% FBS. All cells were grown in a humidified incubator with 5% CO_2 at 37^{o}C. All cells are adherent cells. Subculturing of cells was performed using 0.25% trypsin (Invitrogen Corporation, USA) to detach and split at the ratio from 1:4-1:10 and maintained as the method described above.

Histone deacetylase inhibitors, Trichostatin A (TSA, from Sigma, St Louis MO), Vorinostat (SAHA) and MS275 (Enzo life Sciences, UK) were utilized as experimental drugs. Cells were treated with 50-800nM TSA or 0.625-10um SAHA or 3.125-50uM MS275 dissolved in DMSO, or DMSO only (control) for 24, 48 and/or 72 hours. For molecular assays, cells were treated with 400nM TSA or 5uM SAHA or 30uM MS275 unless otherwise specified.

2.1.2 Preservation of Cell Lines

All cells were preserved in liquid nitrogen. Cell suspension in medium was mixed with 5% DMSO (Sigma, USA). Cells were aliquoted into 2ml plastic cryogenic vial (IWAKI, Japan). The cryovials were put into a Cryo Freezing Container (Nalgene labware, USA) overnight at -80^{o}C and stored in liquid nitrogen the next day.

2.1.3 Quantification of Cell Number

Cells were counted by using hemocytometer (VWR LabShop, USA). Briefly, 10ul of diluted cell suspension was mixed with equal volume of Trypan Blue (Invitrogen Corporation, USA). 10ul stained cells were loaded into each chamber of the hemocytometer and cells were counted under microscopy according to manufacturer's menu.

2.2 In Vitro Cell Assays

2.2.1 Cell Proliferation Assays

Cell proliferation assays were performed using a CellTiter96 Aqueous Nonradioactive Cell Proliferation Assay kit (Promega, USA) following the manufacturer's protocol. The method is colorimetric to determine the number of viable cells in proliferation or in chemo-sensitivity assays. The kit contains solutions of a novel tetrazolium compound [3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS]. Living cells can bio-reduce MTS into a purple formazan product which is soluble in culture medium. Specific machines can measure the absorbance of the formazan at 490nm directly from 96-well assay plates. The conversion of MTS into formazan is performed by dehydrogenase enzymes found in metabolically active cells. In some certain ranges, the absorbance quantity of formazan product measured at 490nm is directly proportional to the number of living cells in culture.

Before proliferation assays, cells were seeded at concentrations of 1-5×10^3 cells per well in 96-well plates. After experimental end points, 20ul MTS reagent was added to each well. After four hour of incubation at 37^oC, cellular proliferative activity was measured using an EnVision 2104 multi-label plate reader (Perkin Elmer, Finland) at 490nm absorbance after 24, 48 and 72hours. All experiments were repeated for at least three independent

times. GI50 was calculated by the formula: [(Td-T0)/(C-T0)] x 100 = -50] using absorbance measurements of [time zero, (T0); control growth, (C); and test growth in the presence of drug at the seven concentration levels (Td)].

2.2.2 Colony Formation Assays

Base layers of 0.6% Low Melt Point Agarose (Promega, USA) in complete culture medium were poured into 6-well plates and allowed to harden at 4°C. 30 000 cells/well were suspended in complete media plus agarose mixture at 42°C and seeded on top of the solidified base layer. The double layer plates were also allowed to harden at 4°C. Plates were incubated at 37°C in for 3-4 weeks, during which complete media was changed every 3 days. After 3-4 weeks, colony numbers were counted and the plates were photographed using the Kodak GL 200 System (EpiWhite illumination). Each assay was repeated 3 times, and the results were averaged over three independent experiments.

2.2.3 Oxidative stress assay

CellROX™ Deep Red Reagent is a fluorogenic probe designed to reliably measure ROS in live cells. The CellROX™ Deep Red dye is cell permeable and nonfluorescent while in a reduced state. Upon oxidation the dye exhibits excitation/emission maxima at 640/665 nm. Cells were treated with the test drugs and incubated for the recommended time. CellROX™ Deep Red Reagent was added at a final concentration of 5μM to the cells, and the cells were incubated for 30 minutes at 37°C. Medium was removed and the cells were washed for three times with PBS. Experiments were repeated for three independent times, and the results were averaged over three independent experiments.

2.3. Gene Transcription Assay

2.3.1 Differential Gene Expression Analysis

GC cell lines were profiled using Affymetrix Human Genome U133 plus GeneChips (HGU133 Plus 2.0; Affymetrix, Santa Clara, CA) and Illumina Human-6 v2 Expression BeadChips (Illumina, San Diego, CA). Gene expression profiles (both Affymetrix and Illumina arrays) were imported into Partek Genomics Suite™ 6.3 (Partek Inc., St. Louis, MO) using default Partek normalization parameters. Probe-level data was pre-processed, including background correction, normalization, and summarization, using robust

multi-array average (RMA) analysis adjusted for probe sequence and GC content (GC-RMA). These values were then $\log_2$ transformed. Background-adjusted, normalized values were then averaged to generate a single value under each gene ID. Differential expression analysis was performed using one-way ANOVA between the HDAC inhibitor sensitive and resistant groups, using a filter of $p<0.05$. False discovery rates (FDR) were calculated using the 'q value' application in Partek software, and the standard was set as <0.3. The gene lists from both Affymetrix and Illumina arrays were overlapped to generate a final candidate differentially expressed gene list.

2.3.2 Quantitative real-time PCR

RNA was isolated from these cells (Qiagen kit) and reverse transcribed to cDNA (First strand synthesis, Invitrogen) followed by Q-PCR. Reaction mixes consisted of 10ul Quantifast SYBR green PCR master mix, 1ul of *RNH1* /*LINE1* primers, 20ng of the template in a final reaction volume of 20ul. Real-time PCR (ABI 7900HT, Applied Biosystems) cycling conditions were 10 minutes at 95°C, followed by 40 cycles of 15 secs at 95°C and 1 min at 60°C. All experiments were performed in triplicate. *RNH1* cycle thresholds were normalized to the *LINE1* repeat element from the same samples, as an endogenous control. Scramble samples from YCC3 or YCC7 were chosen as calibrator and for each analysis a negative control was also prepared using all

reagents except DNA template. The forward and reverse primers of RNH1 and LINE1 were: RNH1_F_P1: 5' ACAACAGGCTGGAGGATGC 3', RNH1_R_P1: 5' CAGTTGTTGCTGAGGTCCAG 3'; RNH1_F_P2: 5' CCTGCAGCTGGTGGAGAG 3', RNH1_R_P2: 5' GGAAGAGCCTCAGGAGATGA 3'; RNH1_F_P3: 5' CGGTTAGCAACAACGACATC 3', RNH1_R_P3: 5' ATCACCCAGCTTGTTGCTG 3'; LINE 1_F: 5' AAAGCCGCTCAACTACATGG 3', LINE 1_R: 5' TGCTTTGAATGCGTCCCAGAG 3'

2.4. Gene Translation Analysis

2.4.1 Protein Extraction

For western blotting, total cell lysates were prepared by incubating cell pellets on ice for 30 mins in 0.5% NP-40 buffer (50mM TrisHCl (pH 8.0), 1mM EDTA, 0.3MNaCl, 10% glycerol, 1mM DTT, 0.5% NP-40 complemented with proteinase inhibitor and halt phophatase inhibitor (Thermo Scientific Pierce, UK)). Cells were then spined at maximum speed for 20 mins at 4^{o}C and supernatants were collected as total cell lysates and immediately frozen in nitrogen liquid, which were then transferred and stored in a -80^{o}C freezer.

2.4.2 Determination of Protein Concentration

Protein concentrations were measured using a BCA assay (Thermo Scientific Pierce, UK) according to the manufacturer's protocol. Briefly, 10 ul of BSA standand from 2mg to 0.125mg (Bio-rad Laboratories, USA) were used as protein standards on a 96 well plate. 10ul water was loaded as 0mg standard while 10ul lysis buffer was loaded as blank. 1ul lysate was loaded for each sample. Samples and standard were loaded in duplicate. Reagent A and reagent B were mixed at the ratio of 5:1 and 200ul of mixture was added to each reaction. Proteins were then incubated in dark for 30 mins. Protein concentrations were measured at absorbance of 562nm using Bio-rad 96 well plate spectrometer reader (Bio-rad Laboratories, USA).

2.4.3 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The SDS-PAGE gel was performed using a Mini-PROTEAN 3 Electrophoresis System (Bio-rad Laboratories, USA). Two concentrations of SDS gels were used in this study depending on the molecular size of the protein to be detected. Proteins with size >40KDa and those <40Kda were separated by 8% and 15% gels respectively. Briefly, separating gels were prepared by mixing appropriate amount of 30% acrylamide/ bisacrylamid solution (37.5:1; Bio-rad Laboratories, USA) with 2.5 ml 1.5M Tris-HCl (pH 8.8), 100ul of 10% SDS, 100ul of 10% ammonium persulfate (APS) (Bio-rad

Laboratories, USA), 6ul of TEMED (Bio-rad Laboratories, USA) and water was added to a total volume of 10ml. The mixture was then poured into gel set cassette. 200ul 100% ethanol was added on the top of the gel immediately.

After the gel was set, overlay was decanted and a 5% stacking gel was poured on top of the separating gel. 5% stacking gel was the mixture of 0.5ml 30% acrylamide/bisacrylamid solution with 0.5ml 1M Tris-HCl (pH 6.8), 50ul of 10% SDS, 50ul of 10% APS, 5ul TEMED and water. A gel comb was inserted properly and the stacking gel was allowed to set before electrophoresis. 40ug of protein samples were mixed with 1X loading buffer (62.5mM Tris-HCl (pH 6.8), 10% glycerol, 2% SDS, 0.05% bromophenol blue) and 2.5% of 2-mercaptoethanol was added right before boiling in 99^{o}C heat block for 5 mins. Samples were allowed to cool down and loaded to each well of SDS gels. Kaleidoscope prestained standards (Bio-rad Laboratories, USA) was loaded as protein standard. Electrophoresis was performed under constant 0.045A per gel until reaching appropriate protein separation in 1X Tris-Glycine-SDS PAGE Buffer (1st base, Singapore)

2.4.4 Gel Transfer

After protein separation, the gel transfer was performed using Mini Trans Blot Cell Systems (Bio-rad Laboratories, USA). The polyacrylamide gel was soaked in 1X transfer buffer: prepared by 1:10 10X Tris-Glycine-SDS PAGE

buffer, 2:10 100% Methanol and 7:10 water. Gel was then placed in between layers of filter membrane (Millipore, USA), Whatman papers and foam pads (Bio-rad Laboratories, USA) and put into holder cassettes in electrode assembly with the membrane facing the anode. The transfer was done in 1X transfer buffer at constant 100V for 90 mins at 4^{o}C.

2.4.5 Western Blotting and Detections

Western blotting was performed on PVDF membrane fractions using the following antibodies and dilutions: 1:200 RNH1 (Santa Cruz Biotechnology, Inc., USA), 1:2000 PARP (Cell Signaling Technologies). 1:200 β-Actin and 1:200 GAPDH (Santa Cruz Biotechnology, Inc., USA) were used as loading control. As secondary antibodies we used the goat anti-rabbit and goat anti-mouse antibodies (Cell Signaling Technologies), conjugated with Horseradish Peroxidase (HRP). Proteins were detected using the Enhanced ChemiLuminescence (ECL) Plus Blotting Detection system (Amersham Biosciences, Buckinghamshire, UK) and were visualized by autoradiography on photographic film (KODAK X-OMAT, New York, USA). Experiments were repeated a minimum of three independent times.

2.5 Gene Modulation

2.5.1 Transfection of shRNA

YCC3 and YCC7 cells were transfected with either specific HuSH-29 shRNAs (OriGene Technologies, USA) targeted to RNH1 or negative control scrambled shRNAs using Lipofectamine 2000 transfection reagent (Invitrogen, USA) in Optimem Medium (Invitrogen, USA) following the manufacturer's protocol. Briefly, 6ul Lipofectamine 2000 transfection reagent was added to 100ul Opti-MEM reduced serum medium (Invitrogen, USA) and they were incubated for 5 minutes at room temperature after mixing. 2ug scrambled or RNH1 shRNA vector was diluted into 100ul Opti-MEM. Diluted Lipofectamine 2000 transfection reagent was mixed with diluted shRNA and incubated for 20 minutes at room temperature to form transfection complexes. Transfection complexes were dispensed into clean wells on 6 well plates and overlayed with 2 ml cell suspension in growth medium. In all of the plasmids the HuSH shRNAs were cloned into OriGene's non-proprietary pGFP-V-RS vector, which allows stable transfection and stable delivery of the shRNA expression cassette into host cells via a replication-deficient retrovirus. The scramble pGFP-V-RS vector offers turbo-GFP expression that facilitates easy monitoring of transfection. The HuSH pGFP-V-RS plasmid vector (Figure 2.1) contains both 5'and 3'LTRs of Moloney murine leukemia virus (MMLV) that flank the puromycin marker expression cassette. The puromycin-N-acetyl transferase gene provides selection of antibiotics puromycin. For YCC3, the

targeted sequence of Clone 1: TACGACATTTACTGGTCTGAGGAGATGGA; Clone 2: TGCTCTGGTTGGCCGACTGCGATGTGAGT. For YCC7, the targeted sequence of Clone 1: TACGACATTTACTGGTCTGAGGAGATGGA; Clone 2: TGGAGAGCTGCGGTGTGACATCAGACAAC. After 72 hours shRNA transfection, cells were incubated in medium containing 1uM puromycin for 4 weeks to establish stable knock-down of RNH1.

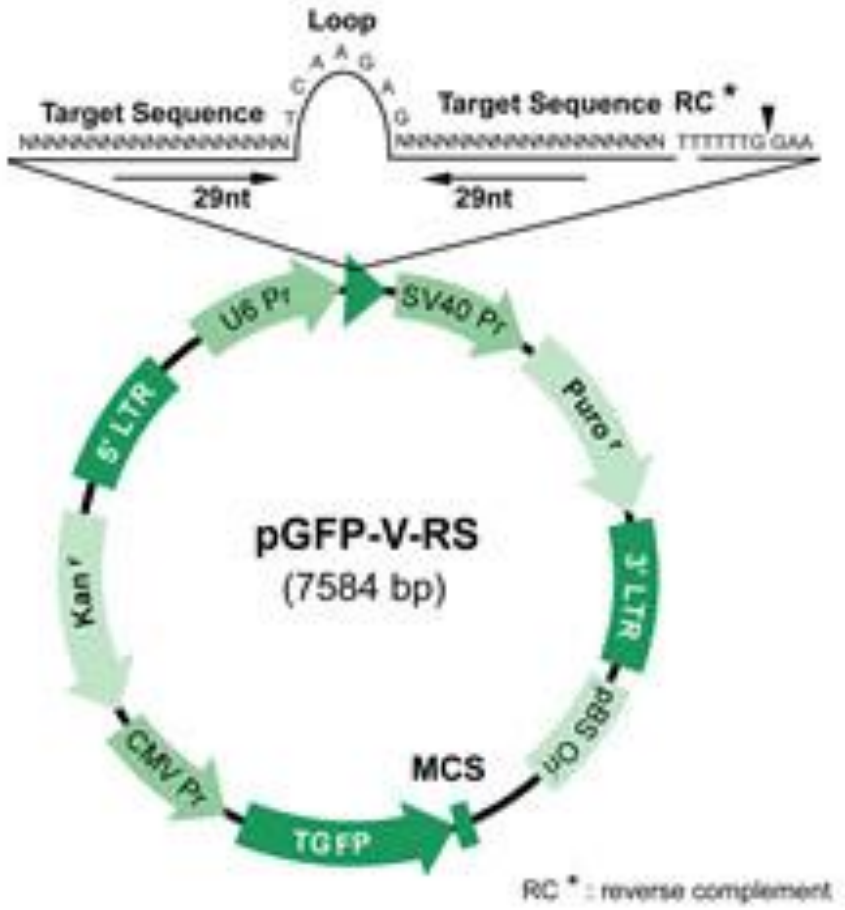


Figure 2.1 The HuSH pGFP-V-RS plasmid vector

2.5.2. Gene overexpression

The full length coding regions of *RNH1* cDNA ORF clone inserted into the pCMV6-AC-GFP vector (Figure2) was purchased from OriGene Technologies, USA. ORFs cloned in this vector will be expressed in mammalian cells as a tagged protein with a C-terminal tGFP tag. Control vectors or fusion-RNH1 vectors were transiently transfected into AZ521 and YCC11 cells using Lipofectamine 2000 transfection reagent (Invitrogen, USA) in Optimem Medium (Invitrogen, USA) following the manufacturer's protocol. Briefly, 6ul Lipofectamine 2000 transfection reagent was added to 100ul Opti-MEM reduced serum medium (Invitrogen, USA) and they were incubated for 5 minutes at room temperature after mixing. 2ug control or RNH1 cDNA vector was diluted into 100ul Opti-MEM. Diluted Lipofectamine 2000 transfection reagent was mixed with diluted cDNA and incubated for 10 mins at room temperature to allow transfection complexes to form. Transfection complexes were dispensed into clean wells on 6 well plates and overlayed with 2 ml cell suspension in growth medium. After 48 hours, cells were subjected for downstream analysis.

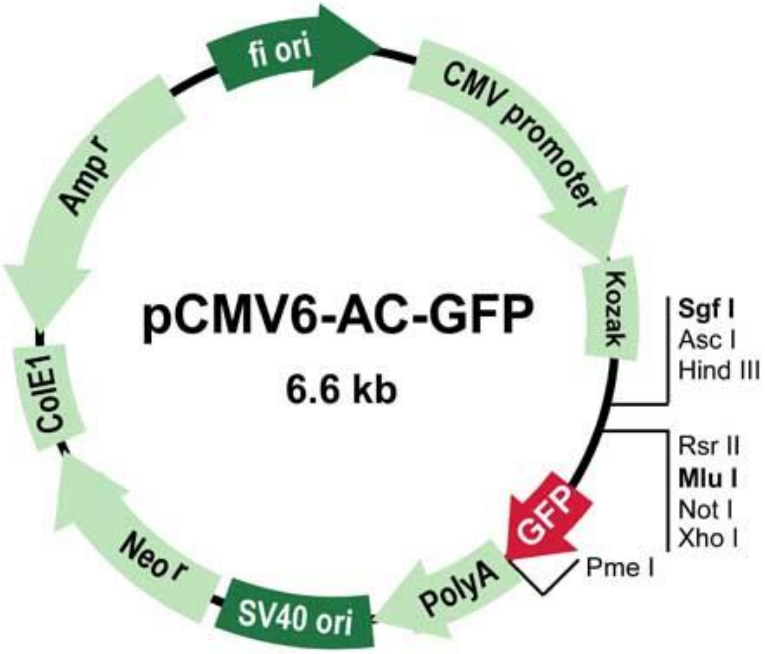


Figure 2.2 The pCMV6-AC-GFP vector

2.7. Statistical Methods.

Unless otherwise specified, all other p-values used in comparisons of two groups were computed using Student's t-test. All p-values are two-tailed.

