Identification of Novel Candidate Tumor Suppressor Genes at 11q and 15q for Esophageal Squamous Cell Carcinoma and Nasopharyngeal Carcinoma via Integrative Cancer Epigenetics and Genomics

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Abstract of Thesis entitled:

Identification of Novel Candidate Tumor Suppressor Genes at 11q and 15q for Esophageal Squamous Cell Carcinoma and Nasopharyngeal Carcinoma via Integrative Cancer Epigenetics and Genomics

Submitted by LI, Jisheng

for the degree of Doctor of Philosophy

at The Chinese University of Hong Kong in July 2010

Inactivation of tumor suppressor genes (TSGs) contributes to the genesis of cancers including esophageal squamous cell carcinoma (ESCC) and nasopharyngeal carcinoma (NPC), two prevalent causes of death in Hong Kong. Apart from genetic abnormalities, epigenetic disruptions including CpG methylation represent another major mechanism for TSG inactivation. Promoter methylation of multiple TSGs was detected in different cancer types, suggesting that it could be utilized as therapeutic target or biomarker for disease diagnosis and prognosis.

TSGs are often located at frequently deleted chromosomal regions and subjected to tumor-specific methylation, making it possible to use an integrative epigenetic and genomic approach combining array comparative genomic hybridization (aCGH) with epigenetic profiling to screen for novel TSGs. Previous aCGH revealed that several loci in 11q22.3, 15q14, 15q21.1 and 15q21.3 underwent frequent copy number loss in ESCC cell lines. Loss of heterozygosity (LOH) of these regions was also reported in other cancers, indicating that TSGs might reside within them. The aim of this study was thus to identify the candidate TSGs in these loci and study their anti-tumorigenic roles. In addition, the tumor suppressive function of *ADAMTS8*, a silenced 11q25 candidate TSG previously identified in our lab via this approach, was also studied.

In brief, mRNA expression profiling of candidate genes in each locus was performed using semi-quantitative RT-PCR in a panel of ESCC and NPC cell lines, normal tissues and immortalized epithelial cell lines. Genes downregulated in cancer

cells but with high expression in normal tissues and immortalized epithelial cells were subjected to promoter methylation analysis using methylation-specific PCR (MSP), bisulfite genomic sequencing (BGS) and pharmacological demethylation treatment. Genes with tumor-specific downregulation and methylation were further selected as candidates and their tumor suppressive roles were verified via functional studies.

In this study, *RAB39* and *WDRX* were identified as candidate TSGs in 11q22.3 and 15q21.3, respectively. Both genes were broadly expressed in normal tissues and immortalized epithelial cell lines, but significantly downregulated and methylated in multiple cancer cell lines. Demethylation treatment with 5-Aza-2'-deoxycytidine restored their mRNA expression, indicating that CpG methylation directly contributed to their transcriptional inactivation. Methylation of *RAB39* and *WDRX* was detected in primary ESCC and NPC, but rarely observed in normal tissues, implicating that their tumor-specific methylation might be used as biomarkers. Ectopic expression of both genes significantly inhibited the clonogenicity of multiple cancer cell lines, supporting their potential roles as functional TSGs. Moreover, *WDRX* repressed WNT/β-catenin signaling, underscoring a possible anti-tumorigenic mechanism for it. In addition, *ADAMTS8* was revealed to inhibit clonogenicity of NPC and ESCC cell lines, acting as a negative modulator for ERK pathway and a potential pro-apoptotic metalloprotease.

In conclusion, *RAB39* and *WDRX*, epigenetically silenced in multiple cancer cell lines, were identified as novel TSG candidates in this study. Meanwhile, the tumor suppressive functions of *ADAMTS8* were further validated, proving the efficiency of this integrative approach. Further study on these novel TSG candidates may help to elucidate the detailed molecular mechanisms for ESCC and NPC, and provide novel therapeutic targets and biomarkers.

通過整合擬遺傳學與基因組學策略在食管鱗狀細胞癌及鼻咽癌中鑒定位於人類 11 及 15 號染色體長臂上的新候選抑癌基因的研究

李際盛

抑癌基因(TSG)失活在包括兩種香港高發癌症-食管鱗狀上皮細胞癌(ESCC)和鼻咽癌(NPC)-在內的多種癌症發生中起關鍵作用。除遺傳學異常外，表觀遺傳學改變也導致 TSG 失活，如啟動子 CpG 甲基化。多種癌症中均特異地檢測到啟動子甲基化所致 TSG 失活，提示 TSG 甲基化可用作癌症早期診斷和預後判斷標志物，或作為新藥物靶點用於癌症治療。

我們採用了一種結合陣列比較基因組雜交(aCGH)和甲基化狀態篩査的“整合擬遺傳學與基因组学策略”來篩選新 TSG。aCGH 顯示 11q22.3, 15q14, 15q21.1 和 15q21.3 內的一些位點在 ESCC 中存在拷貝缺失,上述區域內的雜合性缺失 (LOH) 現象也見於其他癌症中，提示這些拷贝缺失位點內可能存在未知 TSG。本研究目的即是尋找這些位點中的新候選 TSG，並研究其在腫瘤發生中的作用。此外，之前通過這一策略發現 11q25 的 *ADAMTS8* 是一個甲基化沉默的 NPC 候選 TSG，本研究對其抑癌功能也進行了探討。

通過半定量 RT-PCR、甲基化特異性聚合酶鏈反應(MSP)、亞硫酸氫鹽修飾後測序(BGS)及去甲基化藥物誘導等技術，*RAB39*(11q22.3)以及 *WDRX*(15q21.3)被鑒定為新候選抑癌基因。*RAB39* 與 *WDRX* 在多種正常組織及永生化上皮細胞系中正常表達; 但在 ESCC 和 NPC 等多種癌細胞系中表達水平顯著下調或沉默，同時發生啟動子高度甲基化。去甲基化藥物處理沉默細胞系可以恢復 *RAB39* 及 *WDRX* 的表達，提示啟動子甲基化直接導致其表達下調及沉默。*RAB39* 和 *WDRX* 在原發 ESCC 及 NPC 中的甲基化頻率顯著高於正常上皮組織，提示可能被用作這兩種癌症的生物標記。將 *RAB39* 或 *WDRX* 表達載體轉染到多種癌細胞中可顯著抑制其克隆形成能力，同時發現 *WDRX* 能够抑制 WNT/β-catenin 通路活性，顯示它們是潛在的功能性 TSG。此外，對 *ADAMTS8* 進行的功能研究顯示它負向調節 ERK 通路，同時具有潛在的促進癌細胞凋亡的能力。

綜上所述，本研究在 11q 和 15q 鑒定了兩個新候選抑癌基因 *RAB39* 和 *WDRX*，並初步證實它們有抑癌功能；揭示之前篩選到的 11q 候選基因 *ADAMTS8* 通過調節 ERK 通路及細胞凋亡發揮抑癌作用。對這些新候選 TSG 進行更深入的研究有助於進一步揭示 ESCC 和 NPC 的發生機制，並可能發現新的腫瘤標誌物及治療靶點。

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List of symbols and abbreviations

Aza	5-Aza-2'-deoxycytidine
ADAMTS	A disintegrin and metalloproteinase domain with thrombospondin motifs
ATM	Ataxia-telangiectasia mutated
BGS	Bisulfite genomic sequencing
bp	Base pair
C	Cytosine
Ca.	cancer
cDNA	Complimentary DNA
CGH	Comparative genomic hybridization
CGI	CpG island
CLL	chronic lymophoytic leukemia
CpG	Cytosine-phosphate-Guanine
ddH2O	Double distilled water
DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferase
dNTPs	Deoxynucleotide triphosphates
EDTA	Ethylene-diamine-tetra-acetic acid
EGFR	Epidermal growth factor receptor
ERK	Extracellular signal-regulated kinases
ESCC	Esophageal squamous cell carcinoma
EtOH	Ethanol
FISH	Fluorescence in situ hybridization
G	Guanine
GF	Growth factor
GFP	Green fluorescent protein
HCC	Hepatocellular carcinoma
HDAC	histone deacetylase
HNSCC	Head and neck squamous cell carcinoma
kb	Kilo base
kDa	Kilodaltons
KO	Knockout

LB	Luria-Bartani
LOH	Loss of heterozygosity
LOI	Loss of imprinting
MAPK	Mitogen-activated protein kinase
MEK	MAPK and ERK kinase
MGMT	O^6-methylguanine-DNA methyltransferase
MBD	methyl-cytosine binding domain
MBP	Methyl-cytosine-binding proteins
mRNA	Messenger RNA
MSP	Methylation specific polymerase
NCBI	National center for biotechnology information
NPC	Nasopharyngeal carcinoma
NSCLC	Non small cell lung cancer
OD	Optical density
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
RACE	Rapid amplification complementary DNA ends
RB	Retinoblastoma
RCC	Renal cell carcinoma
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
RNAi	RNA interference
RTK	Receptor tyrosine kinase
RT-PCR	Reverse transcription polymerase chain reaction
T	Thymine
TE	Tris EDTA
TF	Transcription factor
TSA	Trichostatin A
TSG	Tumor suppressor gene
TSS	Transcription start site
WDR	WD40-repeat containing
YFP	Yellow fluorescent protein

List of publications

1. **Jisheng Li**, Jianming Ying, Yichao Fan, Longtao Wu, Ying Ying, Anthony TC Chan, Gopesh Srivastava and Qian Tao. *WNT5A* is a tumor suppressor antagonizing WNT/β-catenin signaling and frequently disrupted by CpG methylation in esophageal squamous cell carcinoma. *Cancer Biol Ther*. 2010 Sep 15, Volume 10, Issue 6.

2. Qian Zhang, Jianming Ying, **Jisheng Li**, Yichao Fan, Fan Fong Poon, Ka Man Ng, Qian Tao, Jie Jin. *DLEC1* is a critical 3p22 tumor suppressor for renal cell carcinoma and its aberrant promoter methylation is associated with more advanced tumor stage. *J Urol*. 2010 Jun 21.

3. Wang Y, **Li J**, Cui Y, Li T, Ng KM, Geng H, Li H, Shu XS, Li H, Liu W, Luo B, Zhang Q, Mok TS, Zheng W, Qiu X, Srivastava G, Yu J, Sung JJ, Chan AT, Ma D, Tao Q, Han W. *CMTM3*, located at the critical tumor suppressor locus 16q22.1, is silenced by CpG methylation in carcinomas and inhibits tumor cell growth through inducing apoptosis. *Cancer Res*. 2009 Jun 15;69(12):5194-201.

Chapter 1, Introduction and Literature Reviews

The global burden of cancer was doubled in the last 30 years and it was estimated that there would be 27 million cancer cases worldwide accompanied with 17 million cancer deaths by the year 2030 (Boyle and Levin, 2008). In Hong Kong, an increase of 21% in new cancer cases and 15% in cancer deaths were reported in the ten years from 1998 to 2007 (Hong Kong Cancer Registry, 2009). Cancer has become a major cause of deaths and a serious public health problem with the continual growth and aging of population both in Hong Kong and mainland China. Thus, it's of great value to study the detailed mechanisms of carcinogenesis, with the aim of looking for early diagnosis markers and novel effective therapeutic targets.

Carcinogenesis is a multi-step process involving the abnormalities of various cancer related genes, an essential group of which are the tumor suppressor genes (TSGs), such as famous members *retinoblastoma* (*RB)* and *TP53*. Inactivation of TSGs greatly elevates the chances for pre-cancer cells to enter a status of un-regulated cell growth and is among the most crucial events in cancer initiation. In addition to classical genetic disruptions, epigenetic changes including aberrant promoter CpG methylation represent another group of mechanisms resulting in the inactivation of TSGs in multiple cancers. The importance of cancer epigenetics in carcinogenesis is now widely accepted, with the establishment of a revised conception that cancer is a genetic and epigenetic disease.

In recent years, a lot of novel TSGs have been characterized and it's argued that

there still exist more genes with tumor suppressive functions to be discovered in the human genome (Esteller, 2007). Indeed, identifying new tumor suppressors not only helps us understand the ultimate origins of cancer, but also provides us new clues to develop novel diagnostic and therapeutic strategies. Thus, the major interests of the present study are to identify novel TSG candidates using a strategy of integrative epigenetics and genomics, and to characterize the tumor suppressive functions of the identified candidates. Two prevailing cancers in Hong Kong, esophageal squamous cell carcinoma (ESCC) and nasopharyngeal carcinoma (NPC), are used as cancer models in this study.

1.1 General concepts of tumor suppressor genes

Cancer is a genetic and epigenetic disease of somatic cells involving both the activation of growth-promoting oncogenes and the inactivation of their counterparts, growth-constraining tumor suppressor genes (Knudson, 2002; Momparler, 2003). Since the suppressive roles of TSG were recessive and only became apparent when they were missing or inactivated in normal cells, the pioneer cancer researchers faced more experimental difficulties in studying TSGs compared with oncogenes. As a consequence, earlier studies on cancer related genes focused on oncogenes rather than TSGs, resulting in a delay in its discovery.

With the application of many new techniques in cancer research, especially the somatic cell hybridization, accumulating solid experimental evidence suggested the existence of a group of critical growth-regulating genes, which were often shed by

the hybrids of tumor cells and normal cells when these hybrids reverted back to a tumorigenic status. In another word, in order to develop into full malignancy, cancer cells had to break the anti-growth barriers set by these suppressing gatekeepers, tumor suppressors (Weinberg, 1993). At the same time, progress of human genetics in both theory and technique provided a second clue for the existence of TSG and finally led to the discovery of the first human TSG, the retinoblastoma (*RB*) gene, which was later proved to encode a key transcriptional repressor inhibiting progression from G1 to S phase during cell cycle.

As early as in 1971, Knudson proposed the famous "two-hit" theory of tumorigenesis, postulating that the first hit in the DNA of patient with familial retinoblastoma was inherited in the genome and thus a second hit would rapidly led to cancer at a young age; but in sporadic retinoblastoma two successive hits had to take place before tumorigenesis, explaining why sporadic retinoblastoma usually occurred at an older age (Knudson, 1971). In 1983, the results of a series of genetic analyses (e.g. positional cloning) performed in inherited retinoblastoma pedigrees revealed the 13q14 associated *RB* gene to be the target of the inherited and somatic "hits" in retinoblastoma, and directly verified Knudson's theory as well as the existence of TSGs in human genome (Sparkes et al., 1983).

The successful identification of *RB* also helped to reveal a frequent genetic mechanism recruited by cancer cells to inactivate both copies of TSGs, and led to the establishment of an efficient strategy for TSG identification. Based on the extensive studies in *RB* inactivation, it's learned that while the first copy of TSG was usually

inactivated by a germline mutation, the chromosomal region containing the second wild-type TSG copy was usually replaced by a duplicated copy of the homologous chromosomal region containing the mutant TSG allele, via chromosome non-disjunction or conversion. As a result, the left normal copy of TSG was also inactivated. Actually, this kind of homologous replacement acted as an inactivating phenomenon with a much higher frequency than point mutation or gene knockout in cells. After replacement, the mutant TSG allele as well as its flanking chromosomal region became homozygous, which reduced the heterozygosity of nearby DNA polymorphism markers and led to a status of "loss of heterozygosity" (LOH) in the chromosomal region carrying the TSG. Thus, recurrent LOH of specific polymorphism markers in multiple malignancy samples of the same cancer type pinpointed the presence of TSGs in the nearby chromosomal region. By recruiting genome-wide polymorphism markers to screen for LOH in multiple tumor types, a lot of LOH regions were detected, which directly led to the cloning of some TSGs or provided strong supporting evidence guiding the later characterization of TSGs carried in these regions (Weinberg, 2007).

In addition to LOH, a number of other powerful genetic approaches were also successfully used for TSG identification in both familial and sporadic cancers, including linkage/positional cloning, two-hybrid screening, sequencing regions with heterozygous deletion or translocation, as well as representational difference analysis, which together brought along the fruitful harvest for TSGs in the 1990s (Robertson et al., 1999). By now, dozens of TSGs have been characterized in human genome,

Table 1-1. Major human tumor suppressor genes identified in familial and sporadic cancers. Adopted from text book "The biology of cancer" (Weinberg, 2007).

Name	Location	Familial ca.	Sporadic ca.	Function
RUNX	1p36		Gastric ca.	TF co-factor
HRPT2	1q25-32	Parathyroid tumors, Jaw fibromas	Parathyroid ca.	Chromatin protein
FH	1q42.3	Leiomyomatosis		Fumarate hydratase
FHIT	3p14.2		Many types	Diadenosine triphosphae hydrolase
RASSF1A	3p21.3		Many types	Multiple functions
TGFBR2	3p2.2	HNPCC	Colon, gastric, pancreatic ca.	TGF-β receptor
VHL	3p25	Von Hippel-Lindau syndrome	Renal cell ca.	Ubiquitylation of HIF
hCDC4	4q32		Endometrial ca.	Ubiquitin ligase
APC	5q21	Familial adenomatous polyposis coli	Colorectal, pancreatic, gastric ca.	β-catenin degradation
NKX3.1	8p21		Prostate ca.	Homeobox TF
$p16^{INK4A}$	9p21	Melanoma	Many types	CDK inhibitor
$p14^{ARF}$	9p21		All types	P53 stabilizer
PTC	9q22.3	Nevoid basal cell carcinoma syndrome	Medulloblastoma	Receptor of hedgehog GF
TSC1	9q34	Tuberous sclerosis		Inhibitor of mTOR
BMPR1	10q21-22	Juvenile polyposis		BMP receptor
PTEN	10q23.3	Cowden's disease, breast and gastrointestinal ca.	Glioblastoma, prostate, breast, thyroid ca.	PIP3 phosphatase
WT1	11p13	Wilms tumor	Wilms tumor	TF
MEN1	11p13	Multiple endocrine neoplasia		Histone modification, transcriptional repressor
BWS/CDKN1C	11p15.5	Beckwith-Wiedemann syndrome		$p57^{KIP2}$ CDK inhibitor
SDHD	11q23	Familial paraganglioma	Pehchromocytoma	Mitochondrial protein
RB	13q14	Retinoblastoma, osteosarcoma	Retinoblastoma, sarcomas, bladder, breast, esophageal, lung ca.	Transcriptional repression, control of E2Fs
TSC2	16p13	Tuberous sclerosis		Inhibitor of mTOR
CBP	16p13.2	Rubinstein-Taybi	Acute monocytic leukemia	TF co-activator
CYLD	16q12-13	Cylindromatosis		Deubiquitinating enzyme
CDH1	16q22.1	Familial gastric ca.	Invasive cancers	Cell-cell adhesion

BHD	17p11.2	Birt-Hogg-Dube syndrome	Kidney ca., hamartomas	Unknown
TP53	17p13.1	Li-Fraumeni syndrome	Many types	TF
NF1	17q11.2	Neurofibromatosis type1	Colon ca, astrocytoma	Ras-GAP
BECN1	17q21.2		Breast, ovarian, prostate ca.	Autophagy
PRKAR1A	17q22-24	Multiple endocrine neoplasia	Multiple endocrine tumors	Subunit of PKA
DPC4	18q21.1	Juvenile polyposis	Pancreatic, colon ca.	TGF-β TF
LKB1 /STK11	19p13.3	Peutz-Jegher syndrome	Hamartomatous colonic polyps	Serine/threonine kinase
RUNX1	21q22.12	Rhabdoid predisposition syndrome	Malignant rhabdoid tumors	Chromosome remodeling
NF2	22q12.2	Neurofibroma-position syndrome	Schwannoma, meningioma, ependymoma	Cytoskeleton-membrane linkage

which controlled diverse essential physiological processes, such as proliferation, apoptosis, cell cycle, cell motility, differentiation, DNA repairing and so on (Table 1-1). They acted as brakes for abnormal cell growth, and their loss of function inevitably led to an increase in the carcinogenesis potential of cells and directly accelerated malignant progression in all human cancers (Weinberg, 1995).

As one of the most crucial events during cancer initiation, loss of function or the inactivation of TSGs could be achieved via lots of genetic abnormalities in pre-cancer cells, including point mutations, small deletions, loss of chromosome (monosomy), genetic recombination or any combination of these mechanisms (Robertson et al., 1999). In addition to these classical genetic factors, it was later found that a lot of known TSGs could also be inactivated by epigenetic mechanisms, such as promoter CpG methylation, resulting in the emergence of a brand new field of cancer research, "Cancer Epigenetics" (Esteller, 2007).

1.2 Cancer Epigenetics

The latest definition of epigenetics describes it as a study of structural adaption of chromosomal regions so as to register signal or perpetuate altered activity states (Bird, 2007). Epigenetic information is generally stably inherited during cell division independently of the DNA sequence, existing as alternate states of DNA methylation, histone modification, chromatin structure, and transcriptional activity. Now we know that epigenetic inheritance is essential in a variety of physiological and pathophysiological processes, such as development and carcinogenesis. The notion that epigenetics could be associated with cancer was first suggested as early as in 1983, and research work thereafter proved epigenetic disruptions of tumor suppressor to be a hallmark of cancer (Momparler, 2003; Feinberg and Vogelstein, 1983). Aberrant DNA methylation, histone modification and RNA associated gene silencing, are the major epigenetic changes emerging during cancer development (Feinberg and Tycko, 2004).