Chapter Three: Results Part I

3 Sensitivity of Gastric Cancer Cell Lines to HDAC inhibitors

3.1 Sensitivity of Gastric Cancer Cell Lines to Trichostatin A (TSA)

3.1.1 Selection of Gastric Cancer Cell lines Experimental Panel

17 Gastric Cancer cell lines were chosen for the sensitivity scanning under TSA treatment. The origins and culture media were listed as Table 3. The selection was random, but we aimed cover as many histological subtypes of GC as possible.

Cell lines	Medium	Disease	Source
AGS	RPMI	AC	Stomach
MKN1	RPMI	ASC	Node
MKN7	RPMI	AC	Node
Ist1	RPMI	AC	Liver
N87	RPMI	AC	Liver
TMK1	RPMI	AC	Stomach
AZ521	RPMI	GC	Stomach
SCH	RPMI	CC	Stomach
NUGC3	RPMI	AC	Muscle
YCC-3	MEM	AC	Acsite
YCC-6	MEM	AC	Acsite
YCC-7	MEM	GC	Acsite
YCC-10	MEM	AC	Acsite
YCC-11	MEM	AC	Acsite
YCC-16	MEM	AC	Blood
HS746T	DMEM	GC	Leg
IM95	DMEM	AC	Stomach

Table 3.1 17 Selected Gastric Cancer Cell lines.

The original sources and the culture media of each cell lines were listed. GC, gastrocarcinoma; AC, adenocarcinoma; ASC, adenosquamose carcinoma; CC, choriocarcinoma

3.1.2 Cell Reduction of 17 Gastric Cancer Cell Lines Induced by TSA

Previous studies have suggested that individual gastric cancers can display differing responses to HDAC inhibitor treatment.(112) To identify gastric cancer cell lines exhibiting differential sensitivities to HDAC inhibitor compounds, we treated 17 gastric lines with increasing doses of TSA, a pan-HDAC inhibitor, to determine LC20 values for each line, referring to the drug concentration required to cause 20% cell reduction relative to untreated controls (Figure 1A). 5 cell lines (YCC11, AGS, IST1, SCH and AZ521) were associated with low LC20 values (less than 200nM) relative to the other lines (above 400nM, mostly above 800nM) – these lines were designated as TSA-sensitive lines (Figure 3.1)

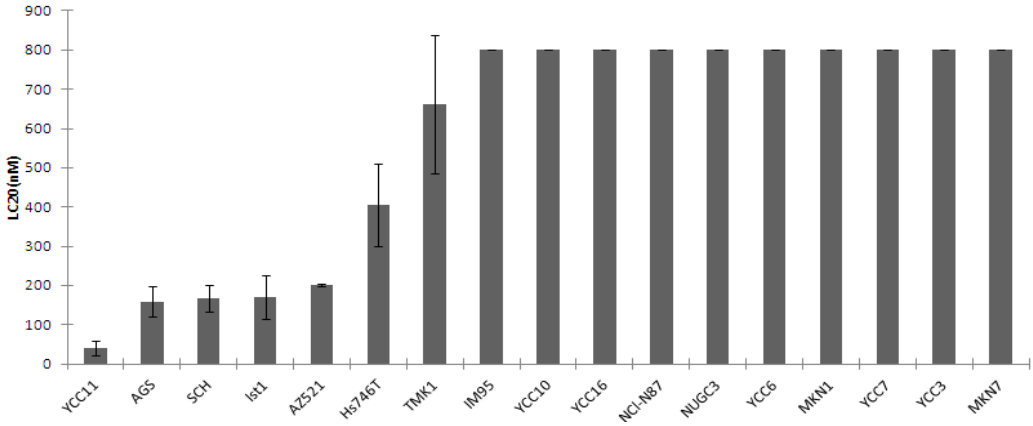


Figure 3.1 Ranking of LC20 values of 17 Gastric Cancer cell panel under TSA treatment.

Y axle is the LC20 value. Triplicate experiments were performed for each cell lines and the average values were calculated and ranked.

3.1.3 Growth Inhibition of 17 Gastric Cancer Cell Lines Induced by TSA

To support the LC20 results, we then compared GI50 values (the drug concentration required for 50% cell growth inhibition) among the selected 17 gastric cancer cell lines under the TSA treatment (0-800nM, 24 hours). (Figure 3.2) MTS assay was performed to GI50 was calculated to evaluate the growth inhibition of each cell lines induced by TSA treatment. Cell lines were ranked according to the GI50 values. Higher GI50 value means higher resistance to growth inhibition induced by TSA treatment. 5 cell lines with highest GI50 values (YCC3, YCC7, YCC10, MKN1 and MKN7) were defined as potential resistant gastric cancer cells to TSA treatment. There was a >10x difference in the GI50 values between the sensitive and resistant groups ($P<0.001$). This result demonstrates that different gastric lines can indeed demonstrate differential sensitivities to HDAC inhibitor treatment.

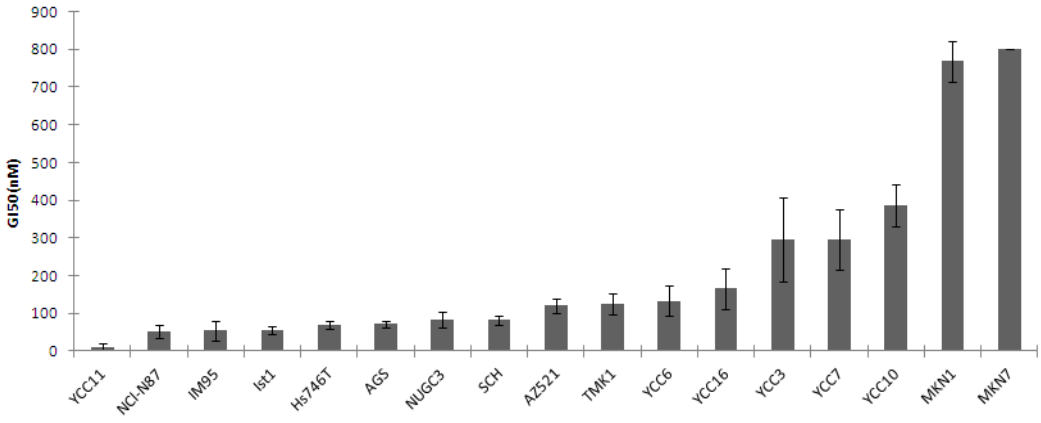


Figure 3.2 Ranking of GI50 values of 17 gastric cancer cell panel under TSA treatment.

Y axle is the GI50 value. Triplicate experiments were performed for each cell lines and the average values were calculated and ranked.

3.1.4 Apoptosis of 10 Gastric Cancer Cell Lines Induced by TSA

Inhibition of cell proliferation by TSA could be due to several alternative and distinct mechanisms, including induction of apoptosis, cell growth arrest, and senescence. To clarify which of these mechanisms might explain the TSA effects, we assessed the TSA-treated cells for the presence of cleaved PARP and caspase 3, well-established markers of apoptosis. Because both death-receptor pathway (extrinsic) and mitochondrial pathway (intrinsic) of apoptosis pathways need PARP and caspase 3 cleavage, cleaved PARP and caspase 3 were used as indicators of TSA inducing gastric cancer cell apoptosis. Western blotting was performed to show the difference of cleaved PARP and caspase 3 induced by TSA treatment between potential sensitive and resistant gastric cancer cell line groups. (Figure 3.3) All the 5 GC cell lines with lower GI50 values showed obvious PARP and caspase 3 cleavage, while gastric cancer cell lines YCC3, YCC7 and MKN7 showed no obvious cleaved PARP or caspase 3.

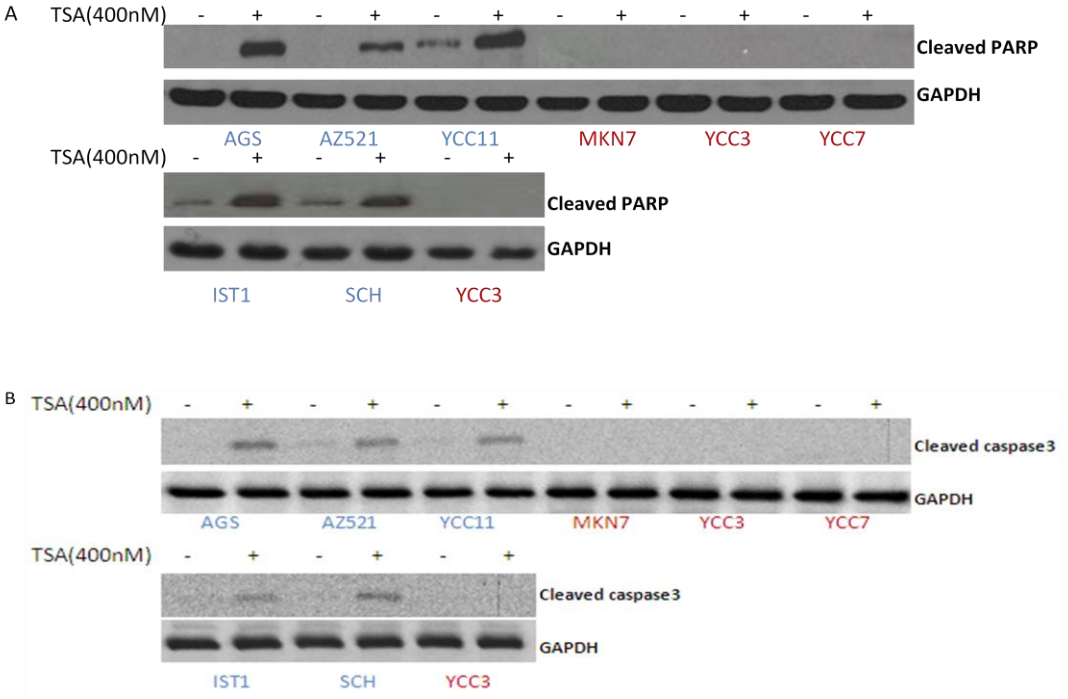


Figure 3.3 Western blot of cleaved PARP (A) or caspase 3 (B) induced by TSA treatment between sensitive and resistant gastric cancer cell line groups.

All the gastric cancer cells were treated by 400nM TSA for 24 hours. Cells treated by same amount of DMSO were taken as negative control. Whole protein extracted from the cell pellets were sent to western blot

3.1.5 TSA treatment of YCC10 and MKN1 with extended time

An experiment with different time points of TSA treatment was performed on MKN1, YCC10 and YCC3. Unlike YCC3, extended TSA treatment (such as 48 hours) could induce obvious growth inhibition and cell reduction of MKN1 and YCC10, although these two cell lines showed high resistance to TSA treatment within 24 hours. (Figure 3.4 A and B) Cleaved PARP was also observed in MKN1 and YCC10. (Figure 3.4 C) From the observation, MKN1 and YCC10 were excluded from resistant gastric cancer cell line group to HDAC inhibitors.

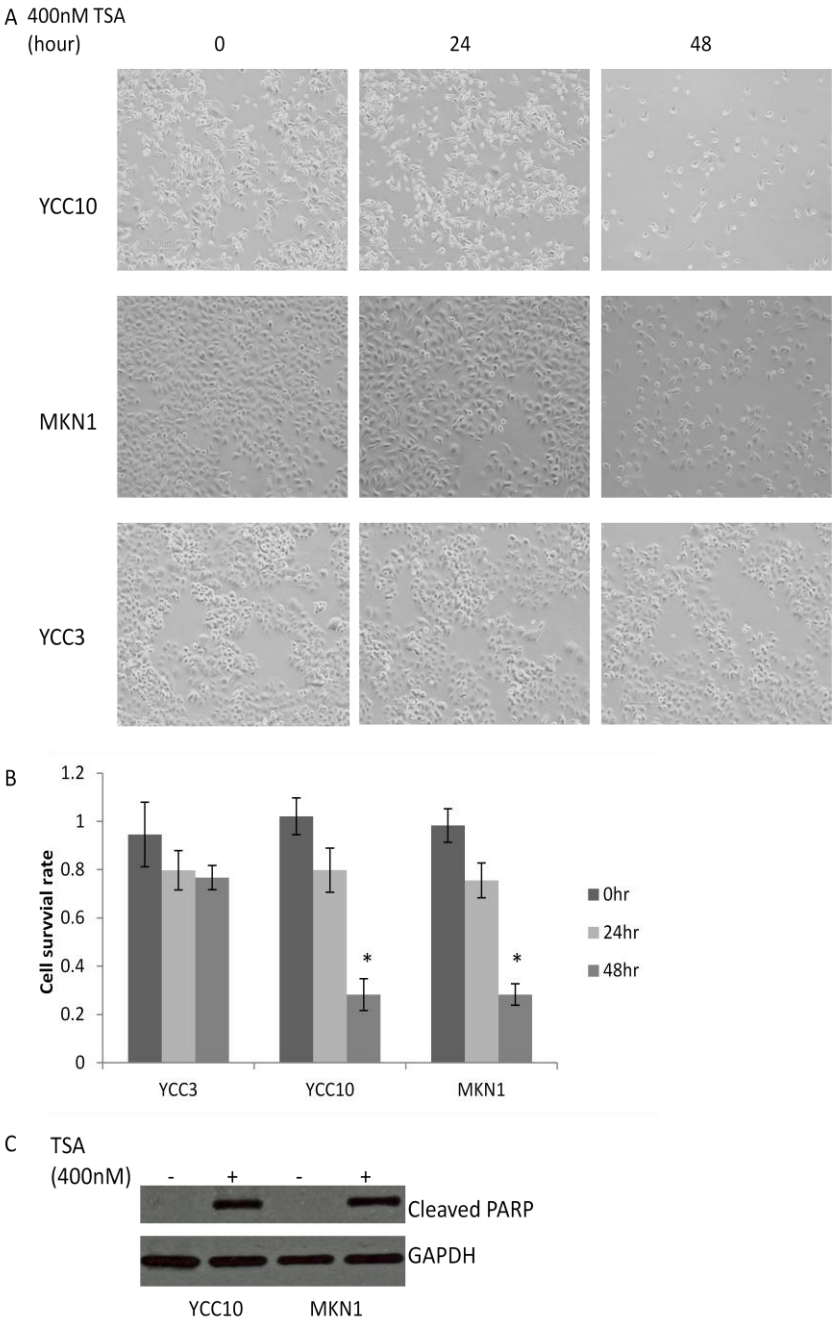


Figure 3.4 TSA treatment of YCC10, MKN1 and YCC3 with different time course.

(A) Photographs showed the growth condition of the three gastric cancer cell lines with TSA treatment of different time course (24 hours and 48 hours). (B) MTS assay was performed to show survival cells after TSA treatment with different time courses. (C) Western blot was performed to YCC10 and MKN1 treated by TSA (400nM, 24 hours) to test the PARP cleavage.

3.1.6 Colony Formation Inhibition of 7 Gastric Cancer Cell Lines Induced by TSA

As a third measure to verify the differential sensitivities of the sensitive and resistant lines to HDAC inhibitor treatment, we conducted soft-agar colony formation assay. As shown in Figure 3.5, after 3 weeks TSA treatment, all the sensitive lines showed obvious decreased colony formation compared to the untreated cells, whereas the colony formation inhibition could be observed in resistant lines (YCC3 and YCC7. MKN7 cells cannot form colony) Difference of colony formation inhibition induced by TSA treatment was compared between sensitive and resistant gastric cancer cell line groups by bar chart. The result suggested that there was a sensitivity difference in 3-dimentional culture environment between gastric cancer sensitive and resistant groups.

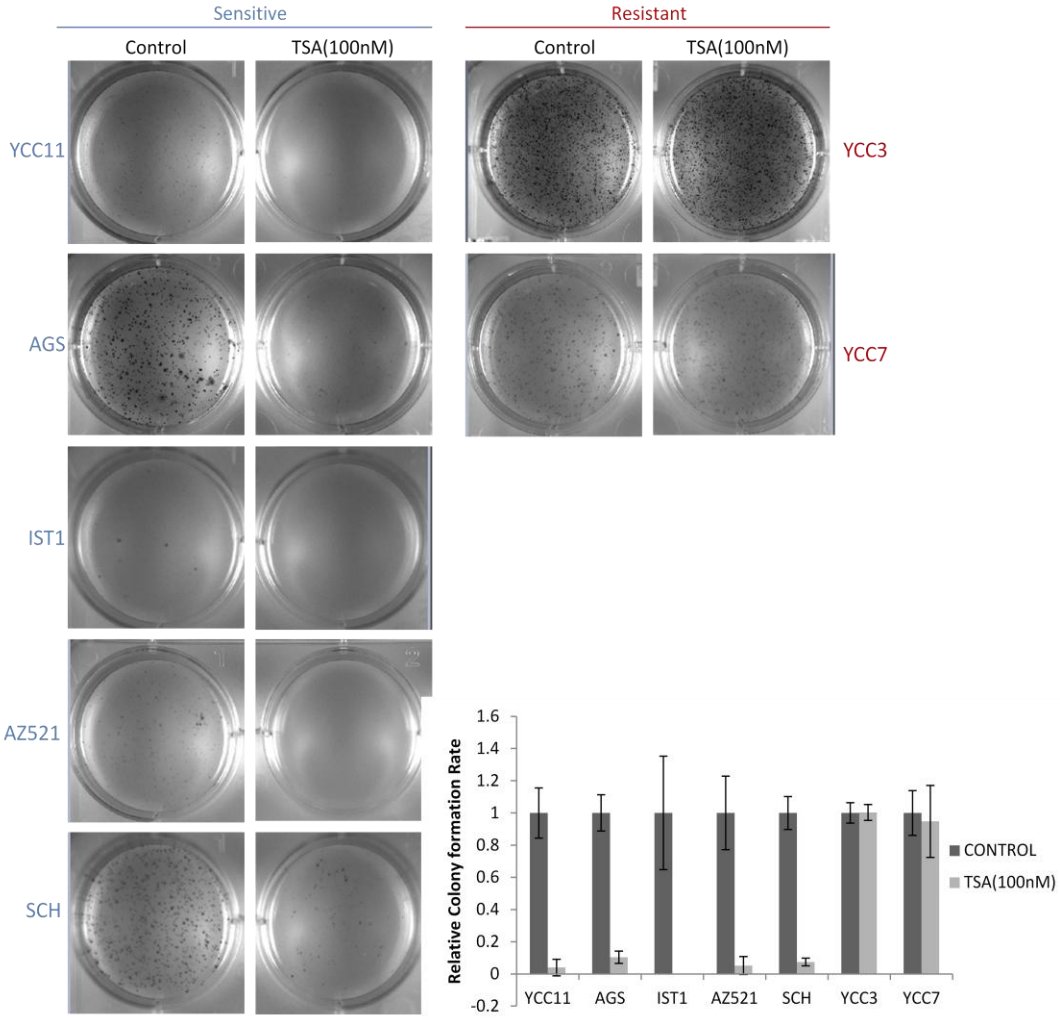


Figure 3.5 Colony formation assay of gastric cancer cell lines treated by TSA.