1.2.1 DNA methylation and carcinogenesis

1.2.1.1 Background introduction of DNA methylation

DNA methylation is the most extensively studied and best understood epigenetic modification by now. As a kind of covalently chemical modification of DNA which is heritable during cell divisions without changing the DNA sequence, DNA methylation refers to the addition of a methyl group to the 5 position carbon (5C) of the cytosine pyramiding ring or to the 6 position nitrogen (6N) of the adenine

purine ring (Clark et al., 1994). However, in eukaryotic cells DNA methylation only occurs at the 5C of cytosine but not at the 6N of adenine. In mammalian genomes, 5-methylcytosine (m5C) primarily occurs at cytosines 5' to guanosines at the 5'-CpG-3' dinucleotides (CpG sites) after DNA synthesis (Ng and Bird, 1999). In humans, CpG methylation is catalyzed by a family of enzymes named DNA methyltransferases (DNMTs), which transfer a methyl group from S-adenosylmethionine to the 5C of cytosine (Fig. 1-1) (Baylin and Herman, 2000).

The human DNMTs family consists of four active members: DNMT1, DNMT3A, DNMT3B, and DNMT3L. DNMT1 is the most abundant DNMT in the majority of mammalian cell types and can predominantly recognize and methylate hemi-methylated CpG. During DNA replication, DMNT1 specifically copies the DNA methylation status in the template DNA strands to newly synthesized daughter strands and thus is responsible for the maintenance of the existed DNA methylation pattern. DNMT1 protein is about 1620 amino acids in length and is composed of a regulatory domain and a catalytic domain, both of which are necessary for the maintenance methyltransferase function of DNMT1 (Yoder et al., 1997; Robertson, 2002; Bestor, 2000). DNMT3A and DNMT3B have similar protein architectures with DNMT1, but are only highly expressed at the stage of embryonic development. They can initiate *de novo* methylation at unmethylated CpG, which is essential for the establishment of genomic DNA methylation patterns during early development (Okano et al., 1999). The other DNMT3 subfamily member DNMT3L also contains DNA methyltransferase motif but is catalytically inactive. Instead, it can positively regulate *de novo* methylation at the imprinted genomic regions and the repetitive

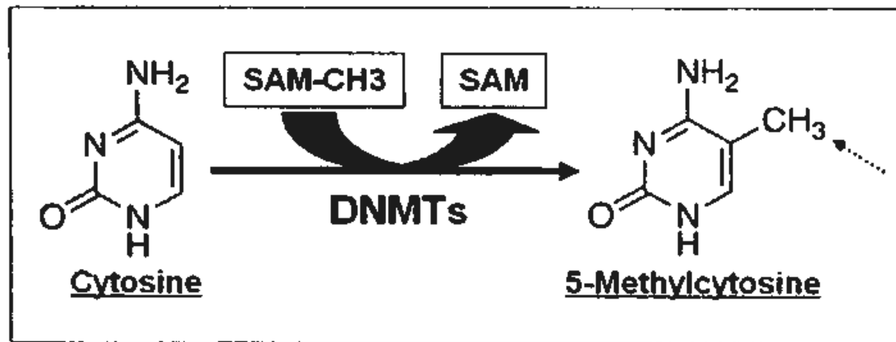

Figure 1-1. Diagram showing the establishment of DNA methylation catalyzed by DNMTs. DNMTs mediate the transferring of a methyl group (CH3) from S-adenosylmethionine (SAM) to the 5-carbon position of cytosine (indicated by dashed arrow).

sequences through enhancing the methyltransferases activity of DNMT3A and DNMT3B, and increasing their DNA binding ability (Hata et al., 2002). While only DNMT1 is abundantly detected in somatic tissues, all four DNMTs are highly expressed in embryo stem cells and most of them are shown to be vital in development. For example, mice embryos lacking both alleles of DNMT1, DNMT3A or DNMT3B would die before or after birth (Robertson, 2001).

The actual occurrence (about 1%) of CpG dinucleotides in human genome is greatly lower than the statistically expected frequency (about 6%). This phenomenon is referred as CpG suppression and is thought to be caused by the spontaneous deamination and changing into thymines of methylated cytosines during evolution (Feil and Berger, 2007). In the haploid human methylome, it's estimated that there are totally more that 28 millions of CpG dinucleotides. In normal conditions, about 70% of them are primarily located in the repeated genomic regions, transposons as well as intrageneic regions, and are kept methylated. On the contrary, the remaining CpGs are kept unmethylated and predominantly exist in clusters called "CpG

islands" (CGIs), which are small stretches of DNA (0.5 to several kb) generally located around the 5' promoter region and the transcription start site of approximately half of all human genes (Baylin and Herman, 2000). Usually a promoter associated CGI is defined as a DNA fragment of at least 500bp in length, with a GC content greater than 55%, and with a CpG occurrence higher than 65% (Takai and Jones, 2002). Although almost all promoter CGIs are commonly protected from methylation, there are actually several normal exceptions in human genome. Fully methylated CGIs could be normally seen at the promoters of the parentally imprinted genes, silenced tissue-specific autosomal genes and silenced genes on the inactivated female X-chromosomes (Baylin and Herman, 2000). This obvious contrast in CpG methylation distribution provides a clue that DNA methylation could regulate gene transcription.

From abundant evidence accumulated over previous studies, now we know that DNA methylation provides a potent intrinsic mechanism for both suppressing gene expression and maintaining chromosomal stability in mammalian genomes. And its capability to regulate gene transcription seems to be the most important underlying foundation for the essential roles that DNA methylation plays in multiple key physiological processes, including cell differentiation, genomic imprinting, X-chromosome inactivation, suppression of repetitive and viral elements, as well as carcinogenesis (Robertson et al., 1996). For instance, DNA methylation established in stem cells during early embryonic development stably alters their gene expression patterns and makes them programmed for further differentiation into various tissues

(Jaenisch and Bird, 2003). Besides, dense methylation of viral elements integrated into mammalian genomes blocks the expression of viral genes and protects hosts from viral infection. Moreover, genomic imprinting and X-chromosome inactivation are actually two excellent examples illustrating the selective suppression of gene expression via DNA methylation. In addition, aberrant methylation of TSG promoters detected in various tumors could act as the second hit during tumorigenesis by transcriptionally inactivating TSG (Baylin et al., 2000). Taken together, these facts highlight the importance of a balanced regulation of gene expression mediated by DNA methylation.

1.2.1.2 Mechanisms of CpG methylation induced gene silencing

The assumption that DNA methylation might affect gene transcription was first proposed early in 1975, based on emerging understandings in mammalian X chromosome inactivation (XCI). XCI refers to the transcriptional silencing of one of the two female X-chromosomes in mammals, which is vital in keeping equivalent levels of X chromosomal gene products between males and females. Based on his studies in XCI as well as DNA modifications, Riggs A. D. proposed a model arguing that: 1) XCI was maintained by DNA methylation in silenced X chromosomal genes, and 2) DNA methylation might be a mechanism recruited by mammalian cells to achieve somatically heritable gene silencing (Riggs, 1975; Holliday and Pugh, 1975). Evidence supporting the gene expression regulating function of DNA methylation later arised from the tissue-specific expression pattern of Rabbit β globin gene, which was correlated with its tissue-specific methylation. Extended study on human and

chicken β globin genes suggested that hypomethylation of promoters was positively correlated with active expression (Waalwijk and Flavell, 1978; van der Ploeg and Flavell, 1980). In addition, findings demonstrating that DNMT inhibitors such as 5-azacytidine could cause DNA hypomethylation, differentiation of mice embryonic cells and re-expression of some silenced gene *in vivo* (e.g. *HGPRT* on inactivated X-chromosome) further highlighted the role of DNA methylation in gene silencing (Santi et al., 1984; Mohandas et al., 1981). Moreover, later studies involving transfection of differentially methylated gene into mammalian cells showed that cells transfected with unmethylated genes had much higher expression level when compared with cells transfected with methylated genes, and proved the transcription-suppressing role of DNA methylation in a much more direct way (Stein et al., 1982; Busslinger et al., 1983). Furthermore, transgenic mouse models further proved that DNA methylation could repress transcription *in vivo* (Siegfried et al., 1999).

Regarding the obvious correlation between DNA methylation and transcriptional silencing, extensive studies have been done to uncover the underlying mechanisms and various models were proposed to address this correlation. According to the present theories, DNA methylation might repress gene transcription by three primary mechanisms reviewed as below:

(1) First of all, methyl-cytosine itself can physically impede the binding of transcription factors (TFs) to gene promoters and thus suppress transcription (Kass et al., 1997). In eukaryotes, the specific binding of TFs to their target DNA sequences

in either gene promoter or enhancer is vital for the initiation and regulation of gene transcription (Barnes and Adcock, 1995). The DNA binding sequences of many TFs contain CpG sites and methylation of these CpGs could prevent the effective binding of TFs to their target DNA sequences. For instance, methylation of a CCGG site in the binding sequence of TF AP-2 within the promoter region of the *proenkephalin* gene dramatically affected the binding of AP-2 and thereby inhibited the expression of this gene (Comb and Goodman, 1990). Extensive studies on other TFs such as c-Myc, CTCF and CREB also provided supporting evidence for this mechanism (Attwood et al., 2002).

(2) Secondly and more importantly, DNA methylation can induce transcriptional silencing via altering chromatin structure (Robertson, 2002). The critical link between DNA methylation and chromatin structure regulation is established by the methyl- cytosine-binding proteins (MBPs). Human MBP protein family is composed of five members including MeCP2, MBD1, MBD2, MBD3 and MBD4, which are all nuclear proteins containing a methyl-cytosine binding domain (MBD) and can specifically bind to methyl-cytosine, with the exception of MBD3. MeCP2 is the best characterized MBP by now, which is shown to be concentrated in the heterochromatin adjacent to centromere (Attwood et al., 2002). On one hand, MeCP2 appears to be able to repress transcription on its own, probably via preventing the binding of basal TFs and the recruitment of transcriptional favorable proteins such as histone acetylase (HAT) and co-activators to gene promoter (Fig. 1-2) (Attwood et al., 2002; Miranda and Jones, 2007). On the other hand, MECP2 can impede transcription indirectly via its association with the Sin3A histone deacetylase (HDAC)

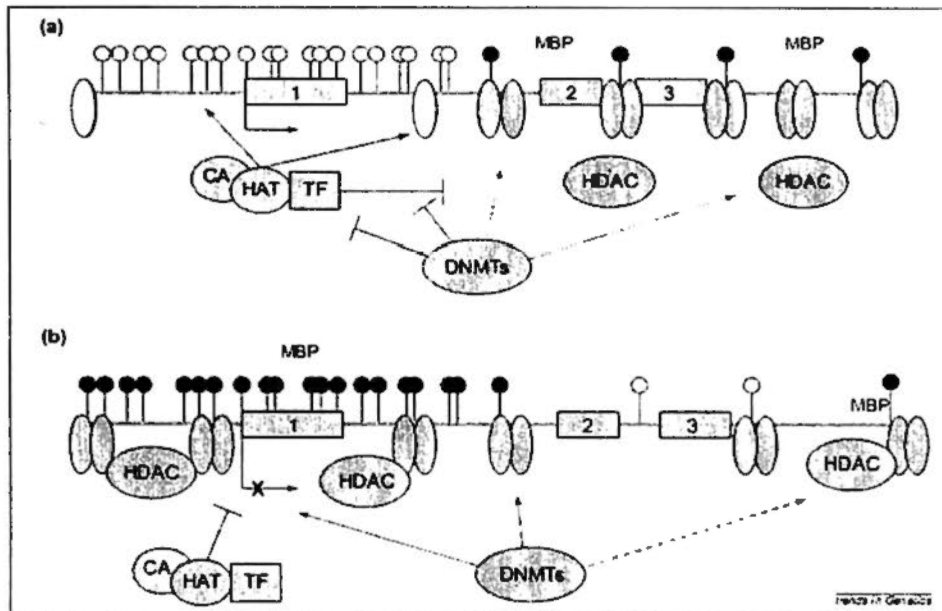

Figure 1-2. Different methylation status and chromatin patterns in the promoter CGIs of an actively expressed gene and a silenced gene. (a) The configuration of an actively expressed gene with an unmethylated CGI spanning promoter and exon1. The chromatin structure of this CGI is transcriptionally favorable, indicated by widely spaced and loose nucleosomes with acetylated histones. This type of CGI can not be accessed by DNMTs, instead it can easily interact with transcriptional initiating complex composed of HAT, TF and co-activator (CA). (b) The configuration of a silenced gene with promoter hypermethylation. Its CGI is fully methylated and contains abundant shortly spaced and compacted nucleosomes, implying a heterogenous chromatin conformation which is against transcription. It can closely interact with DNMTs and the MBP-HDAC complexes, but can not be accessed by the transcriptionally favorable protein complex containing HAT. (Numbered box, exon; white circle, unmethylated CpG; black circle, methylated CpG; white oval, loose nucleosome; grey oval, compacted nucleosome). Adopted from a review paper published in *Trends in Genetics* (Baylin and Herman, 2000).

complex, which is composed of more than seven different protein components and can shape a densely compacted transcriptionally inactive chromatin structure. In addition to the Sin3A complex, there are at least two more MBD interacting HDAC complexes in the human genome. MBD2, HDAC1, HDAC2, and the retinoblastoma protein-binding proteins RbAp46 and RbAp48 together form another HDAC complex named MeCP1, while Mi-2 (a SWI/SNF family chromosome remodeling

protein), MDB3, HDAC1, HDAC2, RbAP46 and RbAp48 form the Mi2/NuRD HDAC complex (Meehan et al., 1992; Zhang et al., 1999).

(3) In addition, DNA methylation can also induce transcriptional repression through influencing the number of nucleosomes at gene promoter and transcription start site. Previous studies on *MGMT* and *MLH1* gene promoters showed that DNA methylation could increase the nucleosome occupancy in the nucleosome free regions *in vivo* (Patel et al., 1997). It was suggested that the interaction of MeCP2 with Brahma, a component of the chromatin remodeling complex SWI/SNF, allowed the binding of this complex to the methylated DNA sequences and in turn caused dense nucleosome occupancy (Harikrishnan et al., 2005). The presence of dense nucleosomes in gene promoter decreased the binding of TFs and RNA polymerase II and finally contributed to transcriptional silencing (Li et al., 2007).

1.2.1.3 Promoter hypermethylation induced TSG silencing in cancers

Considering the potent ability of DNA methylation in regulating transcription, it's indubitable that aberrant DNA methylation could be recruited by cancer cells as a mechanism to achieve gene expression patterns favoring growth, transforming and metastasis. During carcinogenesis, promoter hypermethylation turns out to be a major avenue for cancer cells to aberrantly silence multiple TSGs, resulting in the inactivation of the critical intrinsic growth suppressing mechanisms set by evolution and leading cells to the way towards carcinogenesis (Feinberg, 2004). In the revised

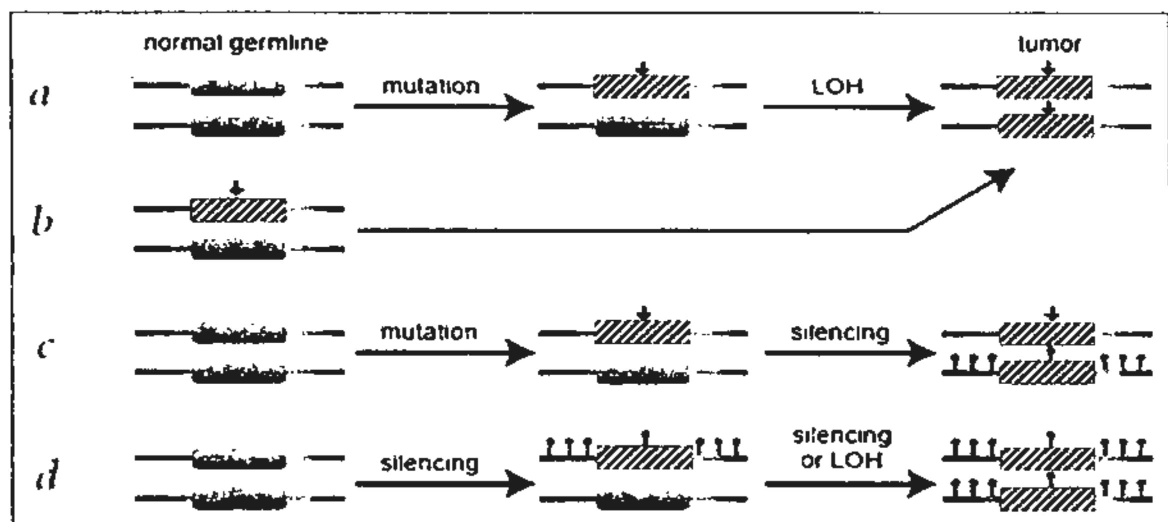

Figure 1-3. Diagram showing promoter methylation as hits of TSG in Knudson two-hit theory. a,b) Illustrations of classical Knudson two-hit theory. a, in sporadic cancers without TSG germline mutations, a first and a second successive inactivating events (vertical arrowhead) are required for tumorigenesis. Shadowed bars indicate inactivated genes. b, in familial cancers with a predisposed mutation (first hit), one more mutational event (second hit) is enough to cause cancer; c) Promoter methylation (vertical bars) induced TSG silencing can act as the second hit following first mutational event to cause cancer; d) Silencing of both TSG copies due to promoter methylation can act as both hits. Adopted from a review paper with author's permission (Balmain et al., 2003).

two-hit model for tumorigenesis, promoter methylation induced gene silencing was regarded as another important mechanism of "hits" in addition to genetic mutational events (Fig 1-3). Surprisingly, promoter methylation of some TSG such as *MLH1* can even act as the first-hit predisposed in the genomes of individuals with multiple primary tumors (Suter et al., 2004). In recent years, the occurring frequency and functional role of promoter methylation in a great abundance of well characterized TSGs or TSG candidates have been extensively studied in various cancers, which further proved the general presence and great contribution of this epigenetic phenomenon in cancer. We can try to illustrate the importance of TSG promoter methylation in cancer in the following aspects: (1) its prevalence in primary

malignancies, (2) the essential pathways and cellular functions affected, and (3) the equivalent selective advantage conferred by this event as that by genetic mutations (Baylin and Herman, 2000).

(1) **Prevalent in cancers**: The first TSG found to be epigenetically disrupted by DNA methylation actually turned out to be the first known TSG, retinoblastoma gene *RB*, which was shown to be frequently inactivated by promoter methylation in sporadic retinoblastoma and even in some hereditary cases as early as in 1989 (Greger et al., 1989). In 1993 a report showing a 92% reduction of *RB* expression level in retinoblastomas with *RB* promoter hypermethylation directly proved the silencing of TSG by DNA methylation (Ohtani-Fujita et al., 1993). Thereafter, several other key TSGs including cyclin-dependent kinase inhibitor $p16^{INK4a}$, mismatch-repairing gene *MLH1* and E-cadeherin gene *CDH1* were all proved to be transcriptionally silenced by promoter methylation in both cancer cell lines and primary cancers of multiple tissue origins (Kane et al., 1997; Gonzalez-Zulueta et al., 1995; Graff et al., 1995). By now, it has been considered that nearly half of the TSGs inactivated in familial tumors due to germline mutations could be epigenetically silenced by aberrant promoter methylation in sporadic tumors. Moreover, TSG inactivation by hypermethylation has been detected in almost all human cancer types, although its occurring frequency in a specific TSG may vary in different tumors (Feinberg, 2004).

(2) Affecting essential pathways: Besides the astonishing prevalence of TSG promoter methylation in human cancers, the genes targeted for inactivation by

methylation are often the key regulators in various cellular pathways controlling essential physiological processes including DNA repairing, cell proliferation, differentiation, adhesion, motility, cell cycle, and apoptosis. For instance, cell cycle controlling gene *p16*^{INK4a} encodes an important CDK4/6 inhibitor, which maintains RB protein in the non-phosphorylation active state and blocks the G1-to-S phase transition. Promoter methylation of *p16*^{INK4a} or *RB* can abolish this essential cell cycle controlling mechanism and convey substantial survival advantages favoring carcinogenesis (Sherr, 1996; Herman et al., 1995). In addition, WNT signaling pathway, which is involved in multiple biological processes including cell proliferation, polarity and migration, can also be disrupted by aberrant promoter methylation of its components (Logan and Nusse, 2004). Several negative regulators in this pathway such as *APC*, *Axin*, and *WIF1* have been shown to be methylated and silenced in multiple tumors, causing the stabilization and nuclear translocation of β-catenin which promoted the transcription of oncogenic target genes including *CCND1*, *c-Myc* and *MMP7* (Esteller et al., 2000c; Suzuki et al., 2004; Koinuma et al., 2006). Moreover, the key genomic surveillance role of p53 can also be indirectly affected by promoter methylation of genes within the p53 network, although *TP53* gene itself is primarily inactivated by genetic mutations instead of epigenetic silencing. For instance, methylation of *p14*^{ARF}, which could stabilize p53 protein via inhibiting MDM2, was found to be frequently methylated in colon cancer and lymphoma, resulting in an indirect inactivation of p53 function (Chin et al., 1998).

(3) Conferring equivalent selective advantage as genetic alterations: *p16*^{INK4a}

is an appropriate example to compare the survival advantages conferred by DNA methylation and by genetic mutations. As one of the most powerful controlling machinery of cell cycling, the *p16^{INK4a}/RB* pathway is disrupted in almost every tumor types (Herman et al., 1995). Actually, the inactivation of *p16^{INK4a}* can be found in almost all common human cancers, due to point mutation, deletion, as well as promoter methylation. Strikingly, in some malignancies of colon and prostate cancer, *p16^{INK4a}* inactivation was found to be achieved by promoter hypermethylation only, without the involvement of classical genetic alterations, while its inactivation by both mechanisms was commonly observed in multiple other tumors, suggesting the comparable role of CpG methylation as genetic alterations in abolishing the *p16^{INK4a}/RB* cell cycle controlling machinery (Herman et al., 1995). Another excellent example is the human *MLH1* gene, which encodes a component of the DNA mismatch repairing complex. Germline mutation of *MLH1* was associated with hereditary nonpolyposis colorectal cancer, which was characterized by a status of microsatellite instability. Interestingly, genetic mutations of *MLH1* were quite uncommon in a distinct group of sporadic colon and gastric cancers characterized by microsatellite instability, although *MLH1* was obviously silenced in most of these tumors. It's later revealed that hypermethylation of *MLH1* promoter was the mechanism responsible for its lack of expression in sporadic microsatellite instable cancers (Aaltonen et al., 1993; Herman et al., 1998). The same scenario happens to *BRCA1*, which is frequently mutated in familial breast cancer but rarely in sporadic ones. Similarly, promoter hypermethylation was found to result in the inactivation of

BRCA1 in sporadic breast cancers (Esteller et al., 2000b).

In brief, TSG promoter hypermethylation and the subsequent tumor-specific TSG silencing is now regarded to be an event as critical as genetic alterations in cancers. Actually, there is considerable evidence demonstrating that epigenetic disruptions might even play a more active role in cancers than genetic alterations. According to the most recent study in colorectal cancer by Stephen B. Baylin in John Hopkins University, nearly 5% of all know genes would undergo promoter methylation in an individual tumor; and there were larger numbers of genes which were subjected to promoter hypermethylation in an individual tumor compared with genes that were genetically mutated; in addition, in individual genes that were found to undergo both genetic and epigenetic changes in cancers, promoter hypermethylation were detected at a higher frequency than genetic mutations (Schuebel et al., 2007). This study further strengthened the vital role DNA methylation played during carcinogenesis.

Moreover, the recognition that promoter hypermethylation might precede and foster genetic mutations highlighted its importance in cancer initiation. It's found that hypermethylation of $p16^{INK4a}$ usually occurred as early as in the pre-cancer cells, which allowed cells to break the critical G1/S cell cycle check point and predisposed cells for further genetic mutations leading to transformation (Nuovo et al., 1999; Belinsky et al., 1998). Besides, germline hypermethylation or "epimutation" of *MLH1*, was reported to act as the predisposing first-hit causing genetic instability in individuals with multiple primary tumors, provided additional excellent supporting

evidence to illustrate the role of TSG methylation in cancer initiation (Suter et al., 2004). These findings suggested that gene silencing mediated by DNA methylation might interact with or even directly enhance genetic alterations during cancer initiation.

1.2.1.4 Hypomethylation and cancer

When realizing the importance of TSG transcriptional silencing caused by CGI hypermethylation in cancer, it's important to realize that hypermethylation is just one of the ways by which DNA methylation contributes to cancer development. Loss of DNA methylation or "hypomethylation" is also closely related to carcinogenesis and is actually the first epigenetic disruption revealed in cancer cells. Concurrently with TSG CGI hypermethylation, nearly all human tumors, both benign and malignant, exhibit a widely spread loss of methylation in the whole genome when compared with the corresponding normal tissues. Moreover, a positive correlation has been revealed between the degree of hypomethylation and the tumor grade or size (Ehrlich et al., 1982; Gama-Sosa et al., 1983).

The proposed mechanisms by which DNA hypomethylation exerts its roles in cancer can be categorized into the following aspects. First, hypomethylation of DNA can lead to aberrant gene activation in cancer cells, especially the oncogenes favoring cell growth and survival, such as *H-Ras* in colon cancers (Feinberg and Vogelstein, 1983). Other critical oncogenic genes activated by hypomethylation in cancers includes cyclin-dependent kinase *Cyclin D2* in gastric cancers, transforming and hypoxia correlated gene *MN/CA9* in renal cancers, metastasis associated gene

S100A4 in colon cancers, as well as tumorigenic viral gene *HPV16* in cervical cancers (Akiyama et al., 2003; Sato et al., 2003; de Capoa et al., 2003; Oshimo et al., 2003; Cho et al., 2001). Besides, a group of genes named cancer/testis (*CT*) genes which are normally expressed only in testis are also activated by DNA hypomethylation in various cancers, although their function remains largely unknown. It's reported that during the development of gastric and liver cancers, hypomethylation of *CT* genes could be detected early in the pre-malignant stages (Cho et al., 2003).

Second, global hypomethylation in cell genome, especially in the repetitive elements and centromeric or juxtacentromeric heterochromatins, destabilizes genome and results in increased level of chromosomal instability, including unbalanced translocation, inversion and LOH, as shown in the *in vitro* and *in vivo* mice models and human malignancies, such as Wilms tumor and ovarian cancers (Eden et al., 2003; Qu et al., 1999; Widschwendter et al., 2004). Subsequently, the resulted genomic mutational events may convey elevated chances for the arising of malignant clones with selective advantages. In addition, it is regarded that loss of imprinting (LOI), which can be induced by hypomethylation, also contributes to tumorigenesis, such as the LOI of insulin-like growth factor-2 (*IGF2*) in Wilms tumors and the pre-neoplastic Beckwith-Wiedemann Syndrome (BWS) (Feinberg et al., 2006).

Obviously, methylation related disruptions in cancers simultaneously include the hypermethylation and hypomethylation of cancer related genes, as well as global DNA hypomethylation in cancer genome. Through both hyper- and hypomethylation,

cancer cells achieve the aberrant silencing of TSG, activation of oncogenes, an unstable chromosomal status as well as loss of imprinting, all of which could potently promote cancer initiation and progression.

1.2.2 Histone modification and carcinogenesis

Histones are a group of eukaryotic nuclear proteins which facilitate the packaging of DNA strands into nucleosomes, the basal structural units of DNA, and act as the primary components of chromatin. Human histones consist of five primary classes that can be further categorized into two sub groups, the core histones (H2A, H2B, H3 and H4) and the linker histones (H1 and H5) (Peterson and Laniel, 2004). Among them H3 and H4 have long N-terminal tails which are essential in the maintenance of chromatin stability, and the lysine and arginine residues in them can undergo covalent modifications including methylation, acetylation, phosphorylation and so on. The numerous combination of different modification of histones actually forms an epigenetic code system named "histone codes", which take part in various biological processes including transcriptional regulation, DNA repairing and chromosome condensation (Bernstein et al., 2005). Considering their roles in regulating transcription, there is no doubt that each of these modifications may have the potential to contribute to carcinogenesis, once the disrupted histone modification pattern lead to the silencing of a TSG or the activation of an oncogene.

Histone acetylation of the lysine resides helps to shape an opening chromatin structure, which is a premise of active transcription; whereas histone deacetylation causes a condensed chromatin conformation repressing transcription. Thus, histone

acetyltransferase (HATs) usually act as transcriptional activators while histone deacetylases (HDACs) act as repressors (Santos-Rosa and Caldas, 2005). Actually, disruptions of a number of HAT genes, such as *p300*, *CBP*, *MOZ*, *MORF* and *NCOA1*, were found in multiple human cancers (Santos-Rosa and Caldas, 2005). For instance, mutation in *CBP* can cause the Rbuinstein-Taybi syndrome, which is associated with substantially increased tumor susceptibility (Petrij et al., 1995). Besides, LOH and somatic mutations of another HAT gene *p300* is associated with human gastric, colon and breast cancers (Zhang and Dent, 2005). On the other hand, overexpression of the HDAC genes, which might result in the transcriptional repression of essential TSGs like *TP53*, has also been detected in cancers. For example, the *HDAC1* gene was reported to be overexpressed in both prostate and gastric cancers (Halkidou et al., 2004; Choi et al., 2001)

Histone methylation associated with both the lysine and the arginine residues of H3 and H4 is a much more complicated post-transcriptional modification of histones, with multiple forms of mono- , or di- or tri-methylation. In contrast to the role of histone acetylation in active transcription, histone methylation can induce either the activation or the repression of transcription, which varies according to the composition of different forms of methylation on different resides. Histone arginine methylation has dual roles in transcription regulation and the corresponding enzyme, arginine methyltransferases seems to play a role in the regulation of tumor cell growth and proliferation. For instance, arginine methyltransferase *CARM1* has been reported to be overexpressed in prostate cancer while *PRMT5* could cause a reduced level of TSG *ST7* and *NM23* (Majumder et al., 2006; Pal et al., 2003). Similarly,

histone lysine methylation also has dual roles in transcriptional regulation. Most lysine methyltransferases are characterized by a catalytic SET (Su-var, Enhancer of Zeste and Trithorax) domain. It's revealed that disruptions of a great number of SET domain-containing proteins are associated with cancer. For example, the K4 (lysine 4) H3 methyltransferase SMYD3 was reported to enhance cell proliferation and overexpressed in most colorectal and hepatocellular carcinomas (Hamamoto et al., 2004). While another K4 H3 methyltransferase coding gene *MLL* (mixed lineage leukemia) was subjected to fusion with various genes and closely associated with the pathogenesis of multiple haematological malignancies (Santos-Rosa and Caldas, 2005). Recently, histone demethylation was also identified to be related with human cancers. For example, the arginine demethylase *PADI4* gene was demonstrated to be expressed in multiple tumors but not the corresponding normal tissues (Chang and Han, 2006). Besides, the lysine demethylase gene *LSD1* (Lysine-specific demethylase 1) that encodes a protein component of the NuRD complexes was shown to suppress breast cancer metastasis (Wang et al., 2009b).

1.2.3 RNA-associated gene silencing and carcinogenesis

The discovery that small exogenous RNA can act as a natural post-transcriptional gene silencing mechanism, a phenomenon named RNA interference (RNAi), is a stunning progress in molecular biology. RNAi is mediated by the microRNAs (miRNAs), a group of short functional RNA molecules with an average length of only 22 nucleotides (Fire et al., 1998). Firstly, the miRNA coding genes are transcribed into products called pri-miRNA hairpins of hundreds or thousands of

base-pairs in length. Then the pri-miRNA is transported out of the nucleus and cleaved in the cytoplasm by the RNase Dicer into short miRNA duplexes. Finally the generated mature single stranded miRNA (the complementary strand is usually quickly degraded) forms the RNA-induced silencing complex (RISC) together with Dicer and other proteins. Via the specific pairing of the miRNA and its target mRNA sequence, RISC is directed to the target transcript to degrade it or block its translation, resulting in the silencing of specific genes (Fire et al., 1998). MiRNAs are well conserved during evolution and are considered to play vital roles in various biological processes including development, cell proliferation, differentiation, apoptosis and so on. Accordingly, disrupted miRNA expression has been frequently detected in human diseases, including cancer (Mattick and Makunin, 2006).

The association of miRNAs with carcinogenesis attracted great interests and has been extensively studied in recent years. A number of miRNAs were identified to exert tumor suppressive or oncogenic roles via their ability to regulate essential pathways during carcinogenesis. For instance, *mir-15* and *mir-16*, two miRNA coding genes located in a frequent deleted region of chromosome 13 in chronic lymophoytic leukemia (CLL) were found to be lost or dramatically downregulated in malignant blood cells and were associated with CLL pathogenesis (Calin et al., 2002). Besides, another miRNA *let-7* acted like TSG by negatively regulating the expression of proto-oncogene *Ras* in cancers. As reported, reduced expression of *let-7* in lung cancer resulted in *Ras* overexpression and was correlated with poor prognosis (Johnson et al., 2005). On the other hand, the *mir-17-92* cluster was found

to be overexpressed in lymphoma and demonstrated oncogenic abilities of promoting cell proliferation, growth and angiogenesis (He et al., 2005). The characterization for the tight relations between miRNAs and cancer are still on-going. Recently, the conception that microRNAs might have the potential to be novel cancer therapeutic targets as well as biomarkers for cancer diagnosis and prognosis has been highlighted (Sassen et al., 2008).

1.3 Clinical implications of DNA methylation

1.3.1 DNA methylation as biomarkers in cancer

DNA methylation is especially suitable for use as biomarkers with several distinct advantages. First, DNA is much more stable than protein or RNA, which makes DNA-based markers easier to be obtained from most types of biological fluid (such as sputum, pancreatic juice and urine) and tissues (even formalin fixed samples). Second, DNA methylation status can be examined by PCR-based analysis with high sensitivity (such as methylation-specific PCR or MethyLight), even with the presence of an abundance of normal DNA. Third, compared with RNA or protein based analysis, the requirements for sample handling are not that strict in DNA methylation analysis (Kristensen et al., 2009).

DNA methylation as diagnostic markers: As discussed before, TSG methylation has been subjected to extensive studies in recent years and a great number of TSGs are identified with promoter hypermethylation in multiple tumors. At the same time, efforts have also been directed towards the revelation of unique

TSG promoter methylation profiles in individual cancers. Previous studies demonstrated another exciting fact that TSG hypermethylation might be an early event during carcinogenesis. For example, promoter hypermethylation of essential TSGs such as *p14*^{ARF}, *p16*^{INK4a} and *MGMT* was found in pre-cancerous adenomas of colon, while *MLH1* hypermethylation was often detected in atypical endometrial hyperplasia (Esteller, 2007). Stunningly, it's reported that *p16*^{INK4a} promoter methylation could be detected in the sputum of smokers as early as three years before the diagnosis of lung cancer (Palmisano et al., 2000). These findings implicate a promising role for TSG methylation as diagnostic marker for early cancer screening in high-risk groups.

DNA methylation as prognosis markers: Because of the unique advantage of DNA methylation analysis that it could be easily assayed with archived clinical materials, translational studies with accumulated patient samples and survival data in recent years successfully demonstrated that a number of TSGs silenced by promoter methylation could be used as potential makers for cancer prognosis. Pioneering studies showed that promoter methylation of a number of TSGs such as *p16*^{INK4a} and *DAPK* were significantly associated with cancer aggressiveness and reduced survival rate in lung and colon cancers (Esteller, 2007). Some other promising candidates, including *APC*, *CHD1*, *CHD13*, *RASSF1A*, *PCDH10*, *MGMT* and *TIMP3*, are functionally related to cancer proliferation, metastasis or angiogenesis (Brock et al., 2008; Brock et al., 2003). A combined methylation profile of these valuable TSGs markers is regarded to be able to elevate the accuracy in prognosis evaluation for

patients (Alaminos et al., 2004).

DNA methylation as a predictor for response to chemotherapy: The best examples by now demonstrating the response-predicting value of TSG methylation in chemotherapy came from the DNA repairing genes undergoing methylation-associated silencing in tumors. The *MGMT* gene mentioned earlier, which encodes a DNA repairing enzyme reversing the addition of alkyl groups to guanine, can resist the DNA attacking effects of alkylating chemotherapeutic drugs including carmustine, nimustine and carbazine. According to a study in gliomas, *MGMT* promoter methylation was significantly associated with responsiveness to alkylating agent carmustine and increased overall survival of patients (Esteller et al., 2000a). Similarly, later studies in glioblastomas also demonstrated that *MGMT* silencing by promoter methylation could be a predictive biomarker for favorable outcome after treatment with DNA alkylating agents (Hegi et al., 2005). In addition to *MGMT*, methylation of other drug-detoxifying genes with DNA repairing abilities such as *BRCA1*, *GSTP1* and *FANCF* can also confer chemo-sensitivity during chemotherapy. On the contrary, the methylation of pro-apoptotic genes such as *APAF 1* and *CASP8* was reported to bring along resistance to multiple anti-cancer chemicals (Glasspool et al., 2006).

1.3.2 DNA methylation as therapeutic targets in cancer

The reversibility of DNA methylation and histone modification offers potential therapeutic targets for novel epigenetic drugs, which may exert anti-cancer effects

via reactivating the epigenetically silenced TSGs. Blocking DNMT activities with nucleoside analogues 5-azacytidine (azacytidine) or 5-aza-2'-deoxycytidine (decitabine) can effectively reverse the promoter CpG methylation of multiple TSGs in cancer cells and show a significant tumor suppressive activity (Bender et al., 1998). These two drugs have already been approved by US FDA for clinical use in the treatment of myelodysplastic syndromes (MDS), a pre-leukemia disease. Recently, a few novel DNMT inhibitors were also demonstrated to have good prospects for future clinical use, such as 5-fluoro-2'deoxycytidine (Zebularine), epigallocatechin-3-gallate (EGCG) and RG108 (Kristensen et al., 2009). Clinical trails also suggested that the combined therapy using DNMT inhibitors and HDAC inhibitors together with other classical anti-cancer drugs can improve the overall survival rate of some malignancies (Gore and Hermes-DeSantis, 2008). Besides, the use of DNMT inhibitors or HDAC inhibitors before traditional chemotherapy is a promising strategy to prevent multi-drug resistance. Since the preceding epigenetic treatment can create an open chromatin conformation, which would thus allow subsequent drugs to access DNA more easily for a potent killing effect (Kristensen et al., 2009). Overall, there is no doubt that efficient epigenetic therapy combined with traditional cancer therapy has exciting prospects for the effective treatment of human cancers in future.

1.4 Identify novel TSGs via an integrative epigenetics and genomics approach

As mentioned earlier, genetic disruptions including mutations, deletions, LOH, copy number changes and chromosome instability which contribute to TSG

inactivation, provided various avenues for the identification of TSGs (Robertson et al., 1999). Considering the importance and prevalence of TSG epigenetic disruptions in cancers, it's reasonable to assume that the TSGs which are preferentially inactivated by epigenetic mechanisms, especially promoter hypermethylation, could alternatively be identified via epigenetic approaches instead of genetic ones (Baylin and Herman, 2000) . Actually, in the last few years, presence of aberrant promoter methylation has been successfully used as a marker for novel TSG screening. Using genome-wide methylation analyzing techniques such as CGI array, MS-RDA (methylation sensitive representational difference analysis) and RLGS (restriction landmark genomic scanning), a number of novel TSG candidates were successfully characterized, including *LOX* in gastric cancer, *SOCS1* in hepatocellular cancer and *BMP3B* in lung cancer (Dai et al., 2001; Kaneda et al., 2002; Yoshikawa et al., 2001).

In our lab, we have been using an integrative approach combining epigenetic and genomic assays in our practice of novel TSG identification in recent years. By array-CGH, we first revealed a number of genomic loci undergoing frequent copy number loss in ESCC and NPC cell lines, which might probably harbor TSGs. Next by analyzing the expression profile and the promoter methylation status of genes within the putative tumor suppressor loci in cancer cell lines, normal tissues, and immortalized epithelial cell lines, we could further identify the cancer-specific epigenetically silenced genes, which might be promising TSG candidates and could be further characterized in following functional studies.

By this approach, our group has identified several novel TSG candidates in both

NPC and ESCC, most of which were later proved to exert tumor suppressive functions in cancer cells. An excellent example highlighting the feasibility and advantage of our approach was the successful identification of *CHD5* gene as an epigenetically silenced TSG candidate in the present lab (Qian Tao et al., data not published). Using engineered mouse models, Bagchi et al. demonstrated that *CHD5* in 1p36, a frequently deleted region in human cancers, was a functional tumor suppressor and implicated *CHD5* deletions in primary malignancies (Bagchi et al., 2007). However, early before they reported *CHD5* as a 1p36 TSG, our lab had already identified this gene as a TSG candidate frequently silenced by promoter methylation in multiple cancers, via screening the expression and methylation status of genes located in a 1p36 copy loss locus which was revealed by array-CGH in multiple NPC cell lines. Other successful examples included *BLU* in 3p21.3, *PCDH10* in 4q28.3, *RASAL* in 12q24.13, and *CMTM3* in 16q22.1, which were all novel epigenetically silenced tumor suppressor candidates identified by this integrative genomic and epigenetic approach (Qiu et al., 2004; Wang et al., 2009a; Jin et al., 2007a; Ying et al., 2006).

1.5 Genetic and epigenetic disruptions in ESCC and NPC

ESCC and NPC, two common malignancies in Hong Kong and mainland China, are selected as cancer models to study in this project. General knowledge about these two cancers and the genetic/epigenetic disruptions revealed for them are briefly reviewed as below.