Potential sensitive gastric cancer lines were indicated by blue color, while resistant lines were indicated by red color. Sensitive lines exhibit decreased soft-agar colony formation after TSA treatment. Lines were compared after 100nM TSA treatment after 3 week

3.2 Sensitivity of 8 Gastric Cancer Cell Lines to Vorinostat (SAHA)

3.2.1 Growth Inhibition of 8 Gastric Cancer Cell Lines Induced by SAHA

To further determine if the cell line sensitivity and resistance profiles might be specific to TSA, or also extendable to other HDAC inhibitor compounds, MTS assays were performed on the 8 gastric cancer cell lines selected from TSA scanning under another hydroxamate HDAC inhibitor SAHA (0-10uM, 24 hours). SAHA is also a pan-HDAC inhibitor, belongs to the hydroxamate class which is similar to TSA and has been applied clinically. GI50s (drug concentration needed for 50% cell growth inhibition) were calculated to evaluate the growth inhibition of each cell lines induced by SAHA treatment. (Figure 3.6) The difference is significant between sensitive and resistant groups ($p<0.001$)

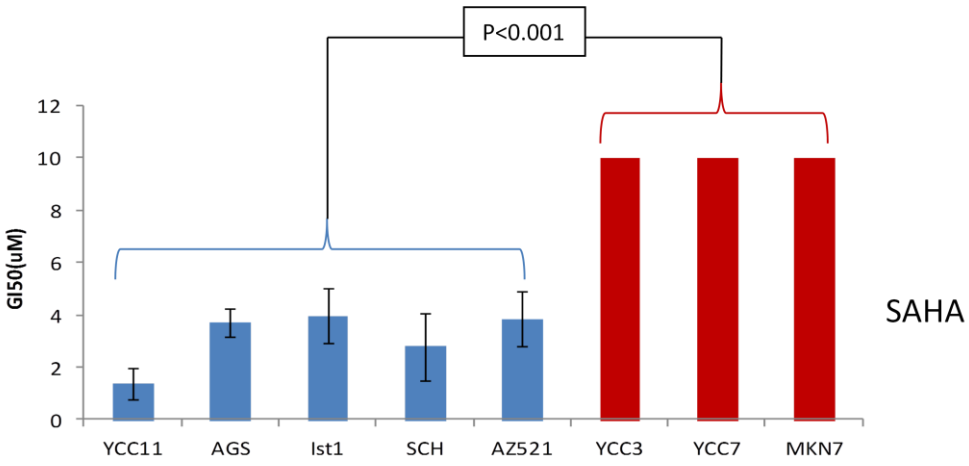


Figure 3.6 Sensitivity difference indicated by GI50 value between sensitive and resistant gastric cancer cell line groups under SAHA treatment.

The treatment concentration range is 0-10uM, and the treatment time is 24 hours. Y axle is the GI50 value. Triplicate experiments were performed for each cell lines and the average values were calculated and ranked.

3.2.2 Apoptosis of 8 Gastric Cancer Cell Lines Induced by SAHA

Western blot was performed to show the difference of cleaved PARP induced by SAHA treatment between potential sensitive and resistant gastric cancer cell line groups. All the 5 gastric cancer cell lines with lower GI50 value showed obvious increased PARP cleavage, while gastric cancer cell lines YCC3, YCC7 and MKN7 showed no obvious increased cleaved PARP induced by SAHA treatment.

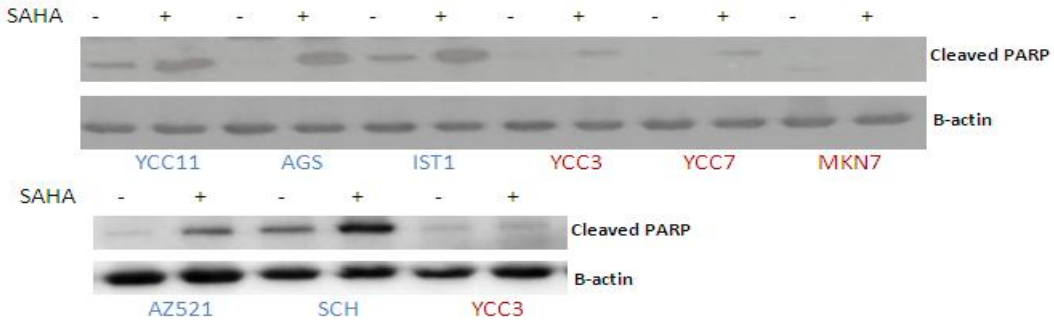


Figure 3.7 The difference of cleaved PARP induced by SAHA treatment between sensitive and resistant gastric cancer cell line groups.

All the gastric cancer cells were treated 5uM SAHA for 24 hours. Cells treated by same amount of DMSO were taken as negative control. Whole protein extracted from the cell pellets were sent to western blot

3.3 Sensitivity of 8 Gastric Cancer Cell Lines to entinostat (MS275)

3.3.1 Growth Inhibition of 8 Gastric Cancer Cell Lines Induced by MS275

MTS assay was also performed to the 8 gastric cancer cell lines selected under benzamide HDAC inhibitor MS275 treatment (0-50uM, 24 hours). MS275 belongs to the benzamide class which is a specific class I HDAC inhibitor. GI50 (drug concentration needed for 50% cell growth inhibition) was calculated to evaluate the growth inhibition of each cell lines induced by SAHA treatment. The difference is significant between sensitive and resistant groups ($p<0.001$).

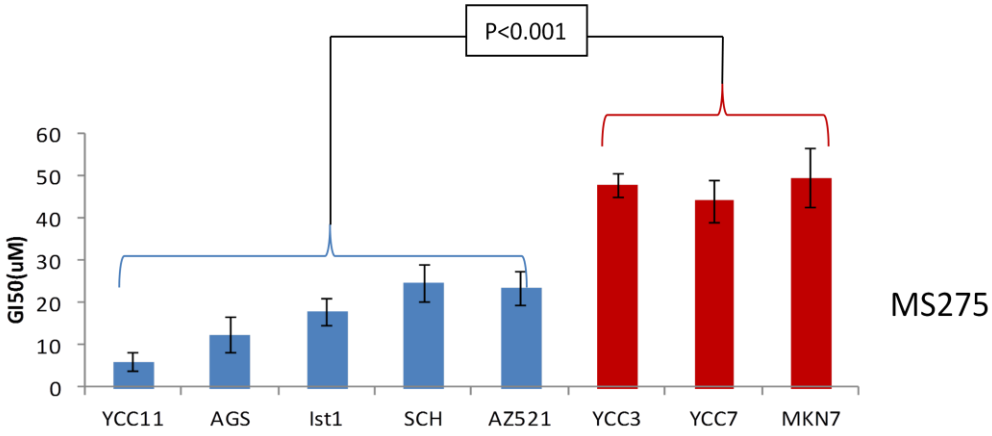


Figure 3.8 Sensitivity difference indicated by GI50 value between sensitive and resistant gastric cancer cell line groups under MS275 treatment.

The treatment concentration range is 0-50uM, and the treatment time is 24 hours. Y axle is the GI50 value. Triplicate experiments were performed for each cell lines and the average values were calculated and ranked.

3.3.2 Apoptosis of 8 Gastric Cancer Cell Lines Induced by MS275

Western blot was performed to show the difference of cleaved PARP induced by MS275 treatment between potential sensitive and resistant gastric cancer cell line groups. All the 5 gastric cancer cell lines with lower GI50 value showed obvious increased PARP cleavage, while gastric cancer cell lines YCC3, YCC7 and MKN7 showed no obvious increased cleaved PARP induced by TSA treatment.

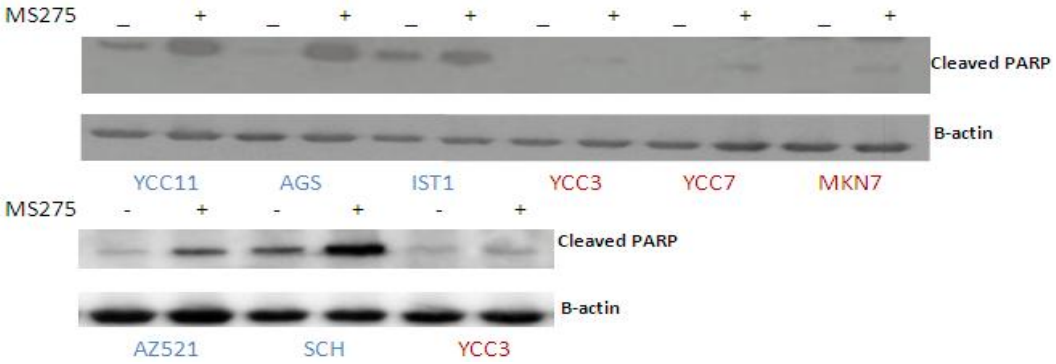


Figure 3.9 The difference of cleaved PARP induced by MS275 treatment between sensitive and resistant gastric cancer cell line groups.

All the gastric cancer cells were treated 30uM MS275 for 24 hours. Cells treated by same amount of DMSO were taken as negative control. Whole protein extracted from the cell pellets were sent to western blot.

3.4 Alterations in histone acetylation status after HDAC inhibitors treatment in gastric cancer cell lines

HDAC inhibitors are believed to act by inhibiting histone deactylase activity, resulting in enhancements of histone acetylation levels and increased gene expression (109). To assess if the TSA-sensitive and resistant lines might be associated with differences in their baseline or treatment-induced histone aceytlation profiles, we determined Histone 3 (lysine 9) and Histone 4 acetylation (lysine 12) levels in 3 sensitive and 3 resistant lines before and after HDAC inhibitor treatment. These experiments were performed at HDAC inhibitors concentrations sufficient to trigger a robust apoptotic response in sensitive lines. H3 and H4 acetylation levels were observed to be similarly increased in both sensitive and resistant cell line groups after HDAC inhibitors treatment. Additionally, *p21* up-regulation by HDAC inhibitor treatment has been widely observed in cancer cells (153, 154). Interestingly, we found that TSA treatment also induced p21 up-regulation in both sensitive and resistant GC cells (Figure 3.10 A). These results suggest that both histone acetylation enhancement and p21 induction are likely not sufficient to trigger growth arrest and apoptosis in gastric cancer cells. Additional factors may thus contribute to the cellular sensitivity of gastric cancer cells to HDAC inhibitors.

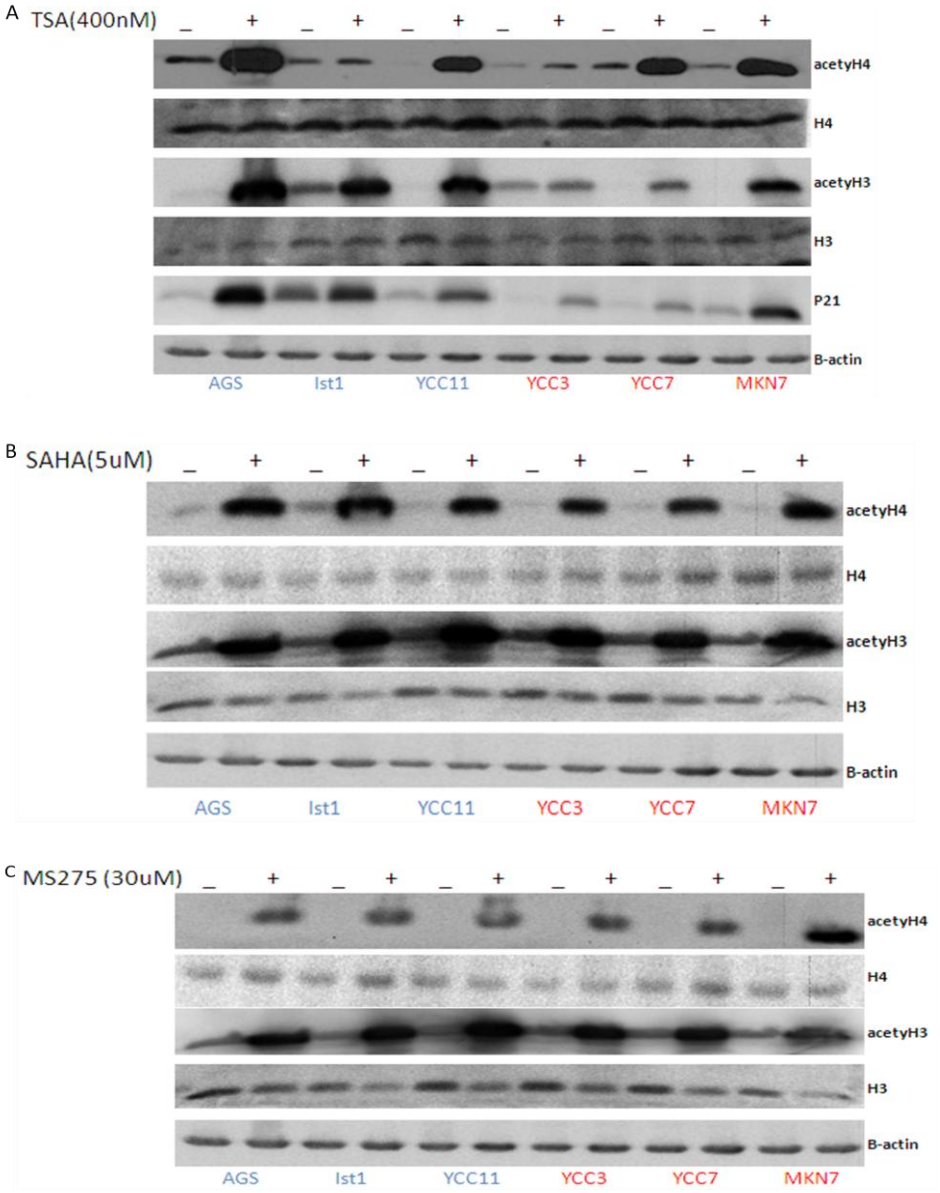


Figure 3.10 Alterations in histone acetylation status after HDAC inhibitors treatment in gastric cancer cell lines.

(A) Sensitive and resistant gastric cancer cell lines were treated by TSA (400nM, 24 hours), and Western blotting was performed to measure global levels of acetylated Histone 3 and 4 as well as unacetylated Histone 3 and 4. P21 protein up-regulation was also tested. (B) Sensitive and resistant gastric cancer cell lines treated by SAHA (5uM, 24 hours) (C) Sensitive and resistant gastric cancer cell lines treated by MS275 (30uM, 24 hours).

Chapter Four: Results Part II

4 Identify RNH1 Contributing to Histone Deacetylase Inhibitors Resistance in Gastric Cancer Cells

4.1 Deterimination of RNH1 as the Potential Gene Related to Histone Deacetylase Inhibitors Resistance in Gastric Cancer Cells

4.1.1 Genomic Analysis of Differently Expressed Genes between Sensitive and Resistant Gastric Cancer Cell Groups

To identify factors regulating the cellular sensitivity of gastric cancer cells to HDAC inhibitors, we examined genes differentially expressed between the sensitive and resistant lines. To reduce the possibility of false-positive results, we adopted an integrated genomic strategy where we combined gene expression profiles of the cell lines from two different commercially available microarray platforms (Affymetrix and Illumina), focusing on genes commonly identified by both platforms. . Through Partek software baseline gene expression level was compared between the two cell groups. On the Affymetrix platform, we identified 1231 genes differentially regulated between HDAC inhibitor sensitive and resistant lines ($p<0.05$; fold change >1.5) and 1165 genes on the Illumina platform at the same significance threshold, representing 514 genes commonly identified by both platforms. Among these genes, we then imposed a q-value (False Discovery

Rate) filter of <0.3 to select the top 7 candidates. These included *RNH1, STAT1, ELF, CXCL5, RAB40B, BLCAP* and *SGPP2* (Figure 4.1), which were all highly expressed in the resistant lines. Notably, among these 7 genes we identified *STAT1*, a gene previously associated with HDAC inhibitor resistance in colon cancer (121) - this re-discovery provides confidence in the robustness of our results.