1) Esophageal squamous cell carcinoma (ESCC) is the sixth most fatal cancer worldwide and accounts for 90% cases of all esophageal cancers (Allen et al., 1997; Parkin et al., 2005). Although the overall ESCC incidence is comparatively low in the Western countries, this malignant disease is quite common in Asian, southern and eastern Africa, and northwestern France (Holmes and Vaughan, 2007; Sarah and David, 2008). In some districts of north and central China the incidence even exceeded 100 cases per 100,000 people per year (Lambert and Hainaut, 2007). Another noticeable fact is that the prognosis for patients with ESCC still remains poor, with a 5-year survival rate of about 10%, though the overall effectiveness of surgical and medical therapies kept improving for human cancers in recent years (Sarah and David, 2008; Holmes and Vaughan, 2007). Thus to elucidate the molecular mechanism in ESCC development with the aim of developing effective prevention and early-diagnosis strategies is of great value for ESCC intervention.

Like in most other cancers, environmental and genetic/epigenetic factors contribute to ESCC pathogenesis. Tobacco, alcohol, hot drink/food, dietary deficiency and achalasia have been considered as major environmental risk factors for ESCC as revealed by epidemiology studies (Sarah and David, 2008; Allen et al., 1997). Cytogenetic and molecular genetic analyses demonstrated that lots of genetic abnormalities were involved in ESCC, including various chromosomal losses and gains. Based on the published literatures, frequently detected chromosomal amplifications in ESCC are located in 1q, 3q, 4q, 5p, 8p, 7q22, 10q21, 11q13 and 20q, while deletions and LOH are commonly detected in 3p, 5q, 9p, 9q, 13q, 17p,

17q and 18q (Shimada et al., 1997; Montesano et al., 1996). As for the affected cancer-related genes, the most frequently activated oncogenes in ESCC include *c-erbB1* (7p12-13), *c-erbB2* (17q21), *c-Myc* (8q24), *cyclin D1* (11q13), *Int-2* (11q13) and *Hst-1* (11q13), which are primarily activated by amplification and over-expression (Lam, 2000). On the other hand, *APC* (5q21), *DLC1* (3p21.3), *MCC* (5q21), $p16^{INK4a}$ (9p21), *RB* (13q14), *TP53* (17p13), and *WWOX* (16q23.2-24.1) are among the most commonly inactivated TSGs in ESCC, due to either genetic mutation (e.g. *TP53*), LOH (e.g. *RB*) or hypermethylation of promoter CGI (e.g. $p16^{INK4a}$) (McCabe and Dlamini, 2005).

Promoter hypermethylation of TSG is a common epigenetic event in ESCC. In addition to the TSGs mentioned above, a number of other well-characterized TSGs and some TSG candidates have also been reported to be inactivated in ESCC, predominantly by promoter hypermethylation instead of genetic lesions (Table 1-2, Page 37). Most of these epigenetically affected genes encode key molecules in various cancer-related signaling pathways, such as cell cycle controlling genes $p15^{INK4b}$ and *FHIT*; pro-apoptosis genes *DAPK*, *UCHL1* and *TP73*; DNA repairing genes *MLH1*, *MGMT* and *CHFR*; cell adhesion regulating genes *CAMD1*, *CDH1*, *CHD13* and *DCC*; Ras negative regulating genes *RASFF1A* and *RASAL*; growth factor receptor genes *RARB* and *ESR1*, and so on (Zhao and Casson, 2008). The list of methylated genes identified in ESCC has kept growing in recent years. There were approximately forty genes shown to be methylated in either primary ESCC or cell lines as summarized by our group in middle 2007. Thus far, key word searching in

the PubMed database demonstrated that about forty additional methylated genes have been reported in ESCC, making the total number of ESCC associated methylated genes to approximate eighty. Most of these newly characterized methylated genes were indicated to have TSG functions in either ESCC or other tumors, such as *TIMP3*, *PLCD1* and *PCAF* (Smith et al., 2008; Hu et al., 2009; Zhu et al., 2009).

2) Nasopharyngeal carcinoma (NPC) is a special head and neck malignancy arising from the epithelial cells of nasopharynx and has distinct characteristics in its geographic and ethic distributions all over the world (Spano et al., 2003). Although NPC is quite rare in other parts of the world even in the neighboring oriental countries, it is a prevalent malignancy in South China, especially in Hong Kong and Guangdong province, with an incidence as high as 30 per 100,000 people per year (Tao and Chan, 2007). Moreover, NPC incidence in oversea descendants of Cantonese people from South China was shown to be significantly higher than the incidence in people of other ethnic origins (Ho et al., 1990). According to data from Hong Kong Cancer Registry in 2002, NPC ranked as the fourth fatal malignancy in Hong Kong, but it was the primary death-causing cancer in local males aged from 20 to 44, further highlighting NPC as a severe heath threat in Hong Kong (Tao and Chan, 2007).

Previous studies demonstrated that NPC pathogenesis was also a multi-step process, similar to the situation observed in other malignancies such as colon cancer. The major etiology factors involved in this process include Epstein-Barr virus (EBV) infection, chemical carcinogen exposure in early ages as well as genetic

susceptibility (Lee et al., 2003). Genetic alterations in NPC have been extensively studied by cytogenetic or CGH studies in the last few years and lots of chromosomal losses and amplifications have been identified. The major chromosomal regions with frequent alterations in NPC were finely summarized in a review paper on NPC by our group (Tao and Chan, 2007).

A great abundance of oncogenes and TSGs with disrupted functions or expression levels have also been correlated with NPC pathogenesis. Key oncogenes overexpressed or amplified in other human cancers are also commonly activated in NPC, including *BCL2*, *CCND1*, *EGFR*, *HER2*, *MDM2*, *ID1*, *Met*, *c-Myc* and *TNFAIP3*. Accordingly, lots of TSGs are shown to be inactivated by both genetic and epigenetic disruptions in NPC, such as *CASP8*, *CDH1*, $p16^{INK4a}$, *CHFR*, *DAPK*, *DLC1*, *MLH1*, *RASSF1A*, *WIF1* and so on (Table 1-2). However, alterations of two essential TSGs *TP53* and *RB* are rather rare in primary NPC. Instead, disruptions of the p53 network and the $RB/p16^{INK4a}$ cell cycle controlling machinery was frequently detected in NPC, which is due to promoter hypermethylation of $p14^{ARF}$ and $p16^{INK4a}$ (Tao and Chan, 2007). Like ESCC, the list of methylated gene identified in NPC has also been growing in recent years. According to a review by Tao et al., before 2007 were nearly 30 genes identified to undergo frequent promoter methylation in primary NPC or cell lines. By now this number has reached nearly 50, with *IRF8*, *PCDH10* and *MIPOL1* as examples of newly identified methylated TSG candidates (Ying et al., 2006; Lee et al., 2008; Cheung et al., 2009), It has been estimated that there existed an average of 100 to 400 methylated cancer-related genes in a given type of tumor

Table 1-2. Summary of major TSGs silenced by promoter methylation in ESCC and NPC. Partially adopted from a review paper (Tao and Chan, 2007).

Gene	Location	Functions	References
APC	5q21-q22	Cell polarity and chromosome segregation	(Kawakami et al., . 2000; Kwong et al., 2002)
TSLC1	11q23.2	Cell adhesion	(Ito et al., 2003; Hui et al., 2003)
CDH1	16q22.1	Cell adhesion, proliferation, metastasis	(Si et al., 2001; Tsao et al., 2003)
CDH13	16q24.2-q24.3	Cell adhesion, proliferation, metastasis	(Sun et al., 2007; Hibi et al., 2004)
$p14^{ARF}$	9p21	Stabilizing p53, cell cycle control	(Kwong et al., 2002; Nie et al., 2002)
$p16^{INK4a}$	9p21	Cell cycle control	(Nie et al., 2002; Xing et al., 1999)
$p15^{INK4b}$	9p21	Cell cycle control	(Xing et al., 1999; Kwong et al., 2002)
CHFR	12q24.33	Cell cycle control	(Shibata et al., 2002; Cheung et al., 2005)
DAPK	9q34.1	Apoptosis	(Kuester et al., 2007; Chang et al., 2003)
DCC (ESCC only)	18q21.3	Cell adhesion, differentiation, Apoptosis	(Park et al., 2008)
DLC1	8p22	Cytoskeleton organization, signal transduction, cell adhesion	(Seng et al., 2007)
DLEC1	3p22-p21.3	Signal transduction	(Ayadi et al., 2008; Daigo et al., 1999)
EDNRB	13q22	Endothelin receptor type B	(Zhou et al., 2007a; Zhao et al., 2009)
FHIT (ESCC only)	3p14.2	Cell cycle control, DNA-damage response	(Tanaka et al., 1998)
MGMT	10q26	DNA repairing	(Kwong et al., 2002; Zhang et al., 2003)
MLH1	3p21.3	DNA mismatch repair protein, cell cycle control	(Guo et al., 2006; Wong et al., 2004)
RASSF1A	3p21.3	Cell cycle control, apoptosis, DNA repair	(Lo et al., 2001; Kuroki et al., 2003)
THY1/CD90 (NPC only)	11q23.3	Cell adhesion, cell cytoskeleton, angiogenesis	(Lung et al., 2005)
TP73	1p36.3	Cell cycle, DNA damage response, apoptosis	(Wong et al., 2003; Alonso et al., 2003)
UCHL1	4p14	Cell growth inhibition, apoptosis	(Muzeau et al., 1997; Mandelker et al., 2005)
WIF1	12q14.3	WNT-signaling pathway inhibitor	(Chan et al., 2007; Lin et al., 2006)

(Esteller, 2007). For NPC, there is no doubt that further studies have to be carried out in order to reveal more epigenetically silenced genes, which can not only expand our understanding in the pathogenesis of this distinct cancer, but also may provide potential biomarkers for non-invasive early diagnosis and prognosis.

1.6 Involvement of 11q and 15q in carcinogenesis

1.6.1 Allelic loss of chromosome 11q and carcinogenesis

Numerous reports indicate that disruption of 11q is an important contributing factor during carcinogenesis, with LOH or deletion of multiple 11q loci frequently detected in lots of cancer types. For instance, 11q loss has been revealed as one of the most frequent cytogenetic characteristics in neuroblastoma. Maris et al. detected LOH of 11q in 44% of 129 primary neuroblastoma samples and defined a common LOH region spanning 11q14-23 (Maris et al., 2001; Breen et al., 2000). Besides, LOH in 11q, especially 11q23, has recently been associated with decreased survival as well as increased metastasis relapses in neuroblastoma patients (Spitz et al., 2006; Attiyeh et al., 2005). Actually, LOH of various loci in region 11q21-24 has also been detected with high frequency in multiple other malignancies including melanoma as well as colorectal, breast, cervical, ovarian and lung cancers (Davis et al., 1996; Tomlinson et al., 1996; Hampton et al., 1994; Carter et al., 1994; Bethwaite et al., 1995; Petzmann et al., 2001). In addition to 11q21-24, allelic loss in 11q13.1, 11q14.3-11q21 and 11q25 has also been reported in lung and breast cancers, as well as retinoblastoma (Petzmann et al., 2001; Boelens et al., 2009; Ganguly et al., 2009;

Gentile et al., 2001).

Available literature on cytogenetic studies for ESCC and NPC demonstrates that 11q loss is also a common phenomenon in both cancers. Allelic losses of 11q were among the most frequent LOH events detected in primary ESCC cases from both China and Japan (Matsumoto et al., 2004; Qin et al., 2008). In the study by Pack et al, an overlapping frequently lost region was mapped to 11q14-22 in 59% of 17 primary ESCC samples (Pack et al., 1999). For NPC, LOH in 11q14-qter was detected in 70% of 20 primary cases from a Hong Kong cohort, while another study in Hong Kong revealed LOH of 11q22-24 in 54% of 52 NPC cases (Hui et al., 1999; Hui et al., 1996). Theses reports suggest that 11q loss might contribute to the pathogenesis of both ESCC and NPC.

The high frequency of 11q loss observed in more than one cancer types implies the presence of important TSGs. Actually, a few important TSGs have already been characterized in several frequently lost 11q loci, including *ATM* in 11q22.3-23.1, *MEN1* in 11q13, *PPP2R1B* in 11q23.2 and *SDHD* in 11q23 (Wang et al., 1998; Stilgenbauer et al., 1997; Vorechovsky et al., 1997; Bystrom et al., 1990; Baysal et al., 2000). Most recently, *CADM1* in 11q23.3 has been identified to be a functional TSG silenced by promoter hypermethylation in non-small-cell lung cancer (NSCLC) and multiple other cancers (Kuramochi et al., 2001; Murakami, 2005; Ito et al., 2003). The great abundance of TSGs identified in 11q until now strengthened the importance of 11q disruptions in tumorigenesis. Hence, it is reasonable to screen for more novel TSG candidates potentially harbored in 11q, regarding the high

frequency of 11q allelic loss detected in multiple tumors.

1.6.2 Allelic loss of chromosome 15q and carcinogenesis

LOH or deletion of 15q were also frequently reported in multiple cancer types including mesothelioma, lung, breast, ovarian, cervical, prostate, bladder, colorectal, as well as head and neck cancers (Poetsch and Kleist, 2006; Beder et al., 2003; Park et al., 2000; Kersemaekers et al., 1998; Ma et al., 2007; Cher et al., 1996; Natrajan et al., 2003; Wick et al., 1996).

For instance, LOH anaysis revealed a frequent allelic loss locus at 15q11.1-15 in 10 of 13 malignant mesotheliomas and in 13 of 24 malignant mesothelioma cell lines, which was further refined to 15q15 using fluorescence *in situ* hybridization (FISH) (Murthy and Testa, 1999; De Rienzo et al., 2001). In lung cancers, LOH at 15q12 was identified in more than 60% of thirty-six cancer cell lines using 399 fluorescent microsatellite markers (Girard et al., 2000). In addition, high incidence (70%) of allelic loss in 15q was detected in metastases from primary breast cancer and a common overlapping lost region at 15q14 was correlated with progression to a metastatic stage of breast cancer (Wick et al., 1996). Besides, LOH in 15q26.2 was also shown to be a frequent genetic event in breast cancer by analyzing 33 pairs of normal and breast cancer DNA samples (Paez et al., 2001). In ovarian cancer, LOH of 15q15-22 was detected in 36% of 37 primary cases (Cliby et al., 1993) and this region was found to be lost to a significantly greater extent in high grade cancers compared to low grade cancers (Dodson et al., 1993). In cervical cancer, LOH was

observed at 15q11-21.1 with a frequency of more than 25% (Kersemaekers et al., 1998). In prostate cancer, frequent LOH (39%) involving 15q11.1-15 was also detected (Cher et al., 1996). In addition, LOH of 15q14-15q15.3 was found in 20 of 51 (39%) bladder transitional cell carcinoma samples, suggesting the 15q15.1 DNA repair gene *RAD51* to be a candidate TSG for this malignancy (Natrajan et al., 2003). Recently, a high-density LOH study with 13 polymorphic markers spanning 15q15.3-q22.1 in 70 cases of sporadic colorectal cancer indicated that 15q21.1 might harbor a TSG involved in colorectal cancer carcinogenesis (Park et al., 2000). Finally, LOH in 15q21.3 was also shown to be an important characteristic for head and neck squamous cell carcinomas with risk of progression (Poetsch and Kleist, 2006).

Above reports provided strong support for the involvement of 15q disruption in carcinogenesis, and suggested that 15q could possibly harbor one or more functionally important TSGs. Actually, by now a number of tumor suppressor candidates have already been mapped to the frequent LOH regions in 15q, including the TGF β pathway genes *SMAD3* and *SMAD6* in 15q21-22 revealed in colorectal cancer, DNA repair gene *RAD51* in 15q15.1 revealed in bladder transitional cell carcinoma, and a cell-to-cell and cell-to-matrix interaction mediator gene thrombospondin (*THBS1*) in 15q21.1 revealed in pancreatic cancer (Park et al., 2000; Natrajan et al., 2003; Jonson et al., 1999).

1.7 RAB small GTPase and carcinogenesis

Small GTPases (small guanosine triphosphatase) are a large group of cytosol monomeric G proteins sharing homology with the alpha subunit of heterotrimeric G

protein (Gα). Unlike Gα which formed a functional "large" G protein with other subunits, small GTPase can function independently via its innate GTPase activity and switch between the GTP-bound activated form and the GDP-bound inactivated form, upon the catalysis by the GDP/GTP exchange factor (GEF) and GTPase-activating protein (GAP) (Fig. 1-4). Human small GTPases consist of more that 150 members and are also referred as Ras super family of small GTPase due to its well known family member Ras. Based on the similarities in sequence and function, family members are categorized into five major subgroups including Ras, Ras, Rho, Rab, Ran and Arf (Wennerberg et al., 2005; Vetter and Wittinghofer, 2001; Colicelli, 2004).

RAB (originally referred as Ras-like proteins in brain) small GTPase family is the largest subgroup of Ras superfamily in human and is comprised of more than 60 members, a number of which remain highly conserved between yeast and human (Stenmark and Olkkonen, 2001). RAB proteins are localized upon the cytosolic face membranes of distinct intracellular organelles, such as Golgi, endoplasmic reticulum and endosome, and are involved in both the exocytic and endocytic pathways that facilitate membrane trafficking, a flowing process of membrane and protein within cells. Via this continuous trafficking, various essential cellular processes are carried out, including sorting of synthesized proteins to their exact destinations, intake of extracellular nutrients and internalization of membrane receptors. By interacting with a group of RAB effector proteins in their GTP-bound activated forms, RAB proteins play an important role in the regulation of all major steps in membrane trafficking,

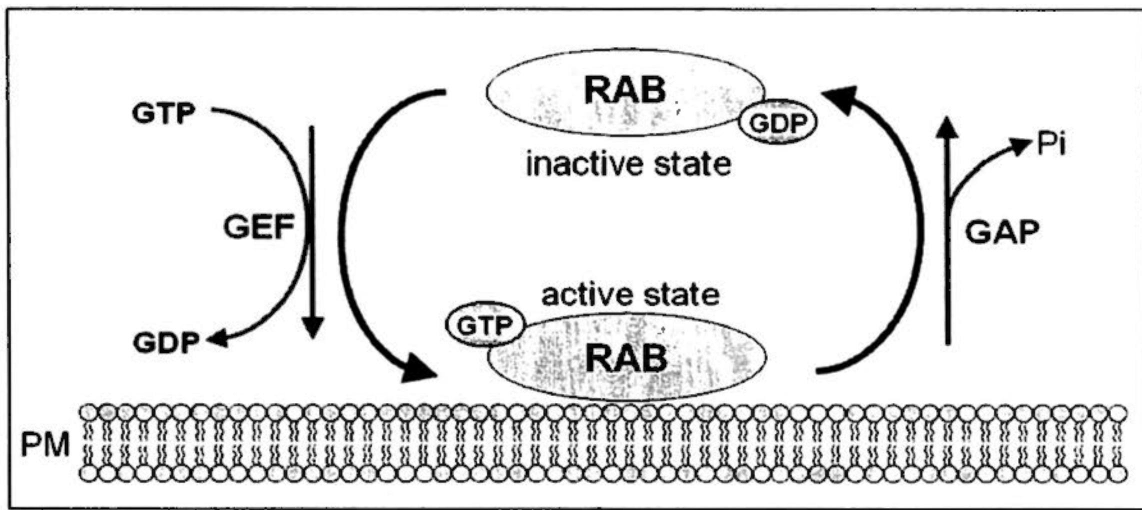

Figure 1-4. Diagram showing the cycling of RAB GTPase. RAB GTPase can switch between the GTP-bound active and GDP-bound inactive forms with different conformations. GEF, GDP/GTP exchange factor, catalyzes the conversion from GDP- to GTP- bound active forms and makes the RAB protein able to interact with effector molecules. While GAP, GTPase-activating protein, facilitates GTP hydrolysis and the conversion from GTP- to GDP- bound inactivated state. Pi, orthophosphate; PM, plasma membrane.

including the budding, delivery, tethering and membrane fusion of transporting vesicles (Touchot et al., 1987; Stenmark and Olkkonen, 2001; Pereira-Leal and Seabra, 2001; Grosshans et al., 2006). Recently, the role of RAB proteins in signal transduction has caught more and more attention. It's indicated that some RAB members could act as key molecules regulating multiple signaling pathways involved in cell proliferation, cell immune response, and compartment fragmentation during mitosis and apoptosis (Delcroix et al., 2003; Bucci and Chiariello, 2006; De Matteis and Godi, 2004).

Alteration of some RAB GTPase, their interacting proteins and regulatory molecules has been associated with a few human diseases. For example, genetic mutations in *RAB27A* or *MYO5A*, the coding gene of a putative *RAB27A* effector can lead to the hereditary Griscelli syndrome which is characterized by partial albinism

with immunological defects or neurological dysfunctions (Pastural et al., 1997; Menasche et al., 2000). Besides, hereditary Choroideremia, a retinal degradation disease leading to blindness, is caused by mutations in the gene encoding the RAB escort protein 1 (*REP-1*), which facilitates the post-translational modification of newly synthesized RAB proteins and delivers them to membranes of appropriate organelles (Seabra et al., 1995; Seabra et al., 1993; Alexandrov et al., 1994).