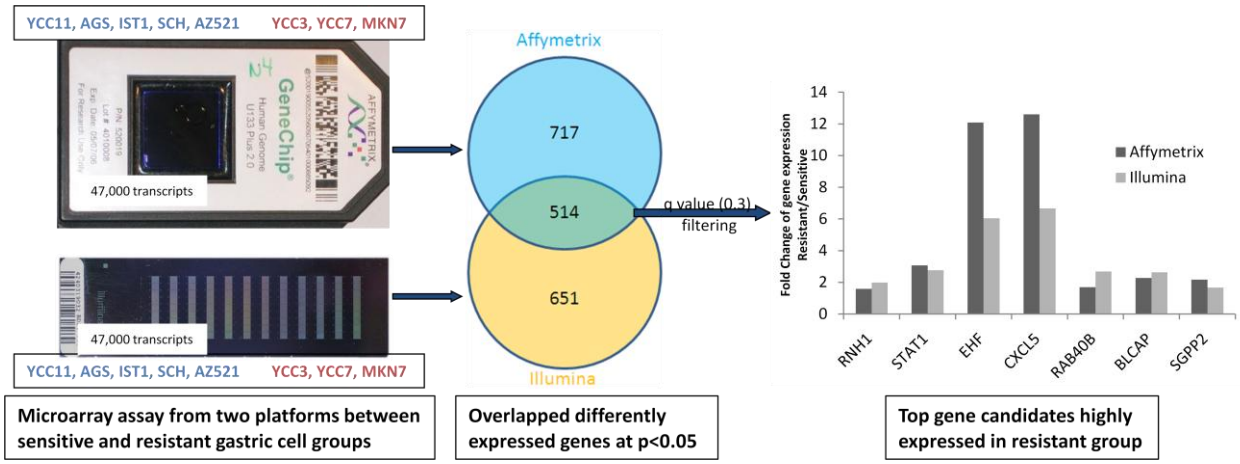
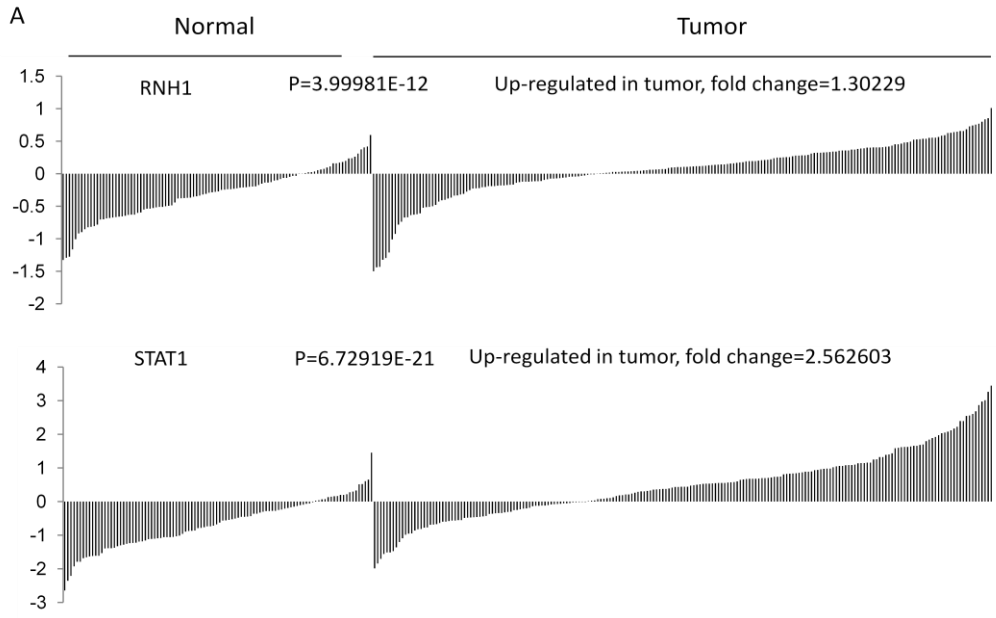


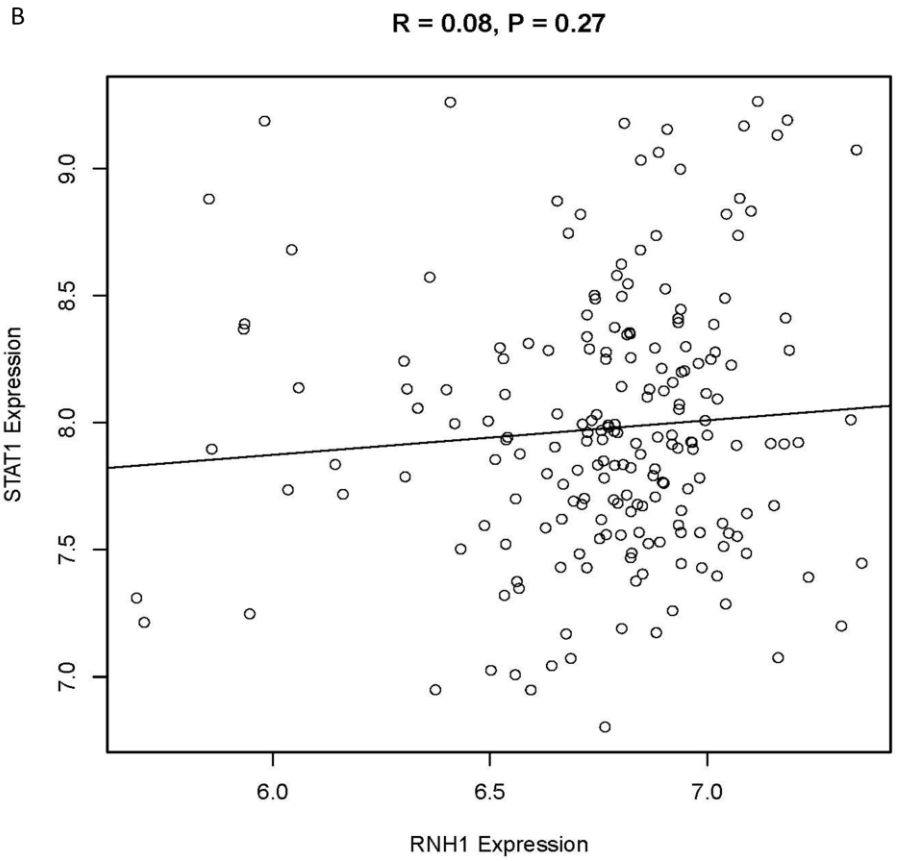
Figure 4.1 Identify differently expressed genes between gastric cancer sensitive and resistant groups.

The baseline gene expression data from both Affymetrix and Illumina platforms were normalized and the comparison was performed to identify the differently expressed genes between sensitive and resistant gastric cancer cell line groups. Around 400 genes in common were identified from both platforms ($p<0.05$). 7 top candidate genes were filtered out at False Discovery Rate (FDR) <0.3 through calculating q value.

4.1.2 Gene STAT1 and RNH1 expression in 300 Primary Gastric Tissue Samples of Singapore Cohort

In order to investigate the expression profile of gene STAT1 and RNH1 in primary gastric tissue to compare the results from cell lines, we analyzed the u133 plus microarray gene expression data from 300 primary gastric tissue samples of Singapore cohort including 100 normal tissue samples and 200 cancer tissue samples. We found that both STAT1 and RNH1 were more highly expressed in cancer tissues than in normal tissues. Setting 2 folds of normal tissue median as the threshold, we found that 27% (54/200) cancer tissue samples for gene STAT1 and 5% (10/200) cancer tissue samples for gene RNH1 showed higher expression. Through Pearson correlation analysis, we found there was no obvious correlation between STAT1 and RNH1 expression in gastric cancer tissues. We also performed gene silencing of STAT1 or RNH1 in two HDAC inhibitor resistant cell lines, YCC3 and MKN7. We found that silencing of these two genes could not affect protein expression of each other. The result suggested that STAT1 and RNH1 could influence HDAC inhibitor sensitivity in two independent path ways.


A
Normal
Tumor
RNH1
P=3.99981E-12
Up-regulated in tumor, fold change=1.30229
1.5
1
0.5
0
-0.5
-1
-1.5
-2
STAT1
P=6.72919E-21
Up-regulated in tumor, fold change=2.562603
4
3
2
1
0
-1
-2
-3


B
R = 0.08, P = 0.27
STAT1 Expression
9.0
8.5
8.0
7.5
7.0
6.0
6.5
7.0
RNH1 Expression

C

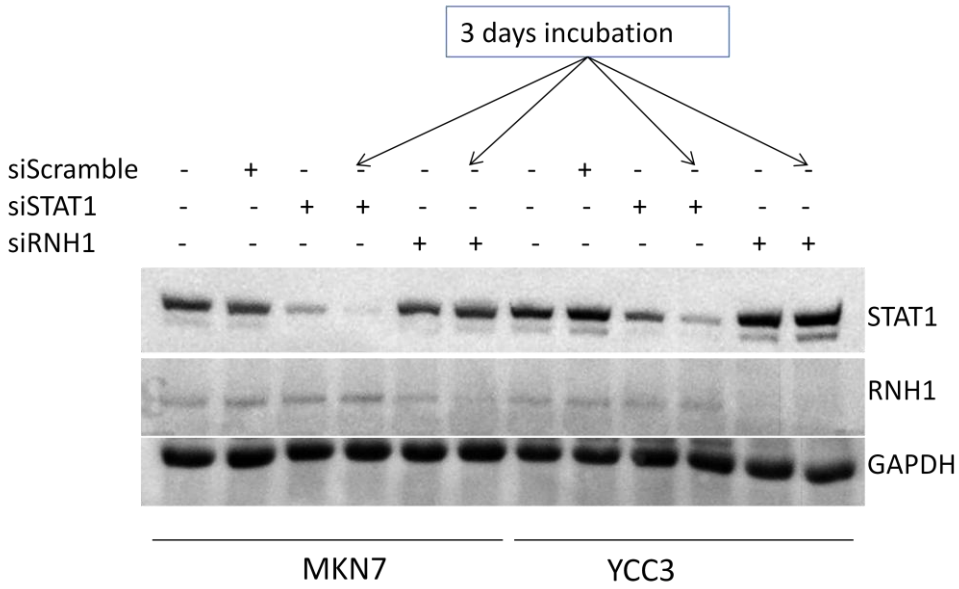


Figure 4.2 Gene expression profiles of STAT1 and RNH1 in 300 primary tissues of Singapore cohort and the correlation between STAT1 and RNH1

(A) Comparison of STAT1 and RNH1 gene expression between gastric normal and tumor tissue. U133 plus raw data were normalized using RMA package, and averaged by gene symbols. T-test was performed between normal and tumor tissue. (B) Correlation of gene expression between STAT1 and RNH1 in primary tissues. Pearson correlation test was performed between STAT1 and RNH1. (C) The effect of gene STAT1 or RNH1 knock-down to each other. STAT1 or RNH1 was silenced in two cell lines YCC3 and MKN7. Cells were collected to be sent to Western blot 24 hours or 72 hours after transfection.

4.1.3 Protein levels of the Top Candidate Genes

We then extended our results from gene expression to the protein level, due to the possibility of an imperfect correlation between gene and protein expression (155, 156). Western blotting assays using antibodies directed to these candidate genes revealed that two genes, *STAT1* and *RNH1* (colored with red in Figure 4.2), exhibited higher protein levels in HDAC inhibitor resistant lines compared to sensitive lines. Compared to STAT1, gene RNH1 showed more dramatic difference between the two cell groups. In contrast, similar protein expression trends were not observed for the other 4 candidates (high-quality antibodies were not available for BLCAP). We thus elected to focus on RNH1 for further characterization.

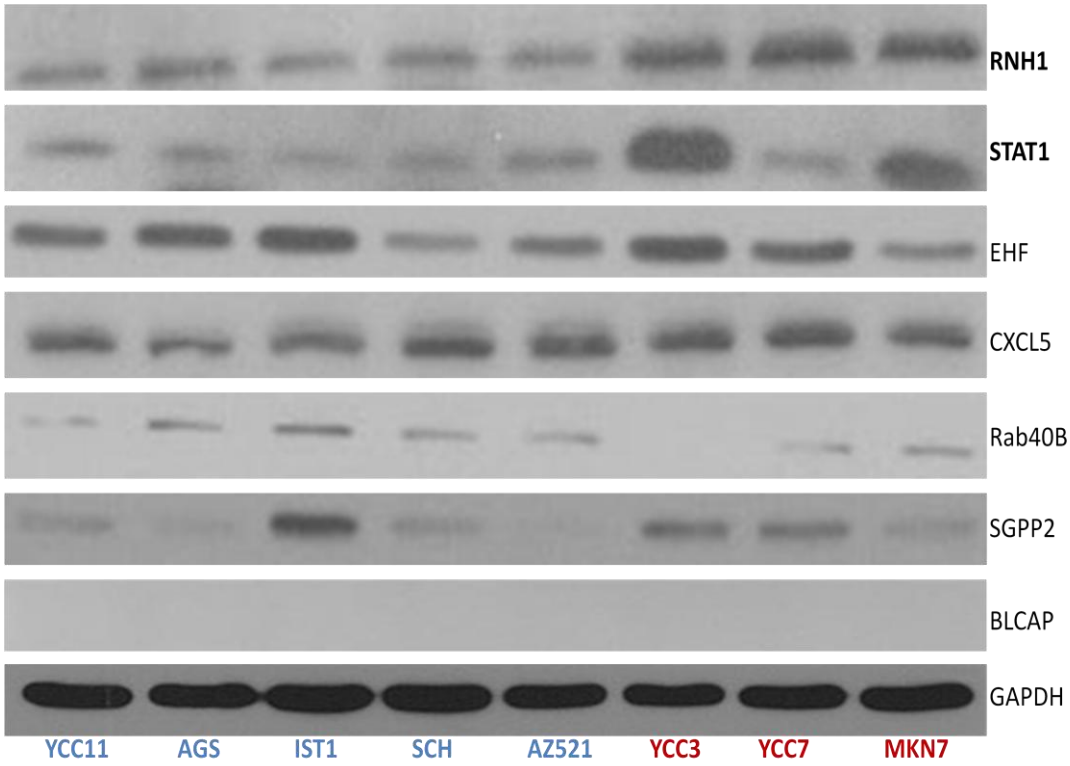


Figure 4.3 Protein levels of the filtered genes in the 8 gastric cancer cell lines.

Western blot was performed to show the protein level of 8 candidate genes in the 8 gastric cancer cell lines.

4.1.4 RNH1 Gene Highly Expressed in HDAC inhibitor-resistance Gastric Cancer Cells

Since the different protein levels of gene RNH1 implies a possible relationship in HDAC inhibitor resistance from selected 8 gastric cancer cell lines, it would be interesting to know if the protein status of RNH1 in other gastric cancer cell lines which are relatively sensitive to HDAC inhibitor treatment. To examine patterns of RNH1 expression in gastric cell lines beyond the original 8 lines used in the microarray analysis, we performed RNH1 Western blotting on an additional 6 gastric lines identified as being sensitive to HDAC inhibitors together with AZ521 and YCC3 as negative and positive control respectively. With the exception of NUGC3 cells, the other 5 lines exhibited lower levels of RNH1 protein expression compared to HDAC inhibitors resistant YCC3 cells (Figure 4.3). These results thus suggest that levels of RNH1 protein expression are negatively correlated to HDAC inhibitor sensitivity in gastric cancer.

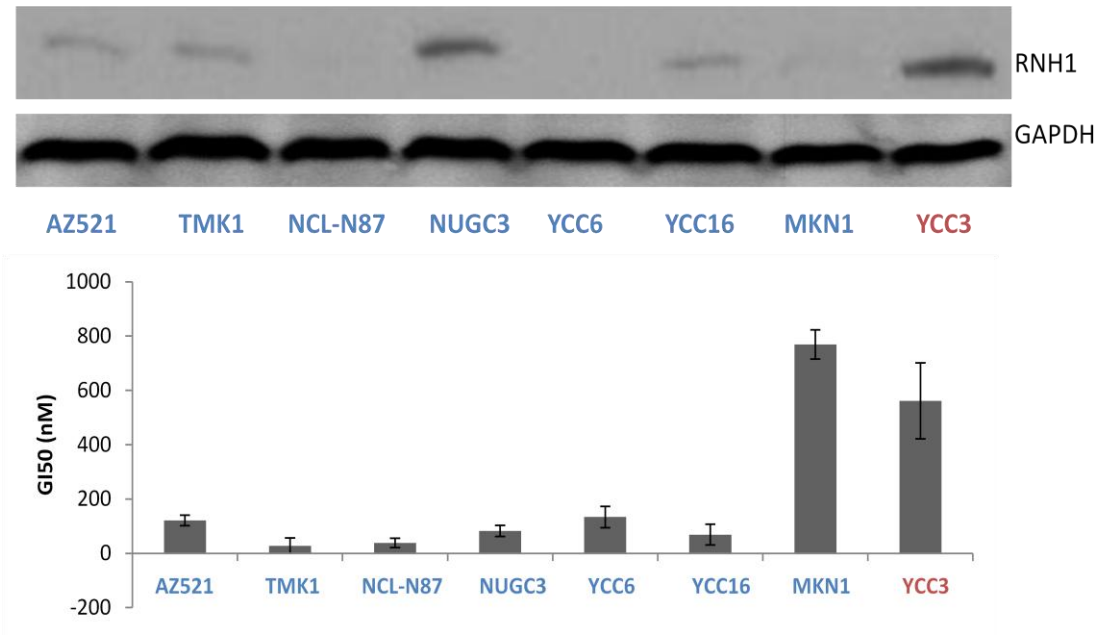


Figure 4.4 The protein level of RNH1 in other gastric cancer cell lines which were relatively sensitive to HDAC inhibitors.

Upper figure showed the protein level of RNH1 of other 7 HDAC inhibitor sensitive gastric cancer cell lines compared to YCC3 cells. Lower figure showed the corresponding GI50 values of each cell lines

4.1.5 RNH1 Protein Level of Gastric Cancer Cells Remain Steady after TSA Treatment

To investigate if HDAC inhibitors could alter RNH1 protein levels in gastric cancer cells either sensitive or resistant to HDAC inhibitor, we treated several gastric cancer cell lines with 400nM TSA for 24 hours. The results showed that TSA treatment could not alter RNH1 protein expression in either HDAC inhibitor sensitive or resistant cell lines. The steady characteristics of RNH1 under HDAC inhibitor treatment supports a functional role of RNH1 in HDAC inhibitor resistance.

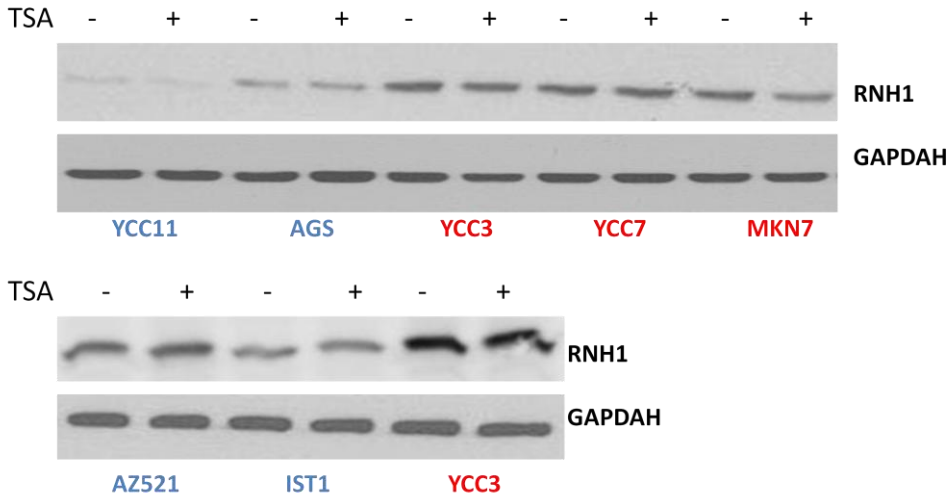


Figure 4.5 the protein level of RNH1 of gastric cancer cells before and after TSA treatment

Both HDAC inhibitor sensitive or resistant gastric cancer cell lines were treated by 400nM TSA for 24 hours. Cytoplasmic protein was extracted and sent to Western blot assay.