However, the potential roles of RAB proteins during carcinogenesis are not discovered until most recently. *RAB25* has recently been identified as an amplified gene in ovarian and breast cancers via high-density array CGH analysis. A significant elevated mRNA level of *RAB25* was detected in 89% ovarian cancers and 67% breast cancers when compared with normal tissues, and was associated with dramatically decreased overall survival of both ovarian and breast cancer patients. Functional experiments demonstrated that by activating AKT signaling and decreasing the levels of pro-apoptotic BCL2 members, *RAB25* could dramatically increase cell proliferation and inhibit apoptosis *in vitro*, while elevate aggressiveness of cancer cells *in vivo* (Cheng et al., 2004). Further studies suggested that *RA25* could enhance tumor cell invasion by directing the localization of integrin-containing vesicles via its association with $\alpha5\beta1$ integrin (Caswell et al., 2007). *RAB25* was the first RAB family member extensively studied for its contribution to tumor progression and aggressiveness. In addition, other members including *RAB1*, *RAB5A*, and *RAB23* were also reported to be elevated in some human cancers such as tongue squamous cell carcinoma, hepatocellular carcinoma and gastric cancer, implicating

the involvement of RAB GTPase in tumor aggressiveness (Fukui et al., 2007; Hou et al., 2008; Shimada et al., 2005).

On the other hand, there is also emerging evidence supporting that some other RAB members are downregulated in cancers and possess potential tumor suppressive activities. For instance, *RAB37* mRNA level was found to be downregulated in 48% (34/71) NSCLC patients and promoter hypermethylation of *RAB37* was detected in 58% (41/71) of patients, suggesting the tumor-specific epigenetic silencing of this gene (Wu et al., 2009). Importantly, decreased *RAB37* mRNA level was significantly associated with metastasis within lung (Wu et al., 2009). Another RAB member, *RAB45*, also known as *RASEF*, was recently identified as a TSG in 9p21, a potential TSG region revealed by segregation study in uveal and cutaneous melanoma families. Moreover, *RAB45* promoter hypermethylation in primary tumors was significantly associated with decreased survival (Maat et al., 2008). Besides, aberrant downregulation of *Rab33A* due to promoter methylation was frequently detected in melanoma and seemed to be an early event prevalent in the pre-cancer melanocyptes of giant congenital nevi (Cheng et al., 2006). In addition, *RAB32* was found to be methylated in 14 of 25 microsatellite instability colon cancers but in none of 23 normal colon specimens, and promoter methylation of *RAB32* was proved to be correlated with its transcriptional downregulation in primary colon cancers (Mori et al., 2004). Although the mechanism by which the downregulation of these RAB members contributes to cancer initiation or progression is still not well elucidated, functional study of some other family members actually shed light on the

anti-tumorigenic role of RAB proteins. Recently, *Rab7* has been shown to regulate the internalization and degradation of nutrient transporters, and in turn trigger nutrient starvation and cell death, demonstrating that it could act as a pro-apoptotic protein and prevent cell-autonomous growth and survival (Edinger et al., 2003).

Considerable evidence suggested that RAB proteins may have multiple roles in cancers, with some members elevated while others downregulated in different malignancies. Interestingly, in the latter case promoter hypermethylation was shown to be the prevailing mechanism resulting in the decreased expression of RAB members in a variety of cancers. Further study in the cancer related epigenetic and genetic disruptions of RAB GTPase, as well as their functional roles during cancer initiation and progression, may provide new insights into the association between cancer and membrane trafficking, a process beginning to enter the spotlight in cancer research.

1.8 WD40-repeat containing proteins and carcinogenesis

WD40-repeat containing (WDR) protein family is comprised of a large group of structurally similar but functionally distinct members, which are mostly found in higher eukaryotes (Smith et al., 1999). It's predicted that there are totally 136 WDR proteins in human and each of them usually contains 4 to 16 repeating WD40 units, which is a short conserved structural motif of approximately 40-60 amino acids initiated in a glycine-histidine (G-H) dipeptide and terminated in a Trp-Asp (W-D) dipeptide (Venter, 2002). The amino acids between the G-H and W-D dipeptides are found to be well-conserved in terms of basic sequence pattern (Clemen et al., 2008).

Table 1-3. Diverse biological functions of WD repeat proteins. Adopted from a review paper (Li and Roberts, 2001).

Function	**Examples of WD repeat family member involved**
Signal transduction	Gβ, RACK1, LIS1, PR55
RNA synthesis and processing	TAFs (TATA box-binding protein associated factors), TUP1
Chromatin assembly	CAF-1 (chromatin assembly factor-1), HIR1 and HIIR2 transcriptional repressors
Vesicular trafficking	Golgi coat promoter or "coatomer", most of α and β COP proteins
Cytoskeletal assembly	MAP (microtubule-associated protein)
Cell cycle regulation	CDC4, CDC20, CDC40, MAD2
Programmed cell death	Apaf-1 (apoptotic protease activating factor-1)
Function unknown	WDR1, WDR3, WDR4

These WD40-repeats altogether form a circularized beta-propeller-like structure, which might act as scaffolds for protein interaction via forming functional complexes with other protein components (Smith et al., 1999). Functionally, WDR proteins are associated with a diverse variety of cellular processes that could be roughly categorized into eight groups, including signal transduction, transcription regulation, cell cycle control and so on, as summarized in Table 1-3 (Li and Roberts, 2001). Although the functions of a number of WDR proteins have been defined, the roles of the majority of WDR members still remained unknown.

Considering the facts that sequences of WDR family members are kept well-conserved among species during evolution and that multiple members are involved in diverse vital processes, it's reasonable to predict that WDR protein might also be related to human diseases. Actually, mutations of a number of WDR protein-encoding genes have been shown to cause a variety of human hereditary diseases. For example: 1) mutations in *LIS1* gene cause lissencephaly, which means

smooth brain; 2) mutations in *CSB* gene cause Cockayne syndrome, a disease characterized by abnormal and slow development after birth; 3) mutations in the *TBL1* gene are associated with late-onset sensorineural deafness; 4) mutations in the *AAAS* gene cause Triple-A syndrome characterized by adrenocorticotropin hormone resistant adrenal insufficiency (Tullio-Pelet et al., 2000; LoNigro et al., 1997; Henning et al., 1995; Bassi et al., 1999).

More recently, it was reported that the genetic and epigenetic disruptions of some WDR genes were associated with tumorigenesis in some malignancies. For instance, *FBW7* (F-box and WD repeat domain-containing 7) gene that encodes a WDR protein containing eight WD40-repeat motifs was identified as an important TSG with mutations and promoter hypermethylation occurring in multiple human malignancies (Welcker and Clurman, 2008; Akhoondi et al., 2007; Gu et al., 2008). *FBW7* acted as the substrate recognition component of the SCF (complex of SKP1, CUL1 and F-box protein)-type ubiquitin ligase which mediated the degradation of a number of proto-oncogenes including *c-Myc*, *cyclin E*, *Notch* and *JUN*. The WD40-repeats in FBW7 protein were especially essential for the recognition of phosphorylated substrates, since mutations in the WD40 formed beta-propeller domain could severely impair the FBW7-mediated c-Myc degradation (Orlicky et al., 2003; Hao et al., 2007; Welcker et al., 2004). One of the WDR family members, WDR6, was found to be the interacting protein of the serine/threonine kinase LKB1. Mutations of the *LKB1* gene led to Peutz-Jeghers syndrome which was associated with elevated incidences of malignancies. It was demonstrated that the co-expression

of *WDR6* with *LKB1* could significantly inhibit tumor cell growth, by up-regulating the stimulatory effect of *LKB1* on the promoter activity and protein level of CDK inhibitor $p27^{Kip1}$ (Xie et al., 2007). In addition, another WDR gene *WDR11* was shown to be disrupted by chromosome translocation in human glioblastoma cells (Katoh and Katoh, 2003; Chernova et al., 2001). After all, considering the abundance and diverse functions of WDR proteins, our understanding for their roles in carcinogenesis still remains quite limited.

1.9 ADAMTS proteins and carcinogenesis.

ADAMTS (a disintegrin and metalloproteinase domain with thrombospondin motifs) proteins are a family of extracellular, multi-domain endopeptidases found in both mammals and invertebrates. A typical ADAMTS protein is usually comprised of a signal peptide, a metalloproteinase catalytic domain, a disintegrin-like domain, a central TSP-1 (thrombospondin type 1-like) repeat and a variable number of c-terminal TSP-1 repeats (Apte, 2004). Human ADAMTS family consists of nineteen members that perform diverse protease-related functions including procollagen processing, cleavage of the matrix proteoglycan, inhibition of angiogenesis and blood coagulation homoeostasis. According to structure similarities and substrates specificity, human ADAMTS proteins can be categorized into seven subfamilies: 1) cartilage aggrecan peptidases ADMATS1, 4, 5, 8 and 15; 2) amino-procollagen peptidases ADAMTS2, 3 and 14; 3) von Willebrand factor peptidases ADAMTS13; 4) GON-ADAMTS proteins ADAMTS9 and 20; 5) ADAMTS6, 7, 10 and 12; 6) ADAMTS16 and 18; 7) ADAMTS17 and 19 (Porter et al., 2005). The physiological

and pathological roles of ADAMTS proteins have been implicated in lots of essential biological processes such as organogenesis, inflammation and fertility, as well as in a series of human diseases including arthritis, asthma and cancers (Porter et al., 2005).

Recently, genetic deletion, downregulated expression level and promoter hypermethylation of some ADAMTS members have been reported in a few cancers, suggesting that these proteases may have tumor suppressor-like function. In addition, *in vivo* studies demonstrated that some members could negatively regulate tumor cell growth via inhibiting of important growth-promoting pathways and suppressing angiogenesis, strengthening the TSG roles ADAMTS proteins might play in cancers (Lo et al., 2007; Viloria et al., 2009; El Hour et al., 2010).

For instance, *ADAMTS9* was identified as a candidate ESCC TSG in a critical TSG locus 3p14.2, which was also a translocation breakpoint in hereditary renal cell carcinoma and frequently deleted in breast cancers (Lo et al., 2007). Downregulation of *ADAMTS9* was detected in ovarian, renal, and breast cancers when compared with normal tissues (Porter et al., 2005). Moreover, epigenetic silencing of *ADAMTS9* by promoter methylation was significantly associated with lymph node metastases in NPC (Lung et al., 2008). Another family member *ADAMTS12* was previously found to act as an anti-tumorigenic protease by suppressing the MAPK/ERK pathway in lung caner and was also epigenetically silenced by methylation in tumor cells during the progression of colon cancer (Moncada-Pazos et al., 2009; Llamazares et al., 2007). Furthermore, a recent study using knockout mice models suggested that *ADAMTS12* exerted tumor suppressive activity and inhibit angiogenesis *in vivo* (El

Hour et al., 2010). Most recently, a novel family member *ADAMTS15* was found to be genetically inactivated in colon cancer in a mutational analysis for 50 primary tumors. Functional studies revealed that *ADAMTS15* could negatively regulate tumor cell growth and invasion through antagonizing the MAPK/ERK pathway. Importantly, immunohistochemistry revealed that the protein level of *ADAMTS15* was negatively correlated with the differentiation grades in colon adenocarcinoma and could be used as an effective differentiation marker (Viloria et al., 2009). In addition to the three members mentioned above, disrupted expression and promoter methylation of several other members such as *ADAMTS1* and *ADAMTS18* were also reported in multiple cancers (Jin et al., 2007b; Lind et al., 2006).

Chapter 2, Aims of this study

Cancer is a complex disease involving abundant genetic and epigenetic disruptions, which result in the malfunctions of multiple tumor suppressor genes and proto-oncogenes. Due to the great diversity of biological processes that TSGs participate in, inactivation of TSGs confers conspicuous favorable advantages to pre-cancer or transformed cells, in aspects of proliferation, invasion, metastasis, and angiogenesis. Identifying novel TSGs and studying their anti-tumorigenic mechanisms can not only help to elucidate the molecular pathogenesis of cancer but also contribute to the development of improved approaches regarding the detection, classification, prognosis, and treatment of cancer.

The discovery of genetic mechanisms recruited to inactivate TSG by cancers brought along a number of techniques to identify TSGs genome-widely, including array-CGH which could efficiently detect genetic loci undergoing frequent copy number loss in cancers. In addition to genetic alterations, epigenetic mechanisms represent another group of mechanisms leading to TSG silencing, such as promoter CpG methylation, which has already been successfully used for the identification of novel TSG candidates. By combining aCGH and methylation status analysis, our lab has been using an integrative genomic and epigenetic approach for TSG identification in both ESCC and NPC, with a number of novel TSG candidates successfully characterized in the last few years.

This study is designed and performed based on previous data in the lab, as a

continuous effort for our practice in identifying novel TSG candidates by this integrative approach. The aims and plans of this study are briefly stated as below:

1) To identify novel TSG candidates in four 11q and 15q copy number loss loci revealed by aCGH in ESCC cell lines: A 3040 BAC clone based 1-Mb high resolution aCGH study was carried out previously to identify copy number changes in the whole human genome in ten ESCC cell lines (Tao et al., unpublished data). As a result, a number of 11q and 15q loci with frequent copy number loss in multiple ESCC cell lines were revealed in 11q22.3, 15q14, 15q21.1 and 15q21.3, respectively. Based on literature review in chapter one, LOH or deletions involving above chromosomal regions have been implicated in multiple other tumors, suggesting that these copy loss loci might potentially harbor TSGs. The first aim of this study is thus to identify novel epigenetically silenced TSG candidates in these four putative TSG loci using combined mRNA-level expression profiling and methylation status analysis. Meanwhile, functional studies such as colony formation assay will be performed to characterize the tumor suppressive roles of the identified candidate genes.

2) To study the tumor suppressive mechanism of a previously identified 11q25 candidate TSG *ADAMTS8*: Previous study focused on a putative TSG locus at 11q25, which was revealed by aCGH in a panel of NPC cell lines (Tao et al., unpublished data), identified several epigenetically silenced genes in multiple cancers including *ADAMTS8*, a member of the ADAMTS metalloprotease family. Two members of this family, *ADAMTS12* and *ADAMTS15*, have recently been

characterized as negative modulators of the ERK pathway in cancers, which exhibited anti-tumorigenic activity both *in vitro* and *in vivo* (Viloria et al., 2009; Llamazares et al., 2007). Interestingly, based on structure and substrate similarities, *ADAMTS8* is categorized into the same subfamily as *ADAMTS15*. Thus it's quite intriguing to explore whether *ADAMTS8* can also act as a potential TSG via repressing ERK pathway, which is the second aim of this study.

Chapter 3, Materials and Methods

3.1 Cancer/immortalized epithelial cell lines and their maintenance

A variety of cancer cell lines arising from different carcinomas were used in this study, including eighteen ESCC cell lines (EC1, EC18, EC109, HKESC1, HKESC2, HKESC3, KYSE30, KYSE70, KYSE140, KYSE150, KYSE180, KYSE220, KYSE 270, KYSE410, KYSE450, KYSE510, KYSE520 and SLMT-1), six NPC cell lines (C666-1, CNE1, CNE2, HK1, HNE1 and HONE1), seventeen gastric carcinoma cell lines (AGS, KatoIII, MKN28, MKN45, NCI87, YCC1, YCC2, YCC3, YCC6, YCC7, YCC9, YCC10, YCC11, YCC16, SNU1, SNU16 and SNU719), four colorectal carcinoma cell lines (HCT116, HT29, SW480 and LoVo), four cervical carcinoma cell lines (C33A, CaSki, Hela and SiHa), three prostate carcinoma cell lines (DU145, LnCap and PC3), thirteen hepatocellular carcinoma cell lines (Hep3B, HepG2, Huh1, Huh4, Huh6, Huh7, Mahlavu, PLC/PRF/5, SNU387, SNU398, SNU423, SNU449 and SNU475), seven lung cancer cell lines (A427, A549, H292, H358, H1299, H1650 and H1975), and nine renal cell carcinoma cell lines (A498, ACHN, CaKi, CaKi-2, HH050, HH244, RCC52, RCC98 and 786-O).

In addition, a few immortalized human epithelial cell lines, exhibiting lots of features of normal epithelial cells, were also recruited in the study as controls, including one nasopharyngeal epithelial cell line NP69, three esophageal epithelial cell lines Het-1A, NE1 and NE3, two breast epithelial cell lines HEMC and HMEpC, a kidney epithelial cell line RHEK-1 and an embryonic kidney epithelial cell line

HEK293.

Above cell lines were conventionally maintained in RPMI160 or DMEM medium, which was supplemented with 10% FBS and 1% both penicillin and streptomycin. The culture dishes or flasks were kept in 37 °C incubator with humidified atmosphere containing 5% CO_2. Cells were subjected to subculture when the confluency reached about 80%. Briefly, cells were detached with 1 ml trypsin solution by incubating at 37 °C for 5-10 minutes and then neutralized with complete growth medium containing FBS. After centrifuging the suspension for 5 minutes at 1,000 to 2000 rpm, the cell pellet was re-suspended and partially seeded into a new culture dish with fresh complete medium.

3.2 Normal and primary tumor tissues

RNA from twenty-two normal adult human tissues including heart, liver, kidney, spleen, pancreas, esophagus, stomach, colon, rectum, larynx, trachea, lung, breast, cervix, ovary, placenta, testis, prostate, brain, bone marrow and lymph node, two normal adult PBMC samples, and nine normal fetal tissues including skeleton muscle, skin, kidney, colon, bladder, brain, stomach, liver and lung, were used in the RT-PCR assay to study the tissue expression patterns of identified genes and were purchased from either Stratagene (Stratagene, CA, USA) or Ambion (Ambion, TX, USA). Achieved DNA samples in the current lab, extracted from normal human esophageal and nasopharyngeal tissues, and from primary tumor tissues including NPC, ESCC and gastric cancer, were also used in the present study.

3.3 Drug treatment for cell lines

Selected cancer cell lines were subjected to demethylating treatment with 5-Aza-2'-deoxycytidine (Aza, Sigma-Aldrich, MO, USA) for the DNA methylation analysis. Cells were seeded at about 30% confluency and grown overnight. The next day Aza was added to freshly replaced medium for a final concentration of about 10-50 μM, with changing for fresh medium containing Aza in the following two days. For the combining treatment of both Aza and HDAC inhibitor Trichostatin A (TSA) (Cayman Chemical Company, MI, USA), TSA was added into fresh complete medium to a finial concentration of 100 ng/ml for 24 hours, following prior three days' treatment with Aza. After treatment, cells were harvested as described before for both DNA and RNA extraction.

3.4 Extraction of total RNA and genomic DNA

RNA and DNA were extracted from cell lines with TRI Reagent (Molecular Research Center, OH, USA). Cell pellets were first collected by trypsinization and centrifugation, and then vortexed in about 1 ml TRI Reagent to reach homogenous. After incubating the mixture at room temperature (RT) for 5 minutes, 0.2 ml chloroform per 1 ml TRI Reagent was then added to the mixture. After vortex, the mixture was incubated for another 5 minutes at RT and then centrifuged at 14,000 rpm for 10-15 minutes at 4 °C. The mixture was then separated into 3 layers: the colorless upper aqueous phase containing RNA, the interphase, and the lower phenol-chloroform phase. The upper layer containing RNA was thus transferred into

a new eppendorf tube and added 0.5 ml isopropyl alcohol per 1 ml TRI Reagent used initially, and then the mixture was incubated for 10 minutes at RT and centrifuged at 14,000 rpm for 10-15 minutes 4 °C to precipitate RNA. Next, RNA pellet was washed with 1 ml 75% ethanol by centrifuging at 10,000 rpm for 5 minutes at 4 °C. Finally, the RNA pellet was air-dried for 10 minutes, dissolved in RNAsecure Resuspension Solution (Ambion, Austin, TX) and stored at -80 °C before use.

For DNA extraction, 0.3 ml 100% ethanol per 1 ml TRI Reagent was added to the left interphase and phenol-chloroform phase. After incubating the mixture at RT for 5 minutes, it was centrifuged at 2,000g for 5 minutes at 4 °C. Next the supernatant was removed and DNA pellet was washed first with 0.1 M sodium citrate in 10% ethanol for 30 minuets at RT, followed by another wash with 1.5 ml 75% ethanol after centrifugation at 2,000g for 5 minutes. After the final centrifugation at 2,000g for 5 minutes at RT, the DNA pellet was air-dried and dissolved in about 500 μl 8 mM NaOH per 10×10^6 cultured cells or 50-70 mg tissues, at 4 °C overnight. DNA was stored at 4 °C before use. The concentration of extracted RNA and DNA was determined according to the optical density readings at wavelength 260 nm in the Beckman DU650 Spectrophotometer (Beckman Coulter, CA, USA).

3.5 Plasmid DNA preparation

3.5.1 Mini-prep isolation of plasmid DNA by alkaline lysis

Single bacteria colony was picked from agar plate and inoculated in 1-2 ml LB medium with appropriate antibiotics, and shaken overnight at 37 °C at the speed of

2000 rpm. Bacteria were harvested by centrifugation at 5,000 rpm for 3-5 minutes. After removing the supernatant, the bacteria pellet was re-suspended by vortex in 150 μl Solution I (50 mM glucose, 25 mM Tris-Cl, 10 mM EDTA, 0.5 mg/ml RNase A) and incubated for 10 minutes at RT. Re-suspended bacteria were then lysed by 300 μl freshly prepared Solution II (1% SDS, 200 mM NaOH) for 3-5 minutes at RT. Then the bacteria lysate was neutralized with 225 μl chilled Solution III (3M potassium acetate, 11.5% glacial acetic acid, pH5.2), incubated for 5-10 minutes at RT and then centrifuged at 14,000 rpm for 10 minutes at 4 °C to remove the precipitates. Following centrifugation, 600 μl supernatant was transferred to a new 1.5 ml eppendorf tube and added with 0.6 volumes (360 μl) isopropanol for 15 minutes' incubation at RT to precipitate DNA. After centrifugation at 14,000 rpm for 10-15 minutes at 4 °C, the supernatant was removed by aspiration and plasmid DNA pellet was then washed by 750 μl 70% ethanol. Finally after centrifugation at 12,000 rpm for 5-10minutes at 4 °C and removal of supernatant, the plasmid DNA pellet was air-dried and dissolved in 20-50 μl ddH_2O.

3.5.2 Midi-prep isolation of transfection grade plasmid DNA by alkaline lysis

Transfection grade large quantity of endotoxin-free plasmid was isolated using the Qiagen Plasmid Midi Kit (Qiagen, Germany). Briefly, 200 μl bacteria start culture was inoculated in 50 ml LB with the presence of appropriate antibiotics, by shaking overnight at 37 °C. Bacteria pellet was then collected by centrifugation at 4,000 rpm for 20-30 minutes and removal of supernatant. Following steps including

re-suspension, lysis, neutralization, column filtering, washing and elution were carried out according to the manufacturer's protocol. Then the eluted plasmid DNA was precipitated by isopropanol, washed by 70% ethanol and air-dried as described in the mini-prep protocols. Finally, the plasmid DNA was dissolved in 250-500μl endotoxin free TE buffer and stored at 4 °C. Before transfection, the concentration of plasmid DNA was measured by spectrophotometer.

3.6 Gel electrophoresis of DNA

To separate PCR products and plasmid DNA, agarose gel was prepared at the concentration of about 1.5-2.0% (w/v) in TAE buffer, containing 4μl ethidium bromide (EB) per 100ml TAE for staining. Before loading, DNA samples were mixed with 6x DNA loading buffer and the gel was immersed in 1 x TAE running buffer in gel tank. After loading the mixed DNA samples into gel wells, electrophoresis was immediately started at 120-125 V for 25 minutes and gel was subsequently photographed under UV by the Gel Documentation System with Quantity One software (Bio-Rad Laboratories, CA, USA).

3.7 DNA sequencing for plasmids and PCR products

DNA sequencing was carried out with the ABI BigDye Terminator v3.1 Sequencing Kit (Applied Biosystems, CA, USA) or commercially ordered at the BGI (Beijing Genomics Institute)-Hong Kong company. Briefly, the BigDye sequencing PCR mixture was prepared at the total volume of 5μl per reaction with 1 μl Big Dye, 0.5 μl of 1.6 μM sequencing primer (plasmid common sequencing primers or gene

specific primers), 1 μl DNA template and 2.5 μl DNase free water. The sequencing mixture was next subjected to 30 cycles of PCR amplifications (96 °C for 15 seconds, 52 °C for 15 seconds and 60 °C for 2 minutes).

After PCR, sequencing products were purified as below. First, 0.5 μl 3M NaOAc and 12.5 μl 95% ethanol were added to the PCR products and the mixture was kept in darkness for 15-20 minutes following a quick vortex. Next the mixture was centrifuged at 14,000 rpm for 10 minutes at RT. After aspirating the supernatant, 100-150μl 70% ethanol was added for washing. Samples were then centrifuged at 14,000 rpm for 5-10 minutes at RT and supernatant was removed again by aspiration. The pellet (invisible) was air-dried in darkness for 1 hour or overnight to allow the sufficient removal of ethanol. After complete air-drying, 12 μl of Hi-Dye (Applied Biosystems, CA, USA) was added to each reaction to dissolve the pellets for 5-10 minutes at RT. Next, the purified sequencing PCR products were transferred to an optical 96 well for sequencing in the ABI 3100 Genetic Analyzer (Applied Biosystems, CA, USA). Finally, the generated sequencing data was retrieved by the ABI Sequence Analysis software (Applied Biosystems, Foster City, CA) and analyzed with the Chromas Lite software (Technelysium Pty Ltd, Tewantin, Australia).

3.8 Semi-quantitative Reverse transcription PCR (RT-PCR)

The process of reverse transcripting total RNA to complementary DNA was performed with the GeneAmp RNA PCR system (Applied Biosystems, CA, USA).

Briefly, 1 μg total RNA in 3.8 μl RNase free water was first denatured at 90 °C for 5 minutes and kept on ice before RT. Then a mixture of other reaction components was prepared with a total volume of 16.2 μl, containing 1 μl 50μM random hexamer, 8 μl 10mM dNTPs, 4 μl 25mM $MgCl_2$, 2 μl 10 x PCR buffer, 0.2 μl 40 U/μl RNase inhibitor (Roche Applied Science, Germany) and 1μl 40U/μl MuLV reverse transcriptase (Applied Biosystems, CA, USA). After mixing RNA and the other components together, the final mixture was kept at RT for 10 minutes, and then subjected to the RT reaction of 1 hour at 42 °C and a followed incubation for 5 minutes at 90 °C. The generated cDNA was immediately used for following PCR procedure or stored at -80 °C before use.

For semi-quantitative RT-PCR, gene specific primers were designed based on the mRNA sequence retrieved from NCBI database, spanning two different exons, with GC content at about 50% for both forward and reverse directions, and generating products of 200 to 500 bp in length. PCR reaction was carried out with GoTaq Flexi DNA Polymerase and GoTaq Green Master Mix (Promega, Madison, WI, USA). Reaction mixture with total volume of 12.5μl was prepared according to formulation shown in Table 3-1.

PCR reaction was performed in a DNA Engine Dyad Thermal Cycler (MJ research, Ma, USA) with the following cycling parameters: an initial denaturing at 95 °C for 2 minutes, 32 cycles (denaturing at 95 °C for 30 seconds, annealing at 55 °C for 30 seconds and extension at 72 °C for 30 seconds) of amplification and a final extension at 72 °C for 10 minutes. *GAPDH* was used as an internal control with a 25

Table 3-1. Components for RT-PCR reaction mixture.

PCR components	Total Volume (μl)	Final concentration
5 x Flexi buffer	2	0.8 x
Forward primer (10μM)	0.75	0.6 μM
Reverse primer (10μM)	0.75	0.6 μM
GoTaq (5U/μl)	0.0625	1.25 U/50μl
RT product	2.5	0.5 μg/50μl
$MgCl_2$ (25mM)	0.5	2 mM
DNase free water	5.9375	
Total volume	**12.5**	

cycles of amplification. The PCR products were then separated by electrophoresis on 1.8% agarose gel containing ethidium bromide, visualized under UV light and photographed under UV by the Gel Documentation System with Quantity One software (Bio-Rad Laboratories, CA, USA).

3.9 5' RACE (rapid amplification of cDNA ends) assay

5' RACE was carried out with the 5'RACE System Kit (Invitrogen, Carlsbad, CA, USA). Briefly, first stand DNA synthesis was performed with a gene specific primer 1 (GSP-1) which is designed based on the exon1 nucleotide sequence from NCBI database, using normal esophageal tissue total RNA as templates, according to the reverse transcription protocol described above in section 3.8. The RT products were treated with RNase H for 30 minutes at 37 °C to remove the RNA templates and then purified with the provided purification cartridges according to manufacturer's instructions. Next the purified cDNA was subjected to tailing with an oligo-dC fragment by treatment with dCTP and TdT (Terminal deoxynucleotidyl transferase) for 10 minutes at 37 °C. The generated tailed cDNA was diluted to 100 folds and

used as templates in the following 35-cycle RACE PCR, which was carried out using the AmpliTaq Gold DNA polymerase (Applied Biosystems, CA, USA), with the primer pair of AAP (abridged anchor primer) and a nested gene specific primer 2 (GSP-2). In order to enhance the specificity of PCR products, another round of nested PCR of 30-35 cycles was carried out with the primer pair of AUAP (Abridged Universal Amplification Primer) and another nested gene specific primer 3 (GSP-3), using the 1:200 diluted products from the preceding PCR. Finally, the PCR products were subjected to agarose gel electrophoresis with EB staining for size checking and the DNA bands in different sizes were excised and collected under visualization by UV, purified by the QIAEX II Gel Extraction Kit (Qiagen, German), and ligated into pCR4-TOPO TA cloning vector (Invitrogen, Carlsbad, CA, USA) for insert sequencing. Finally, the sequence of inserted DNA fragments was aligned with both the cDNA sequence and 5' upstream genomic sequence of the studied gene in order to determine its actual transcription start site (TSS).

3.10 Luciferase activity analysis

For gene promoter activity confirmation, different fragments around the putative promoter were cloned by PCR with high-fidelity DNA polymerase AccuPrime PCR Systems (Invitrogen, Carlsbad, CA, USA) with primers containing appropriate restriction sties. Then PCR products were ligated into promoter-less pGL3-Basic reporter vector (Promega, Madison, WI, USA). The constructed vectors were then verified by sequencing and prepared in large scales using midi-prep protocols as described above. Equal amounts of plasmids containing different promoter fragments were transiently transfected into cells seeded in 24-well plates together with an

internal control vector pRL-SV40 (Promega), using the transfection reagent FuGENE 6 (Roche Diagnostics). At the same time, pGL3-basic empty vector was also transfected as a negative control. For pathway luciferase reporter assay, reporter plasmids such as SRE luciferase reporter (Promega) were co-transfected with expression vector for the studied gene or with empty pcDNA3.1(+) (0.5 μg/well) into cells seeded in a 24-well plate, using FuGENE 6. All transfection was carried out in triplicate.

Forty-eight hours after transfection, cells were washed with 1xPBS and lysed in passive lysis buffer (Promega). Then cell lysates were transferred into an OptiPlate 96-well plate (Perkin-Elmer, Waltham, MA, USA) and luminescence readings were measured in a 1420-multilabel counter luminometer VICTOR3 (Perkin-Elmer, Waltham, MA, USA), by the use of the Dual-Luciferase Reporter Assay System (Promega). Relative luciferase units were measured and normalized against renilla luciferase activity.

3.11 Methylation analysis for identified candidate genes

3.11.1 CpG island analysis

Nucleotide sequences ranging from 1.5 kb upstream to 1 kb downstream of exon 1 of candidate genes were retrieved in the NCBI database. Then the presence of CGI covering the 5' upstream region and/or exon1 of candidate genes were verified with an online tool CpG island searcher (http://cpgislands.usc.edu/) according to the following criteria: CG content > 55%, ratio of the observed CpG to expected CpG > 0.65, minimum CGI length > 500bp.

3.11.2 Sodium bisulfite treatment for genomic DNA

Genomic DNA was extracted and quantified as described in section 3.4, and was modified with sodium metabisulfite for methylation analysis. Briefly, about 1-5 μg genomic DNA in 30 μL TE buffer was first denatured in 0.3 M NaOH, by adding 3.3 μl 3 M freshly prepared NaOH solution. After incubating for 15 minutes at 37 °C, the denatured DNA was bisulfite-modified by adding 333 μl bisulfite solution containing 10 mM hydroquinone and 2.4M sodium metabisulfite (pH 5.0 to 5.2) (Sigma-Aldrich, MO, USA). With overlaying of mineral oil, the mixture was incubated at 55 °C for 4 hours in darkness, with heating to 95 °C for 3 minutes every hour. After bisulfite treatment, DNA was purified using the QIAEX II Gel Extraction Kit (Qiagen, Germany) according to the manufacturer's protocol, and finally dissolved in 50 μl TE buffer for 5 minutes at RT. Then the bisulfite modified DNA was treated in 0.3 M NaOH once more. After incubating at 37 °C for 15 minutes, the mixture was neutralized by adding of 3 M NH4OAc (pH 7.0) and then purified again with the QIAEX II Gel Extraction Kit. The modified DNA was finally dissolved in TE buffer to reach a concentration of 1 μg per 20 μl and stored at -20 °C before use.

3.11.3 Methylation-Specific PCR (MSP)

After bisulfite modification, the unmethylated cytosine was converted into uracil, while the methylated cytosine was unaffected, which meant that the CpG methylation status in CGI could be detected in PCR reactions by primer pairs specifically annealing to and amplifying the unmethylated or the methylated

templates. Two pairs of MSP primers, each covering 2-5 CpG sites within promoter CGI, were thus designed for candidate genes, with one pair of methylated primers (M) to specifically amplify the bisulfite-treated methylated DNA and the other pair of unmethylated primers (U) to amplify the bisulfite-treated unmethylated DNA. It was essential to realize that two strands of DNA became non-complementary after bisulfite treatment, thus the primer-designing should be based on one of the converted strands.

The MSP reaction mixture was prepared according to the formulation shown in Table 3-2, and was carried out using the AmpliTaq Gold DNA polymerase (Applied Biosystems, CA, USA). Each bisulfite converted DNA sample was amplified simultaneously with the methylation-specific (M) primer pair and the unmethylation -specific (U) primer pair, in two independent reactions, to reveal the CpG methylation status in a specific gene. Prior to MSP reactions, both primer pairs were subjected to PCR reaction with unbisulfited DNA as templates, in order to confirm that the unbisulfited DNA left in the bisulfite treated samples would not be amplified by neither the M or the U primer pairs.

MSP reaction was performed in the DNA Engine Dyad Thermal Cycler (MJ research, Ma, USA), which was initiated by a 10 minutes' hot-start procedure at 95 °C, followed by 40 cycles (denaturing at 95 °C for 30s, annealing at appropriate temperature for 30s, and extension at 72 °C for 30s) of amplification, and finalized by extension at 72 °C for 5 minutes. Then PCR products were subjected to electrophoresis and photographing under UV as described before. Some

Table 3-2. Components for MSP and BGS reaction mixture.

Components	Volume (μl)	Final concentration
10 x PCR buffer	1.25	1 x
$MgCl_2$ (25M)	1	2 mM
dNTPs (2.5M each)	1	0.2 mM
Forward primer (10μM)	0.75	0.6 μM
Reverse primer (10μM)	0.75	0.6 μM
AmpliTaq Gold polymerase (5U/μl)	0.0625	1.25 U/50μl
Bisulfite-modified DNA templates	0.5	0.1μg/100μl
DNase free water	7.1875	
Total volume	**12.5**	

representative samples were sequenced to confirm the specificity of MSP primers and PCR conditions, according to DNA sequencing protocols in section 3.7.

3.11.4 Bisulfite Genomic Sequencing (BGS)

Bisulfite Genomic Sequencing was performed to reveal the methylation status of each CpG site within a specific region in the interested CGI. Briefly, a pair of BGS primers was designed based on one strand of the converted DNA, with none or few CpG sites within the primers, to ensure the equal chances in amplifying either the methylated or unmethylated DNA templates. The BGS PCR was performed according to the system used in MSP, but with a final extension step prolonged to 1 hour to ensure the sufficient addition of dATPs to product 3' ends and in turn the efficient TA cloning in later steps. PCR products were electrophoresed and photographed as described before, and the DNA bands were excited and purified with the Costar Spin-X Centrifuge Tube Filter (Cole-Parmer, Vernon Hills, IL, USA). Purified DNA fragments were then ligated into pCR4-TOPO (Invitrogen, Carlsbad, CA, USA) TA cloning vector and transformed into DH5α competent cells according to manufacturer's instructions. Finally, 6-8 bacteria colonies were randomly chosen

for plasmid DNA extraction and sequencing as described in preceding sections. Then the sequencing data were analyzed to reveal the methylation status of each CpG site located in the region defined by BGS primer pairs.

3.12 Construction of expression vectors for the candidate TSGs

3.12.1 Construction of pcDNA3.1(+)-Flag-*RAB39* expression vector

The full length-cDNA of *RAB39* was first cloned from human esophageal cDNA library. Briefly, a pair of *RAB39* gene specific primers flanking the 651 bp of *RAB39* open reading frame (ORF) was designed based on the mRNA sequence retrieved from NCBI database (NM_017516.1). First, 1 μg normal human esophageal total RNA was subjected to reverse transcription by oligo-dT as previously described. Then with the generated cDNA as templates, a cloning PCR was performed using the *RAB39* specific primer pairs (RAB39Fclon and RAB39Rclon) with high-fidelity AccuPrime DNA polymerase (Invitrogen) according to manufacture's protocols. Similarly, another round of PCR was performed with a forward primer RAB39FlagF, containing the Flag tag coding sequence, and RAB39Rclon, using 1:250 diluted PCR products in the first round PCR. Then the second round PCR product was electrophoresed and DNA band was excised, purified by Spin-X tube filter, and ligated into pCR4-TOPO (Invitrogen) vector for sequencing. After confirming the sequence of inserted Flag-tagged *RAB39* ORF, it was subjected to appropriate double restriction enzymes digestion and ligated into pcDNA3.1 (+) mammalian expression vector (Invitrogen) with T4 DNA ligase (Roche). The orientation of inserted *RAB39*

ORF was further verified by digestion with restriction enzymes. Transfection grade endotoxin-free pcDNA3.1 (+)-Flag-*RAB39* plasmid was prepared according to the midi-prep protocol described previously. And successful expression of Flag tagged RAB39 protein in human cells was verified by cell transfection and western blot using the anti-Flag antibody.

3.12.2 Construction of pcDNA3.1(-)-*WDRX* and pEGFP-C2-*WDRX* vectors

The *WDRX* ORF was purchase from American Type Culture Collection (ATCC), which was reversely inserted in pCR4-TOPO vector. First of all, the sequence of the 3309 bp long *WDRX* ORF was verified by DNA sequencing. In order to insert it in to a mammalian expression vector, the ORF was first subcloned into pCR2.1-TOPO (Invitrogen) vector using restriction enzyme Spe I and Not I (New England Biolabs, USA). Then it was further subcloned in the pcDNA3.1(-) expression vector with the use of restriction enzyme Xho I and BamH I (New England Biolabs, USA). For the subcellular localization of WDRX protein and protein level expression verification, the *WDRX* ORF was further subcloned into pEGFP-C2 (Clontech, CA, USA) vector with Xho I and BamH I. The successful expression of the GFP-WDRX protein was verified by western blot using anti-GFP antibody.

3.12.3 Construction of pcDNA3.1(+)-*ADAMTS8*-Flag and pEGFP-N1-*ADAMT-S8* vectors

The full length *ADAMTS8* ORF of 2670 bp in length was previously cloned from the normal human larynx cDNA library in the current lab and was subcloned

into pcDNA3.1 (+) expression vector. In order to detect the *ADAMTS8* expression in the protein level, a Flag-tag was added to the c-terminal of ADAMTS8 protein. Briefly, a cloning PCR was performed using high-fidelity AccuPrime polymerase, with pcDNA3.1(+)-*ADAMTS8* as templates and the primer pairs of ADAMTS8ClonF and ADAMTS8FlagR. As previously described, the PCR products were separated on gel, excised, purified and ligated into pCR4-TOPO vector. After sequencing verification, the Flag-tagged *ADAMTS8* ORF was then subcloned into pcDNA3.1 (+) with BamH I and Xho I, to generate the pcDNA3.1 (+)-*ADAMTS8*-Flag vector. Simultaneously, another PCR was carried out using ADAMTS8ClonF and a reverse prime in lack of stop codon, in order to add a GFP- tag to the c-terminal of ADAMTS8 protein. Similarly, the PCR-cloned ADAMTS8 ORF without stop codon was ligated into pCR4-TOPO, sequenced and then subcloned into the pEGFP-N1 vector, to generate a pEGFP-N1-*ADAMTS8* vector. The successful expression of ADAMTS8-Flag and ADAMTS8-GFP fused protein was verified by western blot using anti-Flag and -GFP antibody, respectively.

3.13 Colony formation assay (CFA) for monolayer cultured cancer cells

Colony formation assay was carried out to evaluate the influence of ectopic expression of identified candidate gene on the anchorage-dependent growth of monolayer cultured cancer cells. Suitable number of cells (1-2 x 10^5/well, varying according to cell size) were plated in 12-well plates and allowed to grow overnight to reach a 70-80% confluency. The next day, cells were transfected with candidate gene

expression vector or empty vector by FuGENE 6 (Roche Applied Science, Switzerland), according to the manufacturer's protocols. In brief, 2.4 μl FuGENE 6 was added to 47.6 μl serum free medium and incubated at RT for 5 minutes. Next, 0.8 μg of plasmid was added to the mixture and incubated at RT for at least 10 minutes. After incubation, the transfection mixture was dripped into wells with freshly changed complete culture medium.