4.2 Deregulation of RNH1 Affects Gastric Cancer Cells Sensitivity to TSA

4.2.1 Knock-down of RNH1 Sensitizes Gastric Cancer Cells to TSA Treatment

To investigate the functional role of *RNH1* on gastric cancer HDAC inhibitor sensitivity, we conducted genetic knockdown experiments. Stable knock-down of *RNH1* was confirmed by qPCR and Western blotting analysis, using two distinct and non-overlapping RNA1 shRNAs in two HDAC inhibitor resistant cell lines, YCC3 and YCC7 (Figure 4.4). Compared to control cells, *RNH1*-silenced cells tended to exhibit decreased growth rates (Figure 4.5Figure) and exhibited significantly lower TSA GI50 concentrations (3-4 fold) in both lines (Figure 4.6A and Figure 4.7A) suggesting that silencing *RNH1* can sensitize gastric cancer cells to TSA treatment. To confirm these findings by other cellular indices, we then conducted cell death and colony formation assays. As shown in Figure 4.6B and Figure 4.7B, *RNH1*-silenced cells YCC3 and YCC7 cells exhibited increased PARP cleavage upon TSA treatment. Moreover, *RNH1*-silenced cells also exhibited decreased colony formation capacities after TSA treatment, by about 2-4 fold (Figure 4.6C and Figure 4.7C). Similar results were observed when TSA was replaced by SAHA or MS275, two other HDAC inhibitor compounds (Figure 4.6 and Figure 4.7). Taken collectively, these results observed in two independent HDAC inhibitor resistant gastric lines suggests that inhibiting *RNH1* can sensitize gastric cancer cells to HDAC inhibitor treatment.

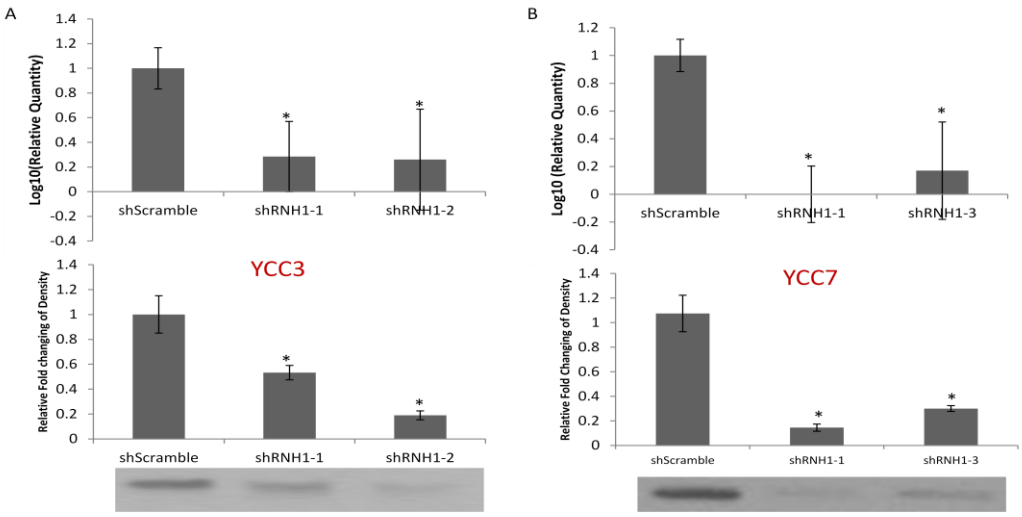


Figure 4.6 Quantification of RNH1 deregulation in gastric cancer cells

(A) Quantitative real-time PCR analysis (upper figure) and quantified western blotting signals (lower figure) of YCC3 cells before and after *RNH1* silencing. * indicates $p<0.05$ between YCC3 cells transfected with a scramble shRNA control and cells transfected with shRNH1-1 or shRNH1-2 (B) Quantitative real-time PCR analysis (upper figure) and quantified western blotting signals (lower figure) of YCC7 cells before and after *RNH1* silencing. * indicates $p<0.05$ between YCC7 cells transfected with a scramble shRNA control and cells transfected with shRNH1-1 or shRNH1-3.

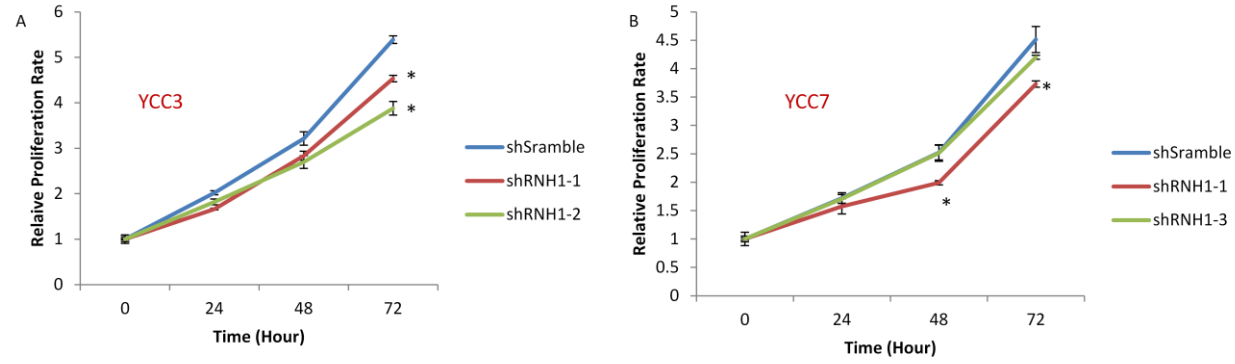


Figure 4.7 Effect of *RNH1* silencing on gastric cancer cell proliferation.

(A) Proliferation assays of YCC3 cells after *RNH1* silencing. * indicates $p<0.05$ between YCC3 cells transfected with a scramble shRNA control and cells transfected with shRNH1-1 or shRNH1-2. (B) Proliferation assays of YCC7 cells after RNH1 silencing. * indicates $p<0.05$ between YCC7 cells transfected with a scramble shRNA control and cells transfected with shRNH1-1 or shRNH1-3

YCC3

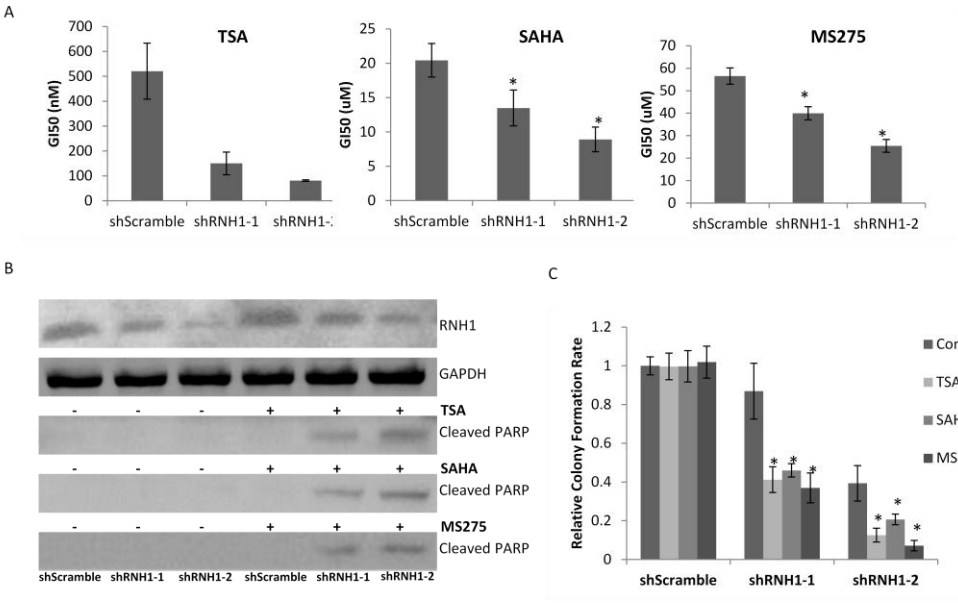

A
TSA
SAHA
MS275
GI50 (nM)
GI50 (uM)
GI50 (uM)
shScramble
shRNH1-1
shRNH1-2
B
RNH1
GAPDH
TSA
Cleaved PARP
SAHA
Cleaved PARP
MS275
Cleaved PARP
C
Relative Colony Formation Rate
Control
TSA
SAHA
MS275
D
shScramble
shRNH1-1
shRNH1-2
Control
TSA
SAHA
MS275

Figure 4.8 Genetic inhibition of *RNH1* sensitizes HDAC inhibitors-resistant cells line YCC3.

(A) Proliferation assays comparing GI50 values of wild-type YCC3 cells with YCC3 cells where *RNH1* was silenced using two independent *RNH1* shRNAs (shRNH1-1, shRNH1-2) or a scrambled shRNA control. *RNH1*-silenced cells exhibited significantly lower GI50 values for TSA, SAHA and MS275, compared to control cells. * $p<0.001$ between scramble shRNA treated and shRNH1-1 or shRNH1-2 treated cells. (B) Apoptosis assays comparing wild-type YCC3 cells with *RNH1*-silenced YCC3 cells before and after HDAC inhibitor treatment. Western blots were performed to monitor PARP cleavage before and after TSA (400nM, 24 hours), SAHA (10uM, 24 hours) or MS275 treatment (30uM, 24 hours). (C) Colony formation assays comparing YCC3 cells with and without stable *RNH1* silencing after HDAC inhibitor treatment. HDAC inhibitor-treated but *RNH1*-silenced YCC3 cells exhibited significant lower levels of colony formation after TSA (100nM, 3 weeks), SAHA (1uM, 3 weeks) and MS275 treatment (5uM, 3 weeks) compared to HDAC inhibitor-treated control YCC3 cells. * indicates $p<0.001$ between control and HDAC inhibitor treated cells. (D) The colony image of YCC3 cells with or without RNH1 silencing before or after TSA, SAHA or MS275 treatment.

YCC7

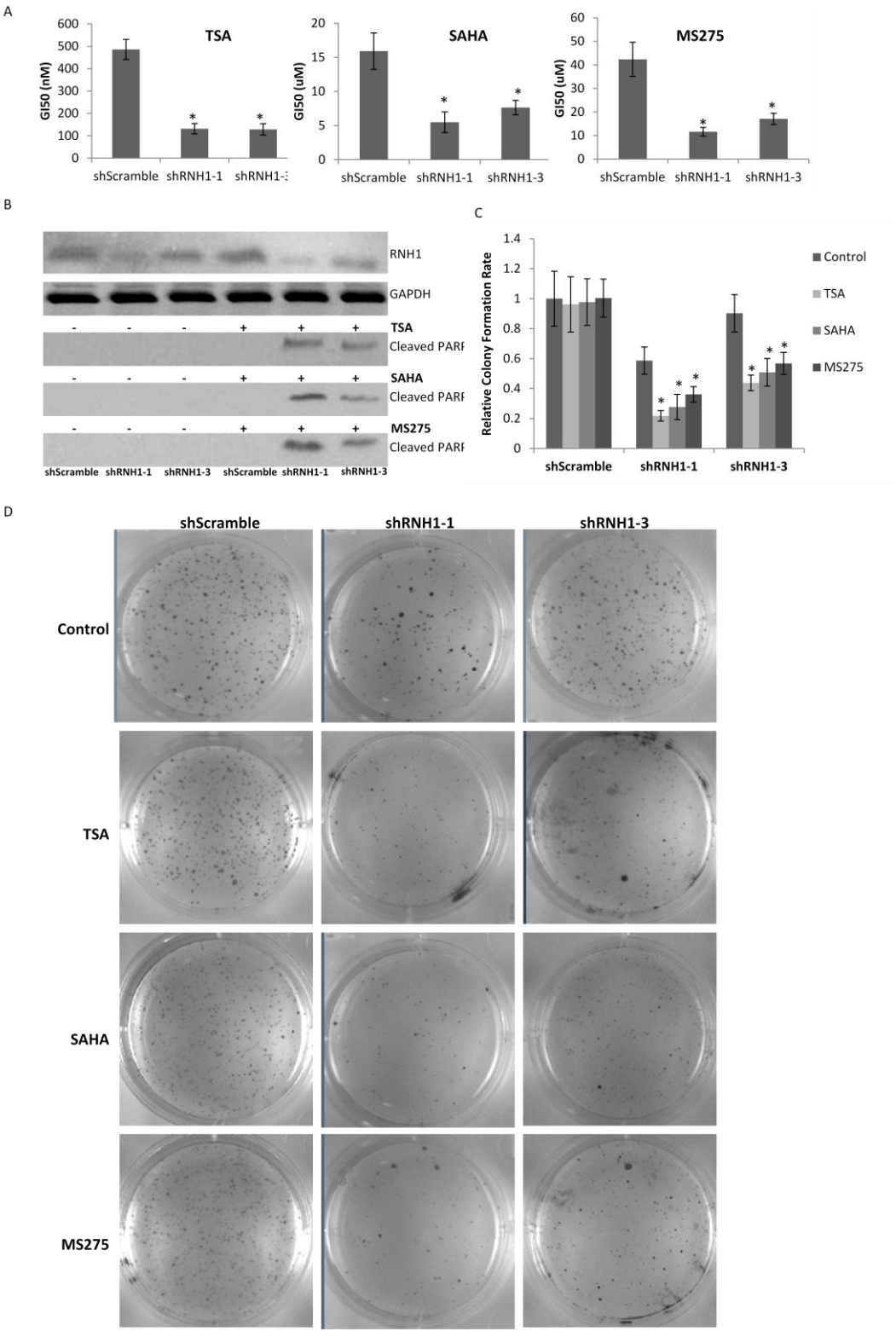

A
TSA
SAHA
MS275
GI50 (nM)
GI50 (uM)
shScramble
shRNH1-1
shRNH1-3
B
RNH1
GAPDH
TSA
Cleaved PARP
SAHA
Cleaved PARP
MS275
Cleaved PARP
C
Relative Colony Formation Rate
Control
TSA
SAHA
MS275
D
shScramble
shRNH1-1
shRNH1-3
Control
TSA
SAHA
MS275

Figure 4.9 Genetic inhibition of *RNH1* sensitizes HDAC inhibitors-resistant cells line YCC7.

(A) Proliferation assays comparing GI50 values of wild-type YCC7 cells with YCC7 cells where *RNH1* was silenced using two independent *RNH1* shRNAs (shRNH1-1, shRNH1-3) or a scrambled shRNA control. *RNH1*-silenced cells exhibited significantly lower GI50 values for TSA, SAHA and MS275, compared to control cells. * $p<0.001$ between scramble shRNA treated and shRNH1-1 or shRNH1-3 treated cells. (B) Apoptosis assays comparing wild-type YCC7 cells with *RNH1*-silenced YCC7 cells before and after HDAC inhibitor treatment. Western blots were performed to monitor PARP cleavage before and after TSA (400nM, 24 hours), SAHA (10uM, 24 hours) or MS275 treatment (30uM, 24 hours). (C) Colony formation assays comparing YCC7 cells with and without stable *RNH1* silencing after HDAC inhibitor treatment. HDAC inhibitor-treated but *RNH1*-silenced YCC7 cells exhibited significant lower levels of colony formation after TSA (100nM, 3 weeks), SAHA (1uM, 3 weeks) and MS275 treatment (5uM, 3 weeks) compared to HDAC inhibitor-treated control YCC7 cells. * indicates $p<0.001$ between control and HDAC inhibitor treated cells. (D) The colony image of YCC7 cells with or without RNH1 silencing before or after TSA, SAHA or MS275 treatment.

4.2.2 Over-expression of RNH1 Enhances Gastric Cancer Cells Resistance to TSA

We then performed the reciprocal experiment by over-expressing wild-type *RNH1* in the HDAC inhibitor sensitive lines AZ521 and YCC11. Ectopic overexpression of RNH1 in these cell lines was confirmed to be within physiological parameters (Figure 4.8), and associated with decreased growth (Figure 4.9). As shown in Figure 4.10 and Figure 4.11, over-expression of wild-type *RNH1* in these lines significantly increased their TSA GI50 values, attenuated production of cleaved PARP, and decreased the effects on colony formation inhibition by TSA. Similar results were observed when TSA was replaced by SAHA or MS275, two other HDAC inhibitor compounds (Figure 4.10 and Figure 4.11). These results provide further evidence that high levels of *RNH1* are sufficient to render gastric cancer cells resistant to HDAC inhibitor therapy.

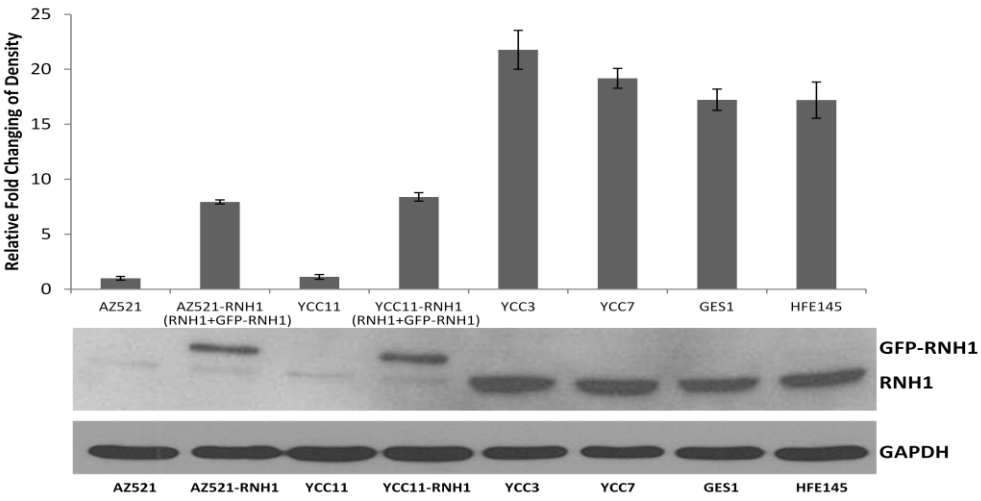


Figure 4.10 Comparison of RNH1 levels among gastric cancer cell lines and normal gastric epithelial cell lines.

RNH1 Western blotting was performed on AZ521 and YCC11 cells before and after *RNH1* over-expression, YCC3, YCC7, HFE145 and GES1 cells. Quantification of Western blotting data was performed using Image J software (http://rsb.info.nih.gov/ij/).

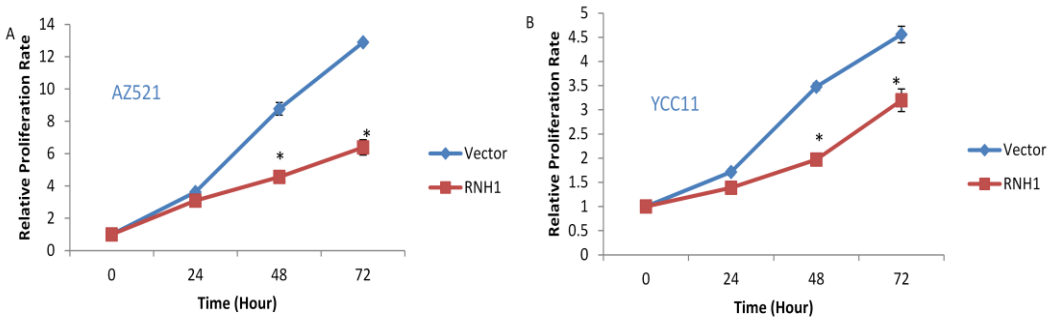


Figure 4.11 Effect of *RNH1* overexpression on gastric cancer cell proliferation.

(A) Proliferation assays of AZ521 cells after RNH1 over-expression. * indicates $p<0.05$ between AZ521 cells over-expressing a RNH1 cDNA to an empty vector control (B) Proliferation assays of YCC11 cells after RNH1 over-expression. * indicates $p<0.05$ between YCC11 cells over-expressing a RNH1 cDNA to an empty vector control.

AZ521

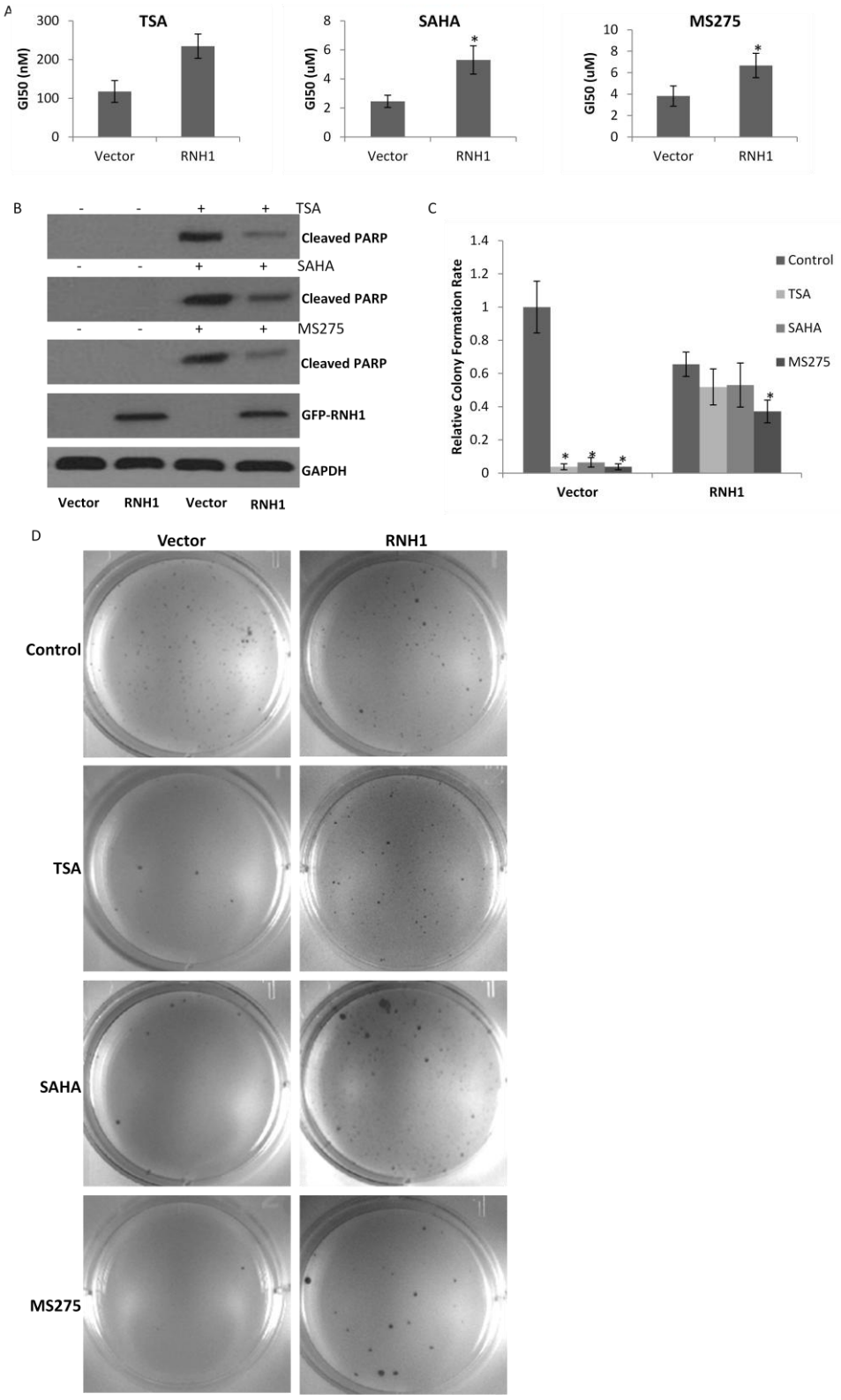

A
TSA
SAHA
MS275
GI50 (nM)
GI50 (uM)
Vector
RNH1
B
TSA
SAHA
MS275
Cleaved PARP
GFP-RNH1
GAPDH
Vector
RNH1
C
Relative Colony Formation Rate
Control
TSA
SAHA
MS275
D
Control
TSA
SAHA
MS275

Figure 4.12 *RNH1* over-expression renders HDAC inhibitor-sensitive cell line AZ521 resistant to HDAC inhibitor treatment.

(A) Proliferation assays comparing GI50 values of parental AZ521 cells to AZ521 cells over-expressing *RNH1* or empty vector transfected controls. *RNH1*-overexpressing AZ521 cells exhibited significantly increased GI50 values for TSA, SAHA and MS275 compared to control cells. * indicates $p<0.001$ between *RNH1*-overexpressing AZ521 and empty vector control cells. (B) Apoptosis assays comparing *RNH1*-overexpressing AZ521 cells to empty vector controls before and after HDAC inhibitor treatment. Western blots were performed to monitor PARP cleavage before and after TSA (200nM, 24 hours), SAHA (5uM, 24 hours) or MS275 treatment (5uM, 24 hours). (C) Colony formation assays comparing *RNH1*-overexpressing AZ521 cells to empty vector control cells after HDAC inhibitor treatment. HDAC inhibitor-treated *RNH1*-overexpressing AZ521 cells exhibited significant more colony formation after TSA (100nM, 3 weeks), SAHA (1uM, 3 weeks) and MS275 treatment (1uM, 3 weeks) compared to HDAC inhibitor-treated control AZ521 cells. * indicates $p< 0.001$ between control and HDAC inhibitor treated cells. (D) The colony image of AZ521 cells with or without RNH1 overexpression before or after TSA, SAHA or MS275 treatment.

YCC11

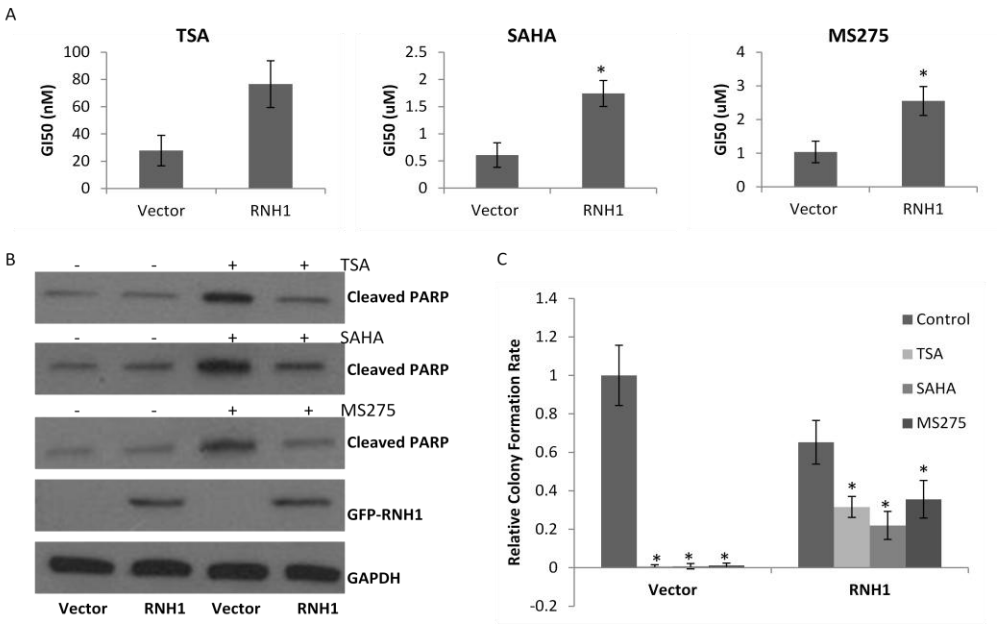

A
TSA
SAHA
MS275
GI50 (nM)
GI50 (uM)
Vector
RNH1
B
TSA
SAHA
MS275
Cleaved PARP
GFP-RNH1
GAPDH
Vector
RNH1
C
Relative Colony Formation Rate
Control
TSA
SAHA
MS275
D
Vector
RNH1
Control
TSA
SAHA
MS275

Figure 4.13 *RNH1* over-expression renders HDAC inhibitor-sensitive cell line YCC11 resistant to HDAC inhibitor treatment.

(A) Proliferation assays comparing GI50 values of parental YCC11 cells to YCC11 cells over-expressing *RNH1* or empty vector transfected controls. *RNH1*-overexpressing YCC11 cells exhibited significantly increased GI50 values for TSA, SAHA and MS275 compared to control cells. * indicates $p<0.001$ between *RNH1*-overexpressing YCC11 and empty vector control cells. (B) Apoptosis assays comparing *RNH1*-overexpressing YCC11 cells to empty vector controls before and after HDAC inhibitor treatment. Western blots were performed to monitor PARP cleavage before and after TSA (200nM, 24 hours), SAHA (5uM, 24 hours) or MS275 treatment (5uM, 24 hours). (C) Colony formation assays comparing *RNH1*-overexpressing YCC11 cells to empty vector control cells after HDAC inhibitor treatment. HDAC inhibitor-treated *RNH1*-overexpressing YCC11 cells exhibited significant more colony formation after TSA (100nM, 3 weeks), SAHA (1uM, 3 weeks) and MS275 treatment (1uM, 3 weeks) compared to HDAC inhibitor-treated control YCC11 cells. * indicates $p< 0.001$ between control and HDAC inhibitor treated cells. (D) The colony image of YCC11 cells with or without RNH1 overexpression before or after TSA, SAHA or MS275 treatment.

4.3 HDAC inhibitor-induced Reactive Oxygen Species (ROS) Production Involved in Gastric Cancer Cell Resistance Contributed by RNH1

4.3.1 TSA Induces Higher ROS Production in HDAC inhibitor Sensitive Gastric Cancer cells

RNH1 encodes a ribonuclease inhibitor protein which is mainly distributed in the cytoplasm (143, 144). Proposed biological roles of RNH1 include modulating the biological activities of cellular ribonucleases and as an oxidative sensor to monitor the redox status of a cell (150-152). Because ROS production has been proposed to play an important role in HDAC inhibitor-induced cancer cell death (133), we hypothesized that RNH1 might contribute to HDAC inhibitor resistance by suppressing drug-induced ROS production in gastric cancer cells. To investigate this possibility, we first measured levels of drug-induced ROS in both the sensitive and resistant gastric cell lines, at early point-treatment time points. As shown in Figure 4.12, TSA induced higher ROS levels in HDAC inhibitor sensitive lines, compared to resistant lines ($p<0.01$). The average level of ROS production in the sensitive group was about 5-fold higher than the resistant group, with the exception of SCH cells which exhibited a relatively low level of ROS production. This result suggests that gastric cancer sensitivity to HDAC inhibitor treatment is associated with their ability of cells to produce ROS upon drug exposure.

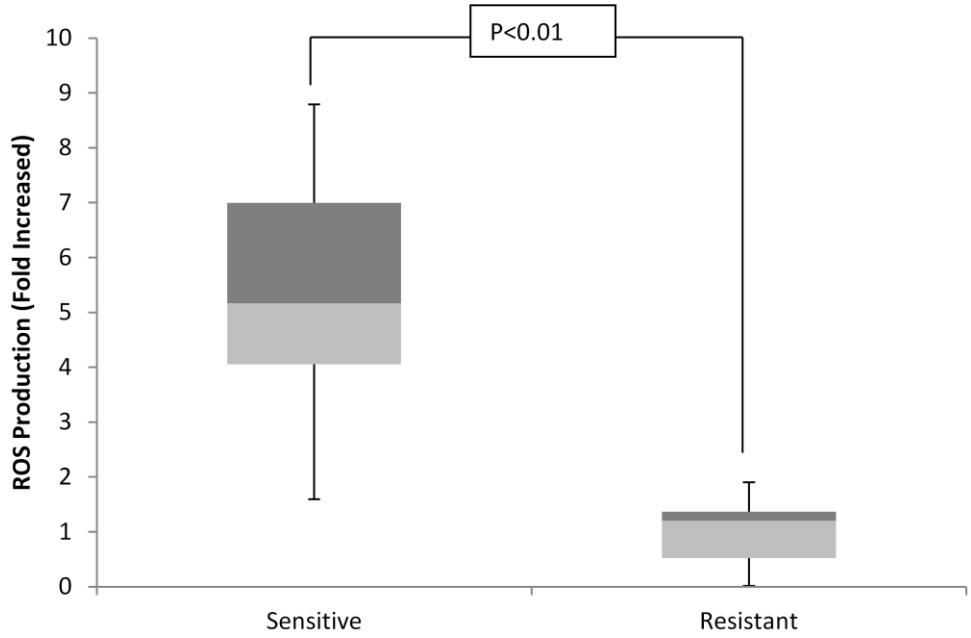


Figure 4.14 Comparison of ROS production after TSA treatment between HDAC inhibitor sensitive and resistant gastric cancer cell line groups.

TSA-inducing ROS production was measured using CellRox ROS detection reagent in the 8 selected gastric cancer cell lines. Middle bar = median, box = inter-quartile range, and bars extend to 1.5x the inter-quartile range.

4.3.2 Deregulation of RNH1 Affects ROS Production Induced by TSA in Gastric Cancer cells

To explore the role of *RNH1* in ROS production induced by HDAC inhibitor treatment, we then compared drug-induced ROS levels between *RNH1*-silenced YCC3 cells and wild-type YCC3 cells, and *RNH1* over-expressing AZ521 cells to parental AZ521 cells. *RNH1*-silenced YCC3 cells produced significantly higher levels of ROS upon HDAC inhibitor treatment, by about 10-17 fold ($p<0.001$) compared to control cells (Figure 4.13). Conversely, *RNH1*-overexpressing AZ521 cells exhibited significantly decreased drug-induced ROS production compared to parental cells ($p<0.01$; ~2-fold) (Figure 4.13). Similar results were observed in both *RNH1*-silenced YCC7 cells (ROS levels increased ~2 fold) and also in RNH1-overexpressing YCC11 cells (ROS levels decreased ~5-fold) (Figure 4.13). These results indicate that the levels of cellular RNH1 likely represent an important determinant in the ability of gastric cancer cells to produce ROS in response to HDAC inhibitor therapy.

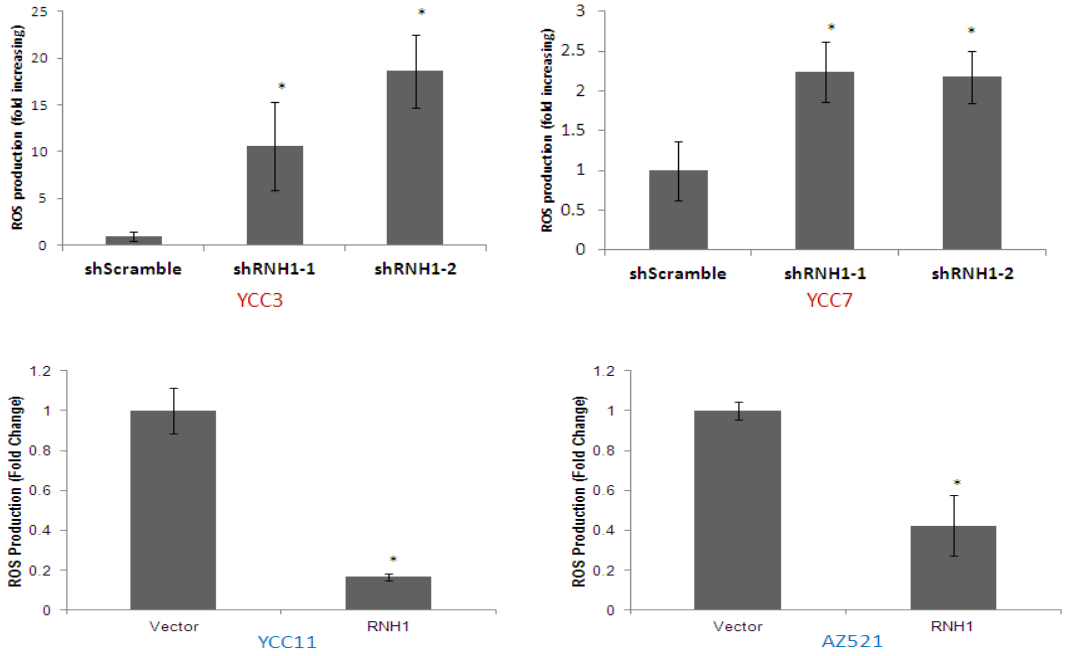


Figure 4.15 Genetic manipulation of *RNH1* levels is sufficient to alter TSA-induced ROS production.

ROS levels were compared between YCC3 or YCC7 and *RNH1*-silenced YCC3 or YCC7 cells, and between AZ521 or YCC11 and *RNH1*-overexpressing AZ521 or YCC11 cells. TSA-induced ROS production was enhanced in *RNH1* silenced cells, and decreased in *RNH1*-overexpressing cells. * indicates $p<0.001$.

4.3.3 ROS Regulators Influence Gastric Cancer Cells Sensitivity to TSA

4.3.3.1 ROS Inducer Enhances the Gastric Cancer Cell Sensitivity to TSA Treatment

To ask if drug-induced ROS might be necessary for HDACi-induced cancer cell death, we then exposed gastric cancer cells to two compounds known to alter cellular ROS levels - β-phenethyl isothiocyanate (PEITC), which is known to increase ROS levels (157), and L-glutathione (GSH), which is known to scavenge and reduce ROS (158). Interestingly, PEITC has been previously reported to enhance the cytotoxicity of HDAC inhibitors in leukemia (142). As shown in Figure 4.14, PEITC co-treatment (5uM) enhanced the ability of TSA to inhibit proliferation in both YCC3 and YCC7 cells (Figure 4.14 A), as well as in *RNH1*-overexpressing AZ521 and YCC11 cells (Figure 4.14 B), at TSA concentrations that would not normally affect cellular proliferation in these cells (200nM TSA). The PEITC/TSA combination also effectively induced much higher ROS production compared to the monotherapy-treated lines.