At 48 hours post-transfection, cells were trypsinized and re-plated into 6-well plates at a suitable density of about 1×10^4 cells per well in medium containing 400 μg/ml G418 (Calbiochem, Germany). The left transfected cells was collected by centrifugation and lysed with TRI Reagent for RNA retraction or lysed with protein lysis buffer for protein collection. RT-PCR or western blot assays were carried out to confirm the ectopic expression of the transfected genes. After selection for about two weeks, with replacing fresh medium with G418 for every 3 days, the surviving cancer cell colonies were washed by PBS, fixed with methanol for 10-15 minutes, stained by gentian violet for 30 minutes, washed with distill water and air-dried. At last the plates were photographed and the colonies were counted (≥50 cells per colony). This assay was performed simultaneously in three wells and repeated for three times.

3.14 Protein preparation and Western blot

Transfected cells were collected by trypsinization and spin, then cell pellets were lysed on ice for 30 minutes in 50 μl (per 1×10^6 cells) chilled Tris buffer (20 mM Tris, pH7.5) containing 137 mM NaCl, 2 mM EDTA, 1% Triton X, 10%

glycerol, 50 mM NaF, 1 mM DTT, and 1x protease inhibitor cocktail (Roche). Then cell lysate was boiled for 10 minutes and centrifuged at 14,000 rpm for 15 minutes at 4°C. The supernatant was transferred to a new eppendorf tube and then subjected to concentration determination using the Bicinchoninic Acid Kit (Sigma) according to manufacturer's protocol.

Then protein lysates were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and electroblotted onto Hybond-P PVDF transfer membranes (Amersham Biosciences, Buckinghamshire, UK). After blocking at RT for 1-2 hours with 5% non-fat milk in Tris buffered saline with 0.1% Tween20 (TBST), the membrane was incubated with primary antibodies at 4°C overnight. After washing with 1x TBST buffer for 3 times (5 minutes each), the membrane was incubated with appropriate HRP-conjugated second antibodies at RT for 1 hour. After another three-time washing with TBST, the blots were detected with Amersham ECL kit (Amersham Biosciences) in dark room.

3.15 Indirect immunostaining assay

Cells were seeded onto autoclaved glass slides placed in 6-well plates, allowed to grow overnight to reach 20% confluency and transfected with Flag-tagged gene expression vectors with FuGENE 6. At 48 hours post-transfection, slides was picked out from wells, washed with 1xTBS, and fixed by freshly prepared 4% formaldehyde in PBS at RT for 15 minutes. Then cells were permeabilized with 0.1% Triton X-100 in PBS for 5 minutes at RT and washed with TBS for 3 times (5 minutes each). Next cells was blocked in 5% normal rabbit serum (NRS) for 1 hour, washed and then

incubated with primary antibody 1:40 diluted in 5% NRS at 4 °C overnight. The next day, slides were washed for three times and incubated with the Texas Red -Conjugated Rabbit anti-mouse secondary antibody 1:20 diluted in 5% NRS at 37 °C for 1 hour in darkness. After a three-time washing, a 1:10,000 diluted 4',6-diamidine-2'-phenylindole dihydrochloride (DAPI, Sigma) (1 mg/ml) was used for nuclear staining for 5 minutes at RT. Finally slides were observed directly under an Axiovert 40 CFL - HBO100 fluorescence microscope (Carl Zeiss, Jena, Germany).

3.16 Statistical analysis

Statistical analysis was carried out using Chi-square test or Student's t test in Microsoft Excel software and $p<0.05$ was considered as statically significant.

Chapter 4, Identification of *RAB39* as a novel epigenetically silenced TSG candidate at 11q22.3

4.1 Identification of a locus with frequent copy number loss in ESCC at 11q22.3

Based on previous aCGH data, an 11q copy number loss locus represented by a BAC clone bA531F16 was detected in 3/10 ESCC cell lines, including EC18, HKESC2 and KYSE520 (tumor/normal signal log2 ratio ranging from -1.1 to -1.5). Bioinformatics searching within the Ensembl Genome Browser (www.ensembl.org/ index.html) mapped bA531F16 to 11q22.3, which spanned ~159 kb and was flanked by another two BAC clones, bA569A20 and bA241D13 (Fig. 4-1). Based on the

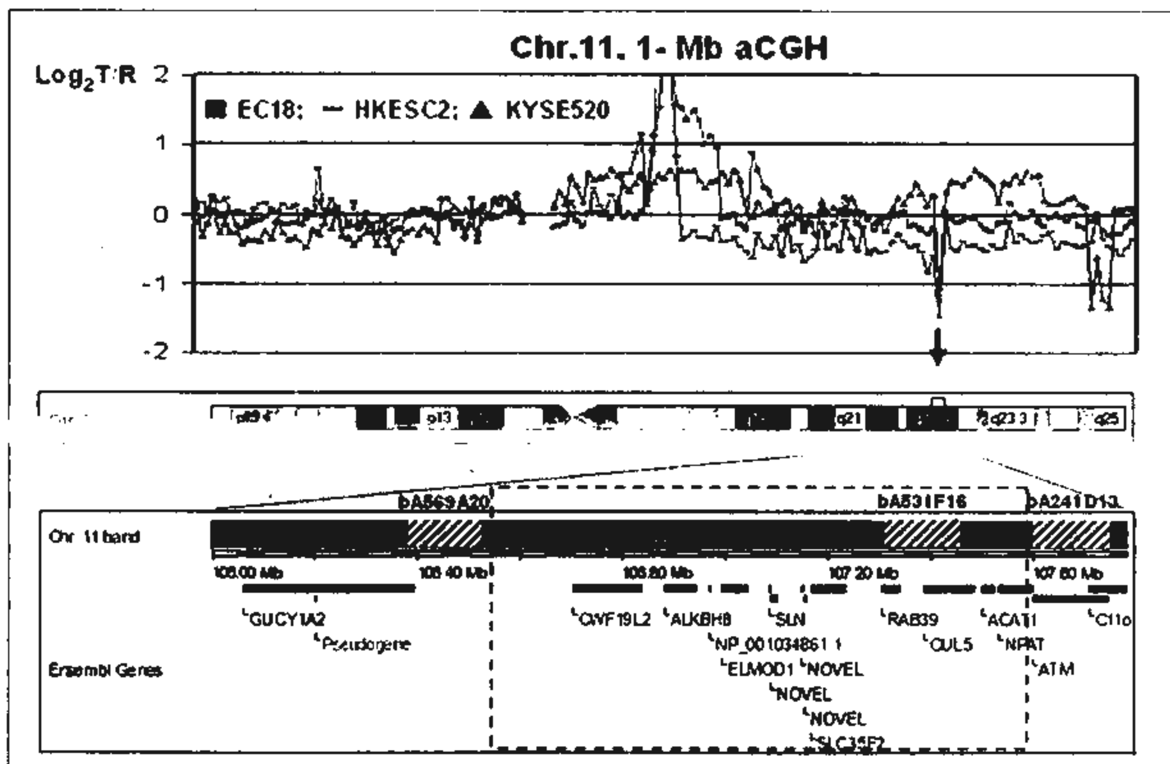

Figure 4-1. Diagram showing the studied copy number loss locus at 11q22.3. Upper panel: aCGH showed a copy number loss locus (arrow) in three ESCC cell lines, EC18, HKESC2 and KYSE520; Lower panel: The copy number loss locus (boxed by dashed lines) was defined by BAC clones bA569A20, bA531F16 and bA241D13 (shadowed bars). Genes residing in this locus were also shown. Partially adopted from Ensembl database (www.ensembl.org/ index.html).

Table 4-1. Information of genes residing in the potential TSG locus at 11q22.3.

Gene	Domain/motif	Protein Function	CGI
CWF19L2	Coiled Coil	Cell communication and Signal transduction	Yes
ALKBH8	N/A	RNA binding;	Yes
SLN	Transmembrane domain	Sarcoplasmic reticulum Ca^{2+}-ATPases; catalyze ATP-dependent transport of Ca^{2+} in muscle cells.	No
RAB39	RAB GTPase	GTPase activity; cell communication and signal transduction	Yes
CUL5	CULLIN	Ubiquitin-specific protease activity	Yes
ACAT1	n/a	Acyltransferase activity	Yes
NPAT	Lissencephaly type-1-like homology motif	Unknown;	Yes
ATM	Phosphoinositide 3-kinase, catalytic domain	Protein serine/threonine kinase activity; Cell communication and Signal transduction, important cell cycle checkpoint kinase	Yes

information retrieved from both Ensembl and NCBI Genbank databases (Build 36.2), totally eight genes located in and around bA531F16 were selected as candidate genes for furhter analysis (Table 4.1).

4.2 Expression profiling for candidate genes at the putative 11q22.3 TSG locus

Based on the phenomenon that TSG is frequently inactivated in cancer cells, the first step to identify the potential TSG in a copy number loss locus should be narrowing down the candidates by screening for the genes with frequent downregulation or even silencing in multiple tumor cell lines. RT-PCR primers were designed for the eight candidate genes listed in Table 4-1 and semi-quantitative RT-PCR was performed for each gene in 8 ESCC cell lines, 3 NPC cell lines and a panel of normal adult tissues including esophagus, larynx, trachea, and testis, with *GAPDH* as internal control, in order to examine their expression profiles (Fig. 4-2)

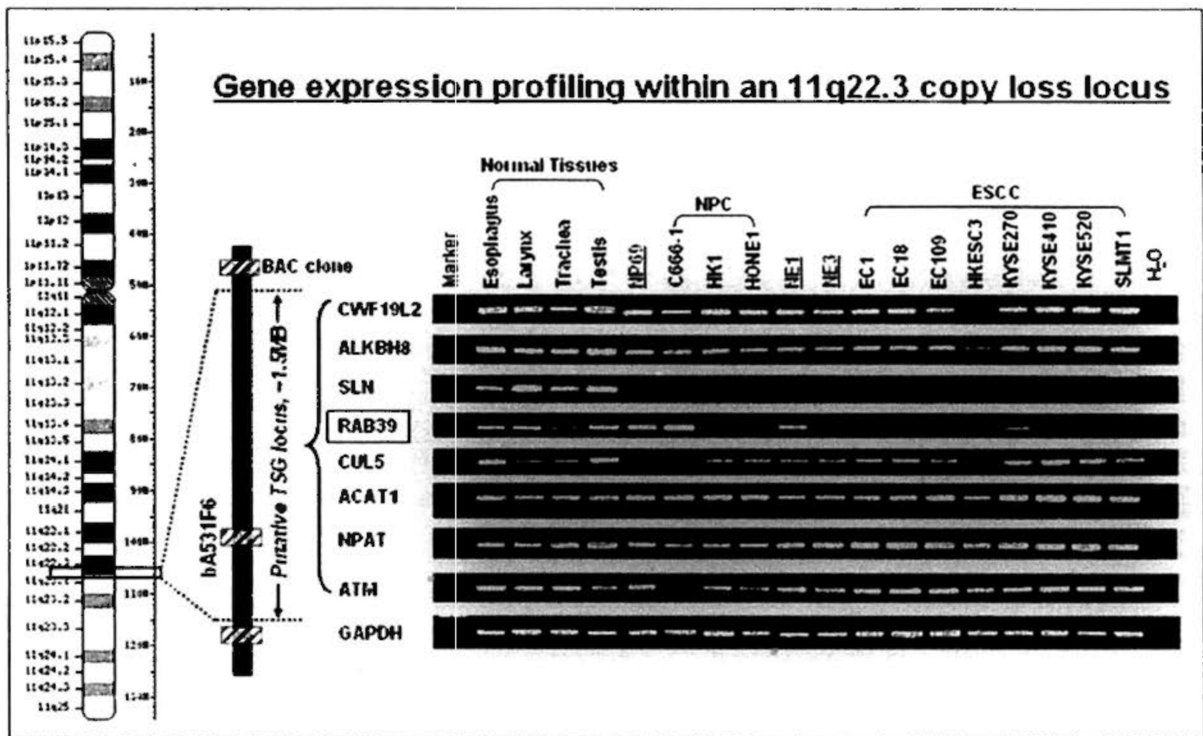

Figure 4-2. mRNA level expression profiling of eight genes residing in the 11q22.3 TSG locus. Left panel: Schematic diagram showing the copy number loss locus, represented by a BAC clone bA531F6; Right panel: gel electrophoresis photo showing the mRNA-level expression of eight genes in four normal tissues, 3 NPC cell lines and 8 ESCC cell lines, as well as 3 immortalized epithelial cell lines NP69, NE1 and NE3 (underlined). *RAB39* (boxed) was selected for further study. *GAPDH* was used as an internal control.

Normal esophageal tissue was used here to verify if these genes were normally expressed in esophagus. However, normal nasopharynx tissue was not used because of the limited amount of this special tissue and the difficulty in obtaining it due to its distinct anatomical position. Instead, normal larynx and trachea were used due to their similarity in embryonic development with nasopharynx. Besides, testis tissue was used as a positive control, since most genes were expressed at mRNA level in testis according to experiences in the current lab. As shown in Fig. 4-2, the mRNA-level expression of two genes, *SLN* and *RAB39*, was demonstrated to be downregulated or silenced in multiple NPC and ESCC cell lines, in contrast to their

abundant expression in normal tissues.

One of the identified downregulated genes *SLN*, sarcolipin, encodes a sarcoplasmic reticulum Ca^{2+}-ATPases in muscle cells, which catalyzes the ATP-dependent Ca^{2+} transport from cytosol into lumen of the sarcoplasmic reticulum. However, it seems to be difficult to associate the known function of *SLN* with cancer. Besides, although *SLN* was highly expressed in both esophagus and upper respiratory tissues, it was neither expressed in the normal immortalized nasopharyngeal epithelial cell line NP69, nor in the immortalized esophageal epithelial cell line NE1 and NE3. Thus, *SLN* was excluded from further analysis. On the contrary, the small RAB GTPase coding gene *RAB39* was expressed in the normal esophagus and upper respiratory tissues as well as in the immortalized cell lines, but was dramatically downregulated in two ESCC cell lines (EC18 and KYSE520), and silenced in the other five ESCC cell lines (EC1, EC109, HKESC3, KYSE410 and SLMT1) as well as in two of three NPC cell lines (HK1 and HONE1). Moreover, recent evidence has implied emerging roles of RAB GTPases in cancer. Therefore, *RAB39* was selected as a target for further analysis in this copy loss locus.

4.3 *RAB39* is downregulated in multiple cancer cell lines

RAB39 is located in the deleted BAC clone bA531F16 and consists of two exons, with a single open reading frame coding a small RAB GTPase protein of 217 amino acids (Fig. 4-3A). By RT-PCR, it was found to be ubiquitously expressed in a variety of human adult and fetal tissues, including esophagus, larynx, stomach, liver, colon, rectum, breast, lung, trachea and so on (Fig. 4-3B).

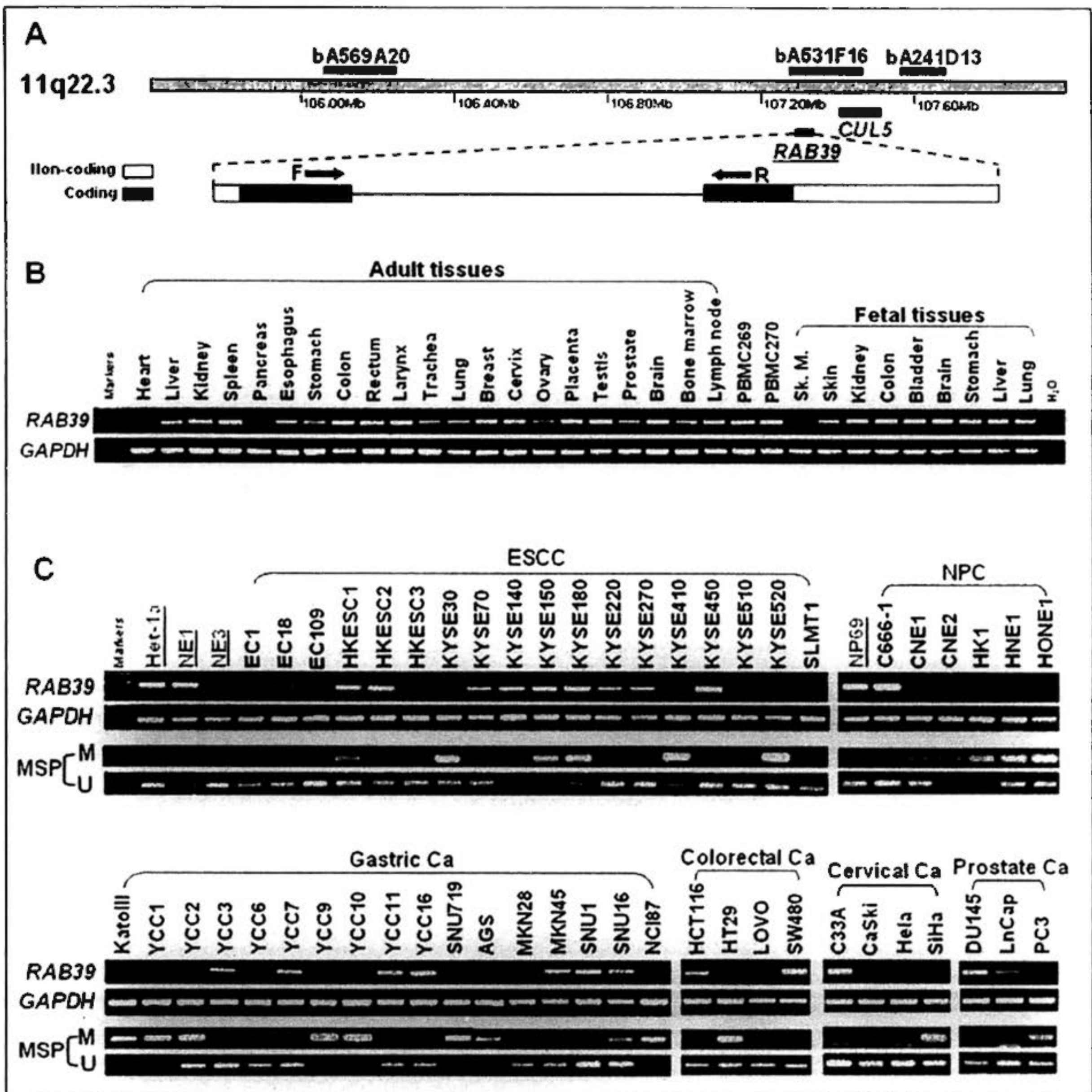

Figure 4-3. *RAB39* is normally expressed in most human tissues, but is down-regulated and methylated in multiple cancer cell lines. A) Upper panel: Schematic diagram showing the relative position of *RAB39* in the deleted BAC clone as well as the structure of its transcript. The forward and reverse RT-PCR primers were indicated with arrows; B) Semi-quantitative RT-PCR result showing the broad expression of *RAB39* in normal human tissues, with *GAPDH* as an internal control. Sk. M., skeletal muscle; C) Results of RT-PCR and MSP for *RAB39* in cancer cell lines. *RAB39* was shown to be downregulated or silenced in multiple cancer cell lines, with *GAPDH* as an internal control. The methylation status of *RAB39* promoter was analyzed using MSP. Promoter methylation was found in the majority of cancer cell lines, such as NPC and gastric cancer, in which *RAB39* was reduced or silenced, but not in the immortalized epithelial cell lines (underlined), suggesting the cancer-specific downregulation and methylation patterns of *RAB39* in these cancers.

Being inactivated during cancer initiation or progression is an important characteristic of TSG. To further prove the inactivation of *RAB39* in cancers, *RAB39* expression level was analyzed in multiple cell lines of other caner types including gastric, colorectal, cervix, and prostate cancers, in addition to ESCC and NPC. As predicted, *RAB39* was revealed to be significantly downregulated or even fully silenced in 12 of 18 ESCC, 5 of 6 NPC, 10 of 17 gastric, 2 of 4 colorectal, 3 of 4 cervical and 1 of 3 prostate cancer cell lines (Fig. 4-3C). On the contrary, *RAB39* was found to be highly or moderately expressed in 4 immortalized epithelial cell lines, including nasopharyngeal epithelial cell line NP69 and esophageal epithelial cell lines Het-1A, NE1 and NE3 (Fig. 4-3C). Taken together, these data revealed a cancer-specific downregulation or silencing pattern of *RAB39*.

4.4 Identification of a new transcriptional start site for *RAB39*

In order to analyze whether the frequently reduced expression of *RAB39* in cancer cell lines was caused by promoter CGI methylation, the transcription start site (TSS) pinpointing the position of *RAB39* exon1 and putative promoter should be validated first. By performing 5' RACE assay, a new TSS of *RAB39* in esophageal tissues was revealed, which was located three nucleotides upstream of the reported *RAB39* TSS according to NCBI database (NM_017516.1) (Fig. 4-4). Accordingly, three 5' nucleotides AGC were added to *RAB39* exon1, without changing the original numbering of *RAB39* exons, nor the approximate position of *RAB39* promoter.