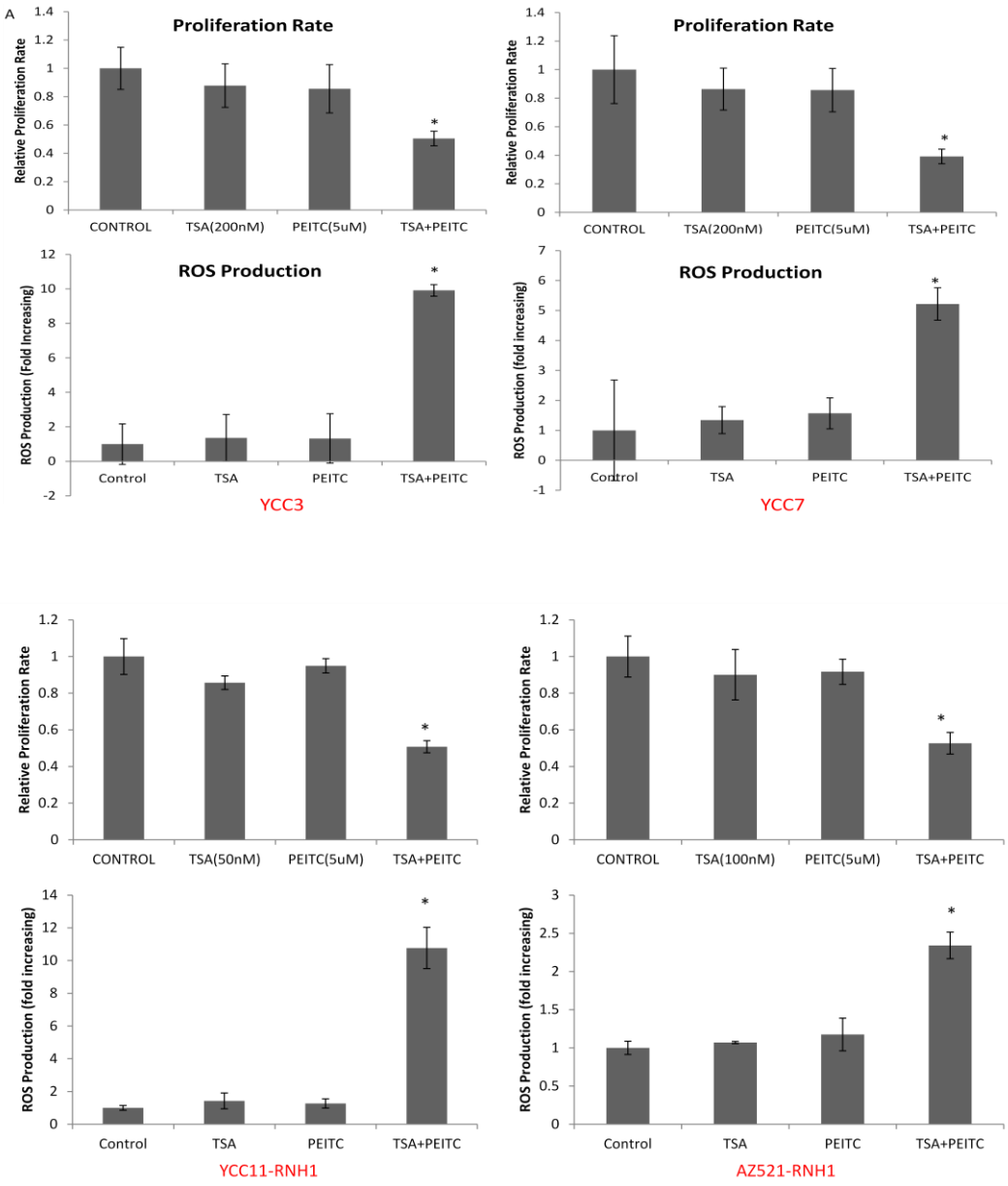
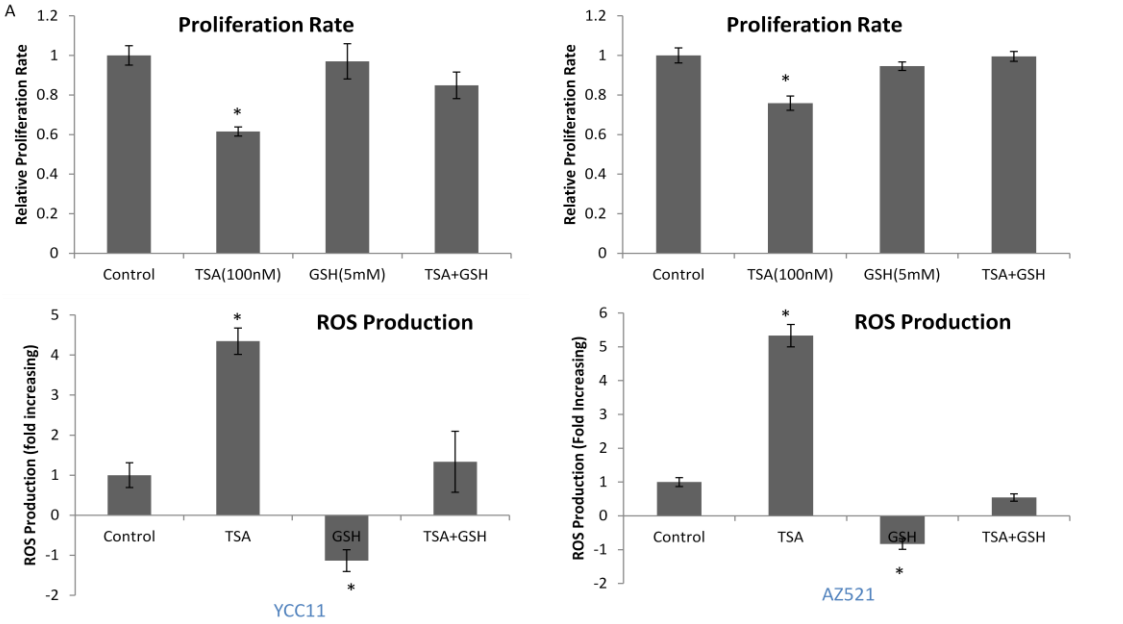


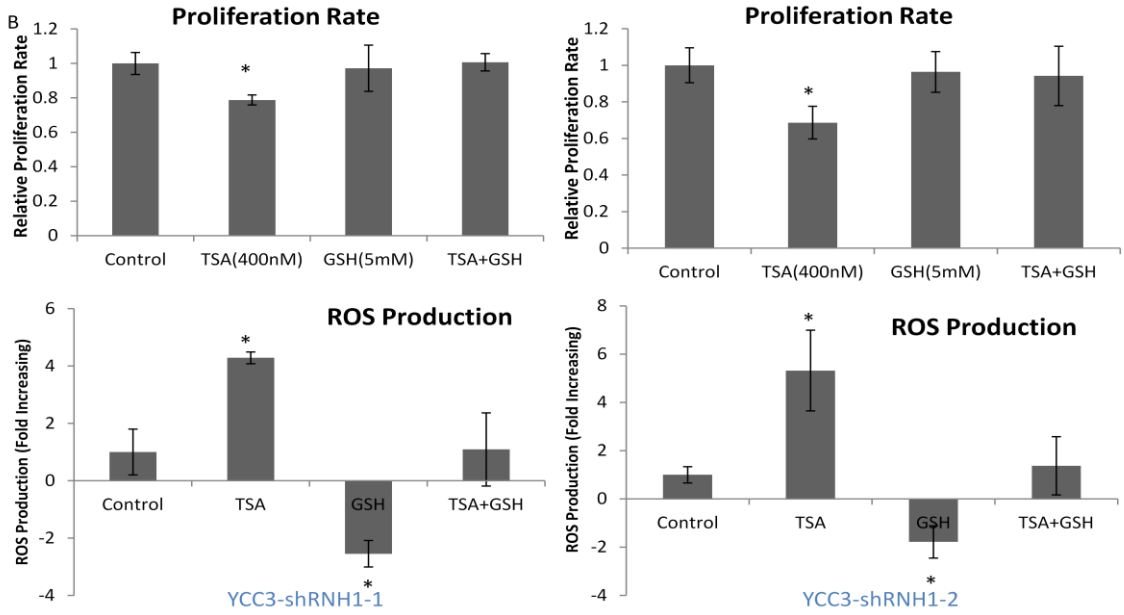
Figure 4.16 TSA/PEITC-treated cells were observed to show significant decreases in cellular proliferation and also significant inductions in ROS levels.

PEITC Resistant cells (YCC3 and YCC7 on top, *RNH1* over-expressing AZ521 and YCC11 cells on the bottom) were treated with TSA, the ROS β-phenethyl isothiocyanate (PEITC), or a TSA/PEITC combination (same concentration as monotherapy). (Top graphs) Proliferation inhibition induced by each treatment. (Bottom graphs) ROS levels induced by each treatment group. * indicates $p<0.01$ between control and TSA/PEITC treated cells

4.3.3.2 ROS Scavenger Enhances the Gastric Cancer Cell Resistance to TSA Treatment

Conversely, we found that pre-incubating cells with GSH rendered them resistant to HDAC inhibitor induced cell death. Specifically, GSH pre-treatment rescued AZ521 or YCC11 cells and *RNH1*-silenced YCC3 or YCC7 cells from TSA-induced growth inhibition, at TSA concentrations normally sufficient to induce robust cell death in the parental cells (200nM for AZ521 or YCC11 and 400nM for YCC3 or YCC7 with RNH1-silencing). This resistance caused by GSH pre-treatment was also associated with a parallel decrease in HDAC inhibitor induced ROS production (Figure 4.15). These results suggest that induction of ROS by HDAC inhibitors was necessary for the induction of proliferation inhibition and cell death in gastric cancer cells.


A
Proliferation Rate
Relative Proliferation Rate
1.2
1
0.8
0.6
0.4
0.2
0
Control
TSA(100nM)
GSH(5mM)
TSA+GSH
*
ROS Production
ROS Production (fold increasing)
5
4
3
2
1
0
-1
-2
Control
TSA
GSH
TSA+GSH
YCC11
Proliferation Rate
Relative Proliferation Rate
Control
TSA(100nM)
GSH(5mM)
TSA+GSH
ROS Production
ROS Production (Fold Increasing)
6
5
4
3
2
1
0
-1
-2
Control
TSA
GSH
TSA+GSH
AZ521


B
Proliferation Rate
Relative Proliferation Rate
1.2
1
0.8
0.6
0.4
0.2
0
Control
TSA(400nM)
GSH(5mM)
TSA+GSH
*
ROS Production
ROS Production (Fold Increasing)
6
4
2
0
-2
-4
Control
TSA
GSH
TSA+GSH
YCC3-shRNH1-1
Proliferation Rate
Relative Proliferation Rate
Control
TSA(400nM)
GSH(5mM)
TSA+GSH
ROS Production
ROS Production (Fold Increasing)
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6
4
2
0
-2
-4
Control
TSA
GSH
TSA+GSH
YCC3-shRNH1-2

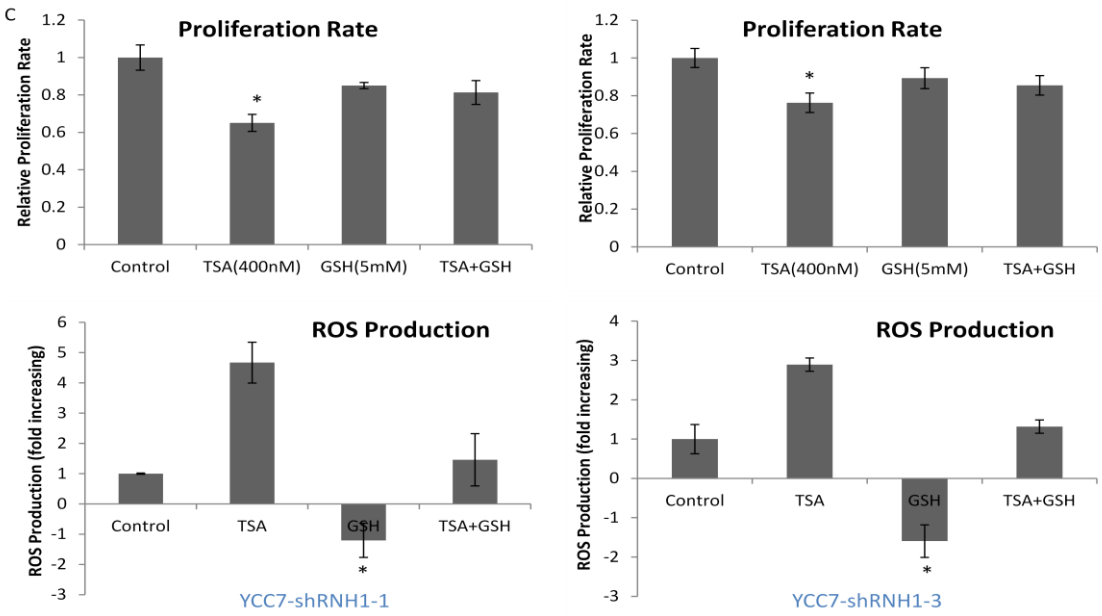

Figure 4.17 GSH/TSA-treated cells were observed to show resistance to TSA-induced proliferation inhibition.

Sensitive cells AZ521 and YCC11 (A), *RNH1*-silenced YCC3 cells (B) and YCC7(C) were treated with TSA, the ROS scavenger L-glutathione (GSH), or pre-treatment by GSH for 2 hours followed by TSA. * indicates $p<0.05$ between control and TSA treated cells.

Chapter Five. Results Part III

5 Extent of RNH1 Significance

5.1 The RNH1 Protein Level and Sensitivity to TSA in Normal Gastric Epithelial Cells

One appealing characteristic of HDACis is that non-cancerous cells typically exhibit high *in vitro* tolerance to these drugs (116). We thus explored if *RNH1* expression levels might be associated with HDACi resistance in normal gastric epithelial cells, and also cancer cells of non-gastric origin. We exposed two normal gastric epithelial lines (HFE145 and GES1) to increasing concentrations of TSA. Both cell lines exhibited GI50 values above 800nM, an even greater level of resistance than YCC3 or YCC7 cells (Figure 5.1). Importantly, both normal gastric lines expressed high RNH1 levels, similar to YCC3 cells (Figure 5.1A). This observation is consistent with the hypothesis of RNH1 contributing to HDACi resistance in normal gastric cells.

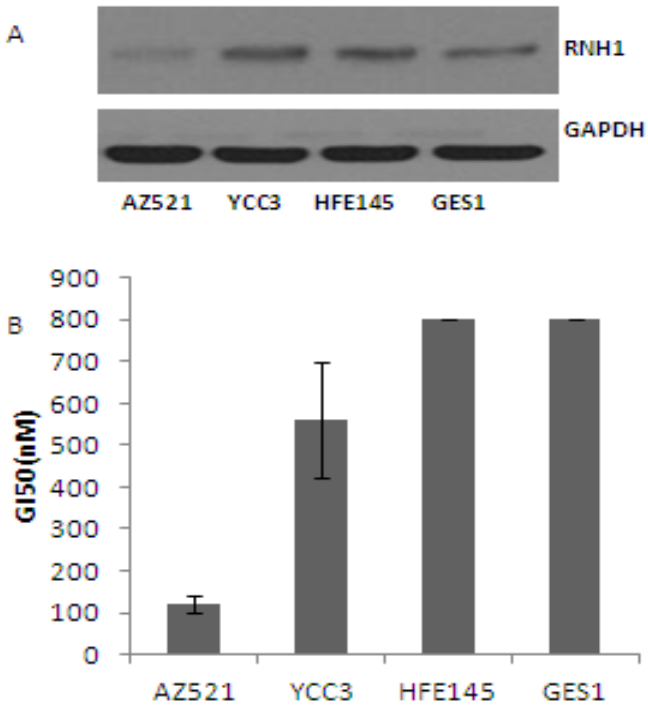

Figure5.1 *RNH1* levels correlate with TSA sensitivity in normal gastric epithelial cells

(A) Western blots showing RNH1 protein levels in normal gastric epithelial cell lines, HFE145 and GES1. AZ521 (sensitive) and YCC3 (resistant) were included as a reference. (B) Proliferation assays showing TSA GI50 values of HFE145 and GES1 cells. AZ521 and YCC3 were included as reference.

5.2 The RNH1 Protein level and Sensitivity to TSA in Cells of Other Cancer Types

Because HDAC inhibitor-induced ROS is not specific to gastric cancer, we examined the correlation between RNH1 expression and HDAC inhibitor sensitivity in cancer cells from other tissue types. We tested four other cancer cell lines -MCF-7 cells (breast cancer), HEPG2 cells (liver cancer), HCT116 cells (colon cancer) and Hela cells (cervical cancer). We found that HEPG2 cells expressed much higher RNH1 protein levels compared to HCT116 and Hela cells, and MCF7 expressed intermediate level of RNH1 protein (Figure 5.2A). The four cell lines were then tested for their sensitivity to HDAC inhibitor treatment. Under TSA treatment, HEPG2 cells exhibited an extremely high GI50 value (>800nM) (Figure 5.2B) and lower levels of cleaved PARP than other cell lines (GI50 <200nM) (Figure 5.2C). The responses of these cell lines to TSA treatment are similar to previous publications testing the same lines (121, 159). The observation that HEPG2 cells, with high RNH1 levels, showing higher resistance to TSA treatment than other cell lines, with low RNH1 levels, imply that RNH1 may also contribute to HDAC inhibitor resistance in other cancer types.

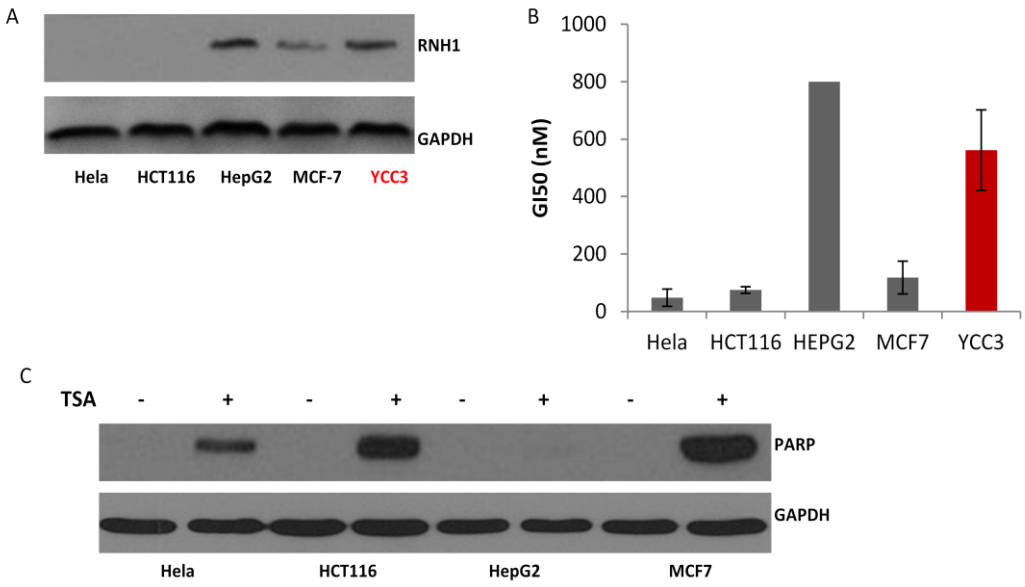

Figure 5.2 *RNH1* levels correlate with TSA sensitivity in cancer cells from other tissues.

(A) Western blots showing RNH1 protein levels in MCF7 (Breast cancer), HepG2 (Liver cancer), HCT116 (Colon cancer) and Hela (Cervical cancer) cells. YCC3 cells were included as a reference. (B) Proliferation assays showing GI50 values of Hela, HCT116, HepG2 and MCF7 cells treated by TSA. (C) Apoptotic assay of Hela, HCT116, HepG2 and MCF7 cells. Western blots were performed to detect PARP cleavage of all the cells treated by TSA.

5.3 The Effect of RNH1 Deregulation on Other Anti-cancer Drugs

In order to test if RNH1 levels also influence the sensitivity of GC cells to other drug treatments, we performed the proliferation assays of YCC3 with/without RNH1 silencing and AZ521 with/without RNH1 overexpression under cisplatin treatment (Figure 5.3). Interestingly, genetic knockdown or over-expression of RNH1 did not seem to influence the sensitivity of gastric cancer cells to cisplatin, although cisplatin is also known to induce ROS production in cancer cells.(160) This result suggests that ROS production plays a more important role in HDAC inhibitor-induced gastric cancer cell death compared to other cytotoxic drugs.

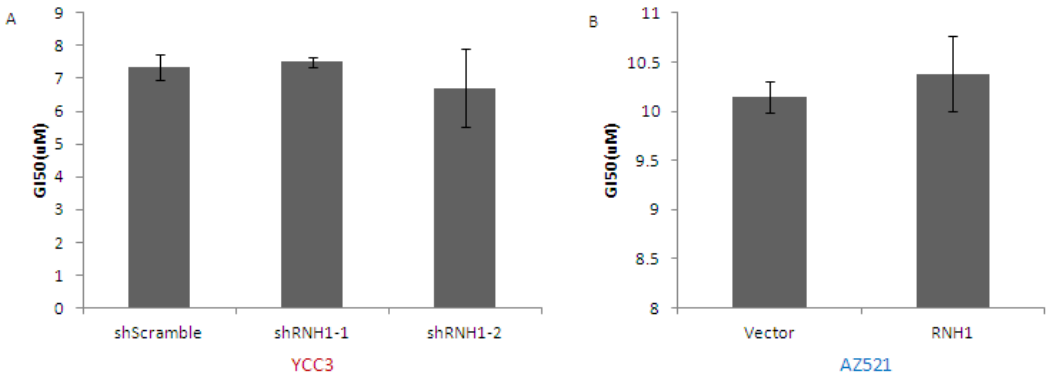

Figure 5.3 RNH1-deregulation does not influence sensitivity of gastric cancer cells to cisplatin treatment.

(A) Proliferation assays of YCC3 cells with stable *RNH1*-silencing. Proliferation assays were performed and GI50 values calculated to examine the effect of *RNH1* knock-down on cisplatin sensitivity. (B) Proliferation assays of AZ521 with *RNH1* over-expression. Cell proliferation assays were performed and GI50 valuse calculated to examine the effect of *RNH1* over-expression in AZ521 treated by cisplatin.

Chapter Six. Discussion

HDAC inhibitors, especially artificially synthesized ones, have been discovered as anti-cancer drug for more than 10 years. One of the most attractive characteristic of HDAC inhibitors is that they selectively induce cell death in transformed cells, while not the normal cells (161). In some hematologic malignancies, HDAC inhibitors such as SAHA, showed favorable clinical outcomes and have been approved for clinical usage (114). Recent publications suggested the feasibility of applying HDAC inhibitor in gastric cancer patients (105) and the potential high sensitivity of gastric cancer cells to HDAC inhibitor treatment (119). However, like other anti-cancer drugs, HDAC inhibitors induce heterogeneous responses in different cancer types, or in different subtypes of the same cancer. Thus it is necessary to understand the resistance mechanism of HDAC inhibitor in cancers, especially solid tumor treatment, which will provide useful information in cancer type and drug selection for clinical application. This project focuses on the different sensitivities of gastric cancer cell lines to HDAC inhibitor treatment and identified some potential genes (RNH1 and STAT1) or signatures (ROS alteration) responsible for the difference by genomic comparison between cell groups with different sensitivities. The significance has also been extended to other cancer types.

6.1 The Sensitivity of Gastric Cancer Cells to HDAC Inhibitors

So far there is no HDAC inhibitor applied clinically in gastric cancer patients. However, some publications suggested the importance of altered histone acetylation in gastrointestinal carcinogenesis, especially in relation to invasion and metastasis. (162) High HDAC expression, especially class I HDACs, are significantly associated with nodal spread and are independent prognostic markers for gastric cancer, (105, 163, 164) which implies considerable therapeutic efficacy of these agents. It has been more than 10 years that people noticed that HDAC inhibitors could induce gastric cancer cell growth arrest or apoptosis. (162) Recently an in vitro chemo-sensitivity assay validated the comparable chemo-response of gastric cancers to HDAC inhibitors and established drugs. (165) The general high sensitivity of gastric cancer cells to HDAC inhibitor treatment was re-proved by our data from TSA treatment. Most gastric cancer cell lines (15 out of 18) showed obvious cell reductions induced under rather low concentrations of TSA treatment (less than 400nM). Among the 18 cell lines screened, only 3 cell lines displayed relatively high resistance to HDAC inhibitors. The fact that most gastric cancer cell lines are sensitive to HDAC inhibitors implies that HDAC inhibitors could be a class of useful cancer therapeutic drug for gastric cancer patients.

6.2 The Heterogeneous Response of Gastric Cancer Cell Lines to HDAC Inhibitors

Besides surgery and radiotherapeutics, the use of chemotherapeutics remain the major option for cancer patients, especially for the patients in late stage. (166) However, one of the most frustrating outcomes of these chemotherapeutics is that patients do not respond or develop resistance. For instance, anti-EGFR (epidermal growth factor receptor) targeted therapy only improved disease control and survival in a subgroup of patients with metastatic colorectal cancer (mCRC). (167, 168) Other examples include gefitinib and erlotinib, the epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors in patients with non–small cell lung cancer (NSCLC) (169, 170). This may also happen to HDAC inhibitor treatment in gastric cancer patients. Using both 2-dimensional (growth inhibition, apoptosis) and 3-dimensional (colony formation) assays, we found that different gastric cancer cell lines displayed significantly different sensitivities to HDAC inhibitors, up to >10-fold in some cases. The differences in sensitivity were common to different HDAC inhibitors (TSA, SAHA and MS275), suggesting that these responses are generalizable to this drug class. Our *in vitro* findings render it plausible that primary gastric cancers may also exhibit similarly heterogeneous responses to HDAC inhibitors *in vivo* like cutaneous T cell lymphoma (CTCL) patients (171, 172).

6.3 HDAC Inhibitors Induce Different Apoptotic Responses among Gastric Cancer Cell Lines

It has been decades since some small-molecule compounds, either naturally existing or artificially synthesized, have been discovered with the capacity to alter HDAC activity and display anti-cancer power. This has led to the development of a series of HDAC inhibitors with different structures designed specifically for various targets. Efforts have focused on increasing their selective ability in promoting cancer cell cycle arresting and/or inducing cell death (173, 174). Cancer cell apoptosis is the one of main indicators for cancer therapy outcome.

HDAC inhibitors have been reported to activate either death-receptor pathways (extrinsic) or mitochondrial pathways (intrinsic) of apoptosis in many cancer models, such as leukemia cells, breast cancer cells or melanoma cells (175-177). The activation of caspases has been reported by early publications to be associated with HDAC inhibitor-induced cell death as the executioners of apoptosis. However, the requirement of caspases in HDAC inhibitor induced apoptosis is controversial (133, 178-180). Since multiple apoptotic pathways in mammalian cell have been characterized, and both apoptotic pathways need caspase 3 and Poly ADP-ribose polymerase (PARP) cleavage. (181-183) So in this project, caspase 3 and PARP cleavage were utilized as indicators to test if gastric cancer cell apoptosis could be induced

by HDAC inhibitor treatment. We found that most gastric cancer cell lines treated by HDAC inhibitors showed PARP and caspase 3 cleavage, which suggests that HDAC inhibitor-induced cell death could be through the caspase-PARP dependent apoptosis pathway. Further studies will be needed for elucidating specific apoptosis pathways responsible for HDAC inhibitor treatment in gastric cancer cells, which is not the key objective of this project.

There was a tremendous difference in caspase 3 or PARP cleavage signal between HDAC inhibitor sensitive and resistant cell lines. The difference in caspase 3 or PARP cleavage was chosen as the major standard to determine the sensitivity groupings of gastric cancer cell lines. According to this standard, MKN1 and YCC10 were excluded from the resistant group, although they showed even higher resistance than YCC3 and YCC7 in growth inhibition induced by TSA within 24 hours. Among the seventeen scanned gastric cancer cell lines, only three lines showed resistance to apoptosis induced by HDAC inhibitors, which further demonstrated that gastric cancer cells were generally sensitive to HDAC inhibitor treatment.

6.4 Candidate genes related to the difference in gastric cancer cell line sensitivity to HDAC inhibitors

In spite of recent development in chemotherapeutics, a major obstacle in the clinical treatment of patients with advanced and metastatic cancer is still intrinsic or acquired resistance during chemotherapeutic treatment (166). Chemo-resistance mechanisms could be related to various signal pathways involving altered expression and/or activity of different signal targets, which protects cancer cells from chemotherapy induced growth arrest and/or cell death effects. Many signaling pathways, such as EGFR, hedgehog, and Wnt/*b*-catenin and signaling targets, including PI_3K (phosphatidyl inositol 3'-kinase)/Akt, nuclear factor-*k*B, Bcl-2, cyclooxygenase-2 (COX-2), survivin, snail, slug, and twist may contribute to this chemotherapeutic resistance (153, 184-194). Previous studies discovered genes which were responsible for HDAC inhibitor resistance in different types of cancer, such as STAT1 in colon cancer cells harboring active RAS (121) and HR23B in cutaneous T-cell lymphoma (CTCL) cells (122). So far there is no report about genes responsible for HDAC inhibitor resistance in gastric cancer cells.

In our genomic analysis, STAT1 appeared in the list of top candidate genes which were differently expressed between sensitive and resistant cell groups. The appearance of STAT1 could be regarded as the evidence that our

bioinformatic analysis could provide biologically plausible outcomes. Compared to STAT1, RNH1 showed a more obvious high expression in protein level in HDAC inhibitor resistant cell lines. Further experiments proved that protein ribonuclease inhibitor, encoded by gene RNH1, contributed to the resistance to HDAC inhibitor treatment in gastric cancer cells, and RNH1 could regulate HDAC inhibitor-induced ROS generation to influence the sensitivity of gastric cancer cells. The effect of STAT1 on gastric cancer cells' resistance to HDAC inhibitors seem uncertain, because we observed that at least one resistant line YCC7 shows a low level of STAT1 (Figure 4.3). The results suggest that the mechanism of gastric cancer sensitivity to HDAC inhibitors could rely on different genes from colon cancer, although the two cancer types share much common characters.

6.5 The Roles of Reactive Oxygen Species (ROS) in Gastric Cancer Sensitivity to HDAC Inhibitors Treatment

ROS contributes to a wide range of pathologies including cancers, cardiovascular diseases, and neurological diseases. The relationship between ROS and gastric cancer has been discussed in the Introduction section. It has been proved by some publications that various HDAC inhibitors stimulate ROS production and their anticancer activity can be inhibited by anti-oxidant treatment. (116, 133, 140, 175). These studies suggest that ROS production may play an important role in cancer cell arrest and /or cell death induced by HDAC inhibitors. ROS stress may induce apoptosis through the intrinsic apoptotic pathway activation (195). In this project, we also found that ROS production by HDAC inhibitors was much higher in sensitive gastric cancer cells than the one in resistant cells. Previous publications focused on the role of thioredoxin (Trx) and its inhibitor, thioredoxin binding protein (TBP-2), in regulating ROS production by HDAC inhibitors. (116, 140) However, in this project, gene RNH1 seemed to play a more important role in explaining the sensitivity and ROS production difference by HDAC inhibitors in gastric cancer cells. This is the first time that RNH1 has been found to be involved in regulation of HDAC inhibitor-induced ROS production.

6.6 The roles of RNH1 in gastric cancer cell sensitivity to HDAC inhibitors

RNH1 has been found in the cytosol of many cell types. (144) RNH1 inhibits both secretory and intracellular ribonucleases, but it has not been detected in extracellular fluids, such as plasma, saliva, and urine. (196, 197). The literature about the biological role of RNH1 is full of conflicting observations. RNH1 biosynthesis seems to correlate positively with cell proliferation; increased RNH1 levels have been found in rat liver after treatment with 2-acetamidofluorene to induce tumors (198) and in developing neonatal rats(199). Other studies, however, found that high RNH1 levels decreased angiogenesis and tumor formation in mouse xenografts. (200) A recent publication showed that knock-down of RNH1 expression in non-invasive BIU-87 bladder cancer cells promoted its growth rate and metastasis ability. (201)

Several studies suggested that RNH1 could protect cells against oxidative damage. Overexpression of RNH1 in rat glial cells conferred protection against hydrogen peroxide-induced stress, and injection of RNH1 into mice also conferred protection from per-oxidative injuries of the liver induced by exposure to carbon tetrachloride. (202) RNH1 has also been found to contributes to intracellular redox homeostasis in normal, primary endothelial HUVE cells, and malignant HeLa cells indicated by decreased GSH

levels as well as increased oxidant-induced DNA. (152) Our experiments show that RNH1 is involved in HDAC inhibitor-induced ROS generation and cell death in gastric cancer cells, and the idea was reinforced by the synergistic effect of the ROS inducer, B-phenethyl isothiocyanate (PEITC), with TSA in high RNH1 expressing cells, and the rescuing effect of L-glutathione (GSH) from TSA in low RNH1 expressing cells. RNH1 seemed rather specific in regulating cell sensitivity to HDAC inhibitor treatment, because the effect could not be seen in cisplatin treatment, although cisplatin also could induce ROS generation in cancer cells (160). We considered that some other molecules may be involved in and might explain the specificity of RNH1 to HDAC inhibitor treatment.

6.7 Conclusions

In conclusion, the RNH1 gene was identified as a contributor to HDAC inhibitor resistance in gastric cancer cells through genomic analysis of differently expressed genes between sensitive and resistant cell groups, as well as following functional verification. We propose that RNH1 mediates this effect through its ability to regulate HDAC inhibitor-induced ROS levels. Our results suggest that ROS production plays a more important role in HDAC inhibitor-induced gastric cancer cell death compared to other cytotoxic drugs.

HDAC inhibitors could be a promising option of chemotherapy for gastric cancer, although no clinical trial has been performed so far. Exploiting the possible mechanism of HDAC inhibitor sensitivity in gastric cancer cells could help understand the rationale and provide supportive information for future possible clinical applications, which may also help to explain and overcome the relatively low response rate of HDAC inhibitors as single agents applied in other solid tumors (109).

6.8 Future Perspectives

This study has demonstrated the RNH1 can contribute to HDAC inhibitor resistance in gastric cancer cells. In this session, we will suggest further investigations to follow up on the existing findings of this project.

1. *In vivo* validation of RNH1 contributing to HDAC inhibitor resistance in gastric cancer

Although we proved the role of RNH1 contributing to HDAC inhibitor resistance at the gastric cancer cell culture level, it is necessary to establish further *in vivo* evidence of this RNH1 effect in some animal models, such as xenograft growth inhibition in a nude mouse model. Since we already have stable RNH1-silenced cell lines YCC3 and YCC7, the next step of this project would be to establish xenografts of YCC3 or YCC7 cells with /without RNH1-silencing in nude mouse model, then observing the different xenografts for growth inhibition induced by SAHA or MS275 treatment between control and RNH1-silenced groups. (TSA is not suitable to be clinically administrated for its short half life in blood and high toxicity.

2. Investigating further mechanism of RNH1 influencing ROS production induced by HDAC inhibitor treatment

Due to the fact of RNH1 contains a high content of reduced cysteines, it is easy to hypothesize that RNH1 could influence HDAC inhibitor-induced ROS production by interacting with ROS molecules directly as a buffering system. However, our observation that RNH1 could not influence cell growth inhibition by another anti-cancer drug, cisplatin, seems to put doubt on this deduction. Before the observation denies the hypothesis, several questions need to be answered: (a) Does ROS production play an important role in gastric cancer cell apoptosis induced by cisplatin treatment? (b) Could RNH1 deregulation also influence ROS production in gastric cancer cells treated by cisplatin? (c) Are there other genes involved in the RNH1 regulation of ROS production in gastric cancer cells?

To answer questions (a) and (b), similar experimental methods, such as the effect of PEITC or GSH on cisplatin-induced apoptosis and oxidative stress assays, could be performed on cisplatin treated gastric cancer cell lines similar to HDAC inhibitor treated cells. For question (c), different gene expression comparisons could be performed between cell lines before and after RNH1 gene deregulation to filter out possible candidate genes related to RNH1 regulating ROS production.

3. Investigating significance of RNH1 contribution to HDAC inhibitor resistance on other types of cancer

RNH1 is distributed in various types of tissues in the human body (144), so it is feasible to expect that the role of RNH1 in gastric cancer may also be observed in other types of cancer. In our primary study (Figure 5.2), liver cancer HEPG2 cells expressed high levels of RNH1 and also showed higher resistance to HDAC inhibitor treatment than the colon cancer cell line HCT116 and Hela cervical cancer cells with extremely low RNH1 expression. Interestingly, MCF7 breast cancer cells with relatively high levels of RNH1 are still sensitive to HDAC inhibitor treatment, which reminds us the role of RNH1 in HDAC inhibitor sensitivity could be diverse according to different tissue localizations. More experimental evidence should be involved to verify the detailed character of RNH1 in this event.

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