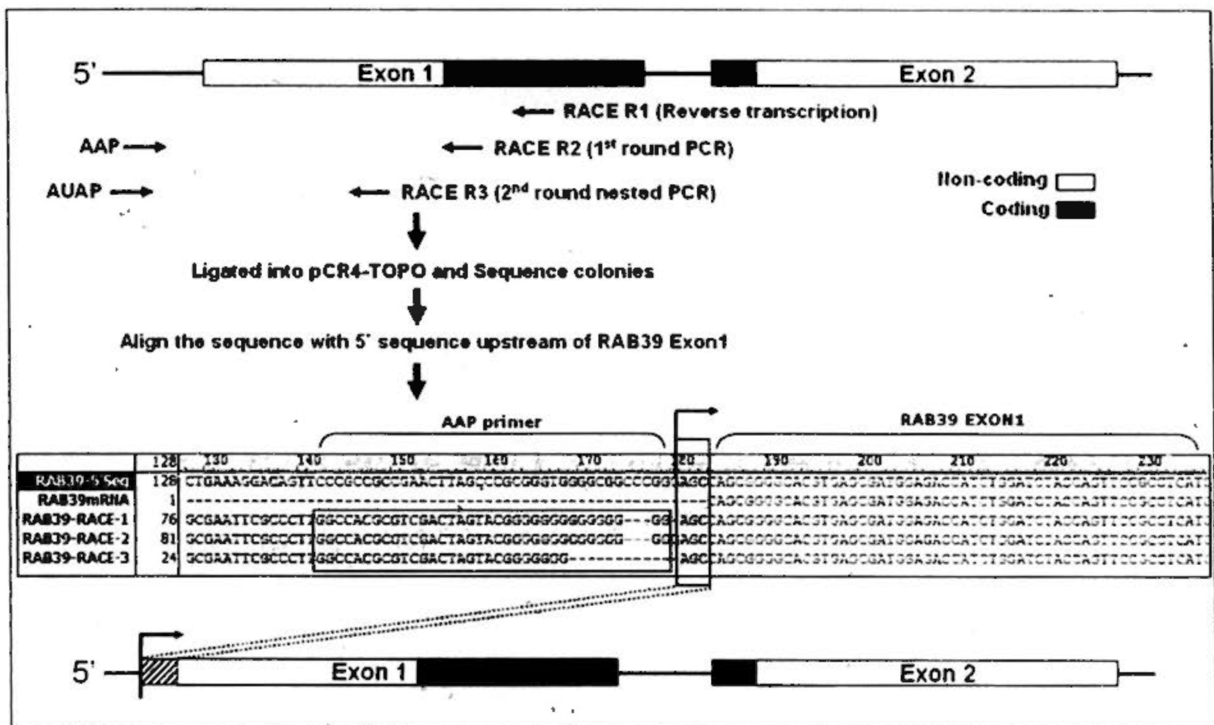

Figure 4-4. General procedures and result of *RAB39* 5' RACE assay. Upper panel: Relative positions of primers used in the 5' RACE assay were indicated by horizontal arrows. The reverse transcription was carried out with gene specific primer RACE R1, first round RACE PCR was performed with AAP and gene specific primer RACE R2, and the second round nested PCR was performed with AUAP and gene specific RACE R3. AAP, Abridged Anchor Primer; AUAP, Abridged Universal Amplification Primer; Middle panel: Diagram showing the alignment of *RAB39* 5' upstream sequence, *RAB39* exon1 sequence and the sequences of inserted fragments in three pCR4-TOPO vectors, which were derived from the second round RACE PCR. The AAP primer and the newly identified 3 additional nucleotides (AGC) preceding *RAB39* exon1 were boxed, and the renewed TSS was indicated by curved arrow; Lower panel: Schematic diagram showing the new TSS as well as the additional nucleotides preceding the original *RAB39* exon1.

4.5 Confirmation of the transcription initiating ability of *RAB39* promoter

In order to assess whether the putative promoter region in the upstream of *RAB39* TSS could initiate transcription, the -1214-+47 and -232-+47 (according to NM_017516.1) DNA fragments were cloned from normal human PBMC DNA and then ligated to luciferase reporter vector pGL3-Basic (Fig. 4-5B). Transcription

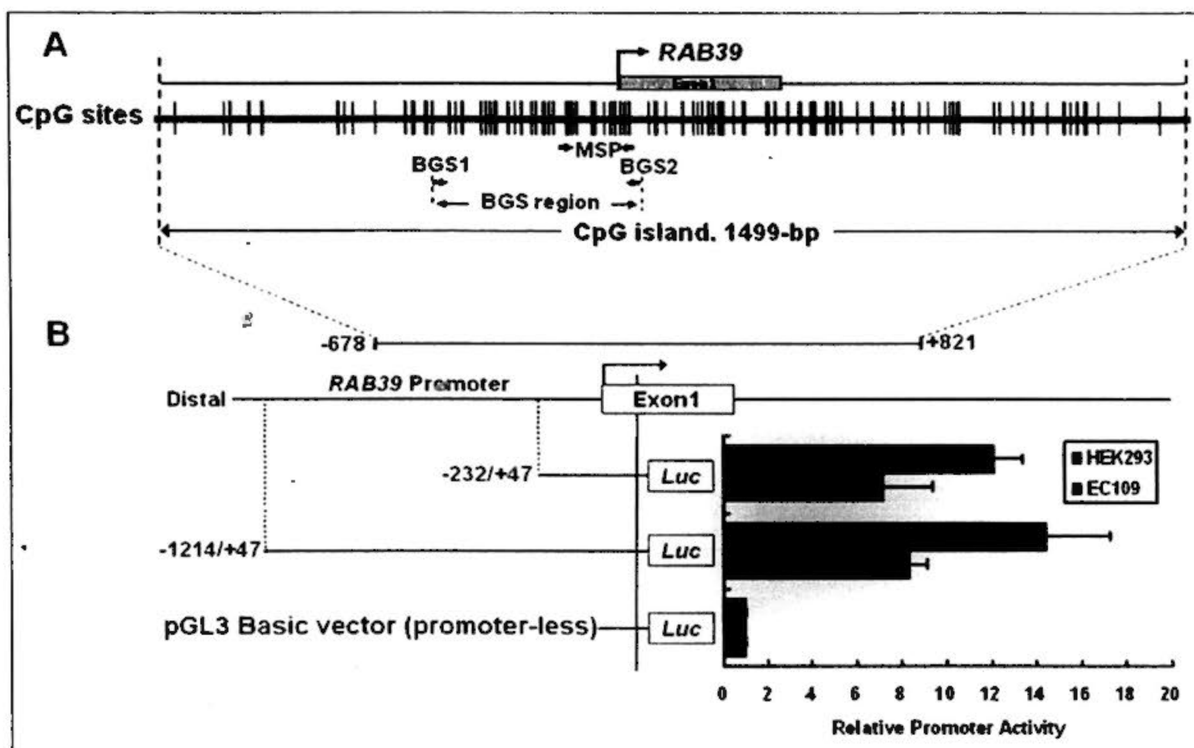

Figure 4-5. The structure and relative position of *RAB39* promoter CGI, and results for *RAB39* promoter-luciferase reporter assay. A) Both *RAB39* putative promoter region and exon1 were spanned by a 1499bp CGI. The relative positions of primers used for MSP and BGS analysis, and the region analyzed by BGS were indicated by arrows; B) Luciferase activities of two *RAB39* promoter constructs, relative to the promoter-less empty vector were shown. Data were means and standard deviations of three independent assays. Both *RAB39* promoter constructs could drive the transcription of the luciferase reporter gene, although the efficiency differed between cell lines.

initiating activities of both fragments were tested by transiently transfecting the plasmids into HEK293 and EC109 cell lines. Results demonstrated that both fragments could lead to significant increase of luciferase activity in two cell lines used (Fig. 4-5B), indicating that the putative *RAB39* promoter was functional with transcription initiating capability *in vitro*.

4.6 Methylation status of *RAB39* promoter in tumor cell lines

4.6.1 Promoter CGI characterization for *RAB39*

Since *RAB39* was downregulated in multiple cancer cell lines and had a

functional putative promoter validated by both 5'RACE assay and luciferase reporter assay, it was reasonable to elucidate whether its downregulation and silencing in cancer cells was due to promoter CGI hypermethylation. Bioinformatics analysis revealed that there was a typical CGI spanning *RAB39* promoter and exon1, from -677 to +822 (CGI Searcher, http://cpgislands.usc.edu/) (Fig. 4-5A), which fulfilled the premise to study the relation between *RAB39* promoter methylation and its tumor-specific downregulation.

4.6.2 MSP reveals RAB39 promoter methylation in multiple tumor cell lines

The methylation status of *RAB39* promoter CGI was analyzed using MSP in multiple cancer cell lines. Several pairs of MSP primers targeting the methylated or unmethylated alleles of *RAB39* pormoter CGI were designed and tested. The optimal methylated (M) or unmethylated (U) pairs of primers with best amplicfation efficiency and speicficity were used for MSP anaylsis in multiple cell lines. In addition, the seletcted M and U primer pairs were excluded from amplifying unbisultifted DNA. MSP results suggested that *RAB39* promoter was methylated in multiple downregulated tumor cell lines derived from different cancer types, including ESCC, NPC, as well as gastric, colorectal, cervical, and prostate cancers (Fig. 4-3C, Page 79). In most cancer cell lines studied, promoter methylation status of *RAB39* was inversely correlated with its mRNA level. Notably, methylation was not detected in any of the four immortalized epithelial cell lines, including Het-1A, NE1, NE3, and NP69, which suggested a tumor-specific methylation manner for

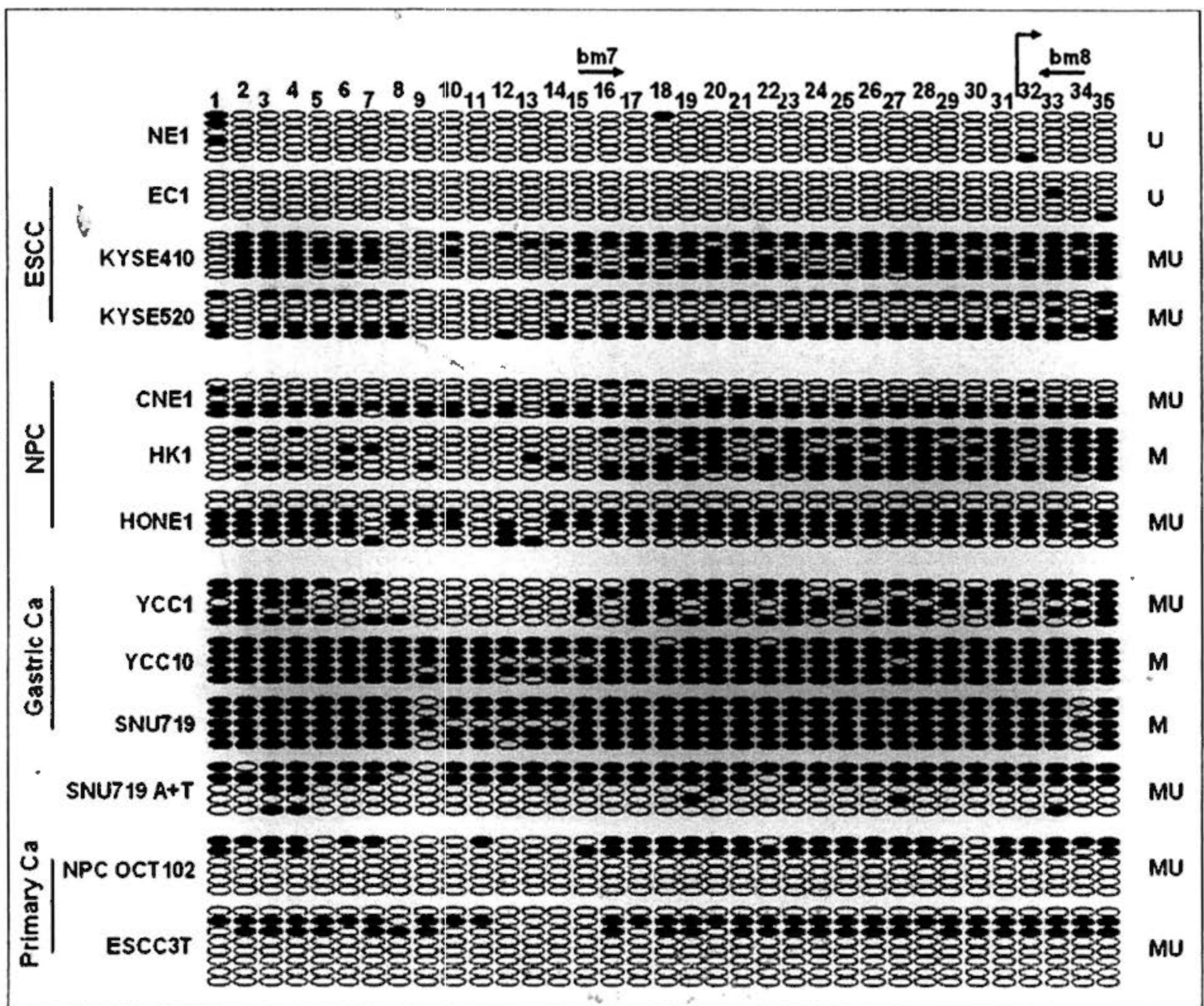

Figure 4-6. *RAB39* promoter methylation revealed by BGS. The methylation status of 35 CpG sites in *RAB39* promoter CGI was analyzed in some representative immortalized cell lines, cancer cell lines of different origins, cell lines treated by Aza and TSA, as well as tissues from primary tumors. The right panel indicated *RAB39* methylation status revealed in MSP. Relative positions of MSP primers and TSS were tagged by arrows. One row of circles indicated an allele of *RAB39* promoter CGI. Open circle, unmethylated CpG; Filled circle, methylated CpG.

RAB39. The frequencies of *RAB39* promoter methylation in cell lines of six cancer types were briefly summarized in Table 4-2 (Page 87).

4.6.3 BGS confirms the tumor-specific methylation of *RAB39* promoter

To validate the result of MSP analysis and analyze the methylation status in detail within part of the *RAB39* promoter CGI, high resolution BGS assay was

performed in some representative cell lines, including immortalized epithelial cell lines, methylated and unmethylated cancer cell lines. As expected, few CpGs within the BGS region were methylated in the immortalized cell line NE1 and the unmethylated ESCC cell line EC1 (Fig. 4-6). In contrast, in the methylated cell lines revealed by MSP, BGS proved that CpGs in the studied CGI were extensively methylated, even almost fully methylated in cell lines without unmethylated alleles detected by MSP, such as YCC10 and SNU719 (Fig. 4-6). The BGS results not only validated the methylation status demonstrated in MSP analysis, but also indicated that the CpG methylation in the downregulated cell lines was not a phenomenon limited to specific CpGs which were targeted by MSP primers, instead, methylation was ubiquitously detected in most CpG sites all over the *RAB39* promoter CGI.

4.7 Restoration of *RAB39* expression by pharmacological demethylation

To further verify the direct association between *RAB39* promoter methylation and its tumor-specific downregulation, a variety of cancer cell lines with reduced or silenced *RAB39* expression along with promoter methylation were selected for pharmacological treatment with DNMT inhibitor 5-aza-2' deoxycytidine (Aza) alone, or together with HDAC inhibitor Trichostatin A (TSA). TSA was used here because it was shown to act synergistically with DNMT inhibitor in the reactivation of methylated cancer-related genes (Cameron et al., 1999). Results showed that upon treatment with Aza, *RAB39* expression level was dramatically restored or elevated in multiple methylated cancer cell lines, and the combined treatment with TSA

increased *RAB39* expression to an even higher level in both HK1 and KYSE520 cell lines, when compared to treatment with Aza alone (Fig. 4-7A). Further MSP analysis proved that after Aza or Aza plus TSA treatment, the amount of unmethylated *RAB39* alleles was dramatically increased, which was further validated by BGS analysis in the SNU719 cell line treated with Aza plus TSA (Fig. 4-6, Page 84). Taken together, these data indicated that promoter methylation mediated the transcriptional inactivation of *RAB39* in cancer cells.

4.8 *RAB39* is methylated in primary ESCC, NPC and gastric cancers

In order to further validate the involvement of *RAB39* hypermethylation in carcinogenesis, the methylation status of *RAB39* was examined in a panel of primary ESCC, NPC and gastric cancers, as well as in normal tissues. *RAB39* methylation was detected in 10/44 (23%) ESCC, 8/45 (18%) NPC, and 4/19 (21%) gastric cancers by MSP (Fig. 4-7C). Some samples were selected for further validation by BGS (Fig. 4-6, Page 84). In contrast, much weaker methylation was detected only in 3/44 (7%) paired surgical marginal esophageal tissues (Fig. 4-7C), 0/7 normal esophageal and 1/10 normal nasopharyngeal epithelial tissues from healthy individuals (Fig. 4-7B), with significantly lower frequencies of methylation than those in primary tumors. These data demonstrated that *RAB39* promoter methylation was tumor-specific, not only in the studied cancer cell lines, but also in primary tumor tissues.

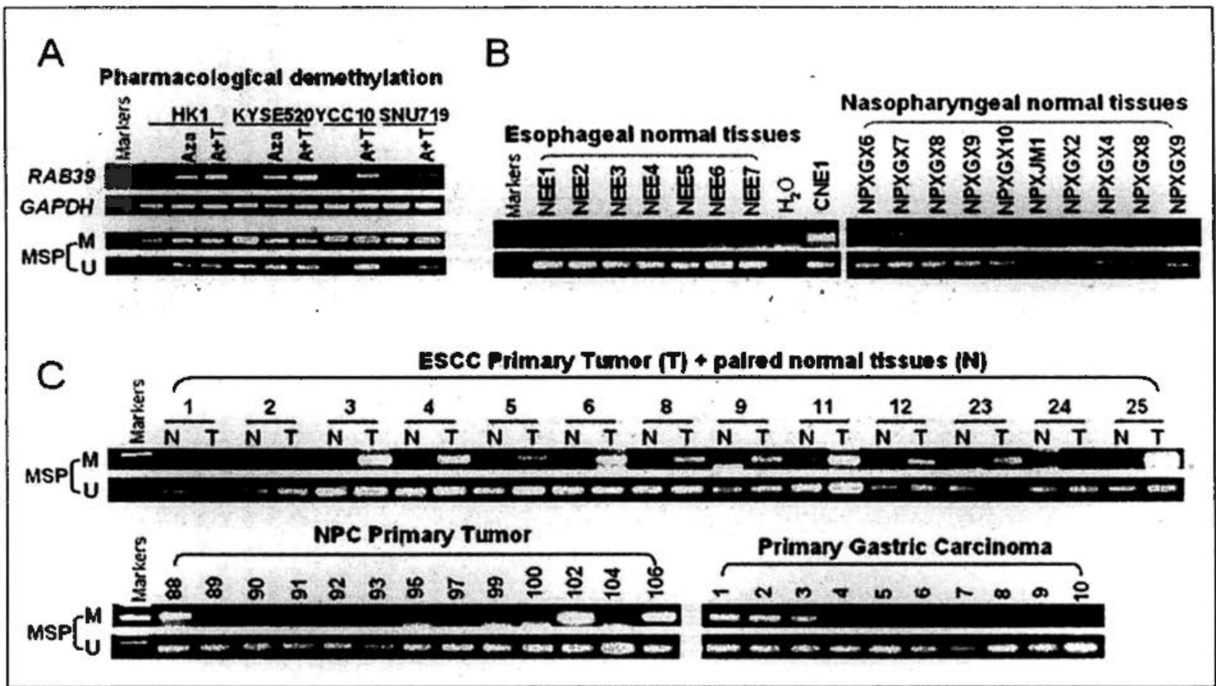

Figure 4-7. *RAB39* reactivation upon pharmacological demethylation treatment and its methylation pattern in primary tumors. A) Pharmacological treatment induced the demethylation of *RAB39* CGI and restored its expression, as analyzed by MSP and RT-PCR. A, Aza; T, TSA; M, methylated; U, unmethylated; B,C) Representative methylation studies of *RAB39* in normal tissues, primary NPC, gastric cancer, and paired ESCC malignant (T) and surgical marginal (N) tissues, as analyzed by MSP. M, methylated; U, unmethylated.

Table 4-2. Summary of the frequencies of *RAB39* promoter methylation in carcinoma cell lines and primary tumors.

Samples	Promoter methylation (%)
Carcinoma cell lines	
Nasopharyngeal	5/6 (83%)
Esophageal	7/18 (39%)
Gastric	9/17(53%)
Colorectal	1/4
Cervical	1/4
Prostate	1/3
Immortalized normal epithelial cell lines	
NP69, HET-1A, NE1, NE3, HMEC, HMEpC	0/6
Normal Esophageal Tissues	0/7
Normal Nasopharyngeal Tissues	1/10
Primary Tumor Samples	
ESCC	10/44 (23%)
NPC	8/45 (18%)
Gastric Carcinoma	4/19 (21%)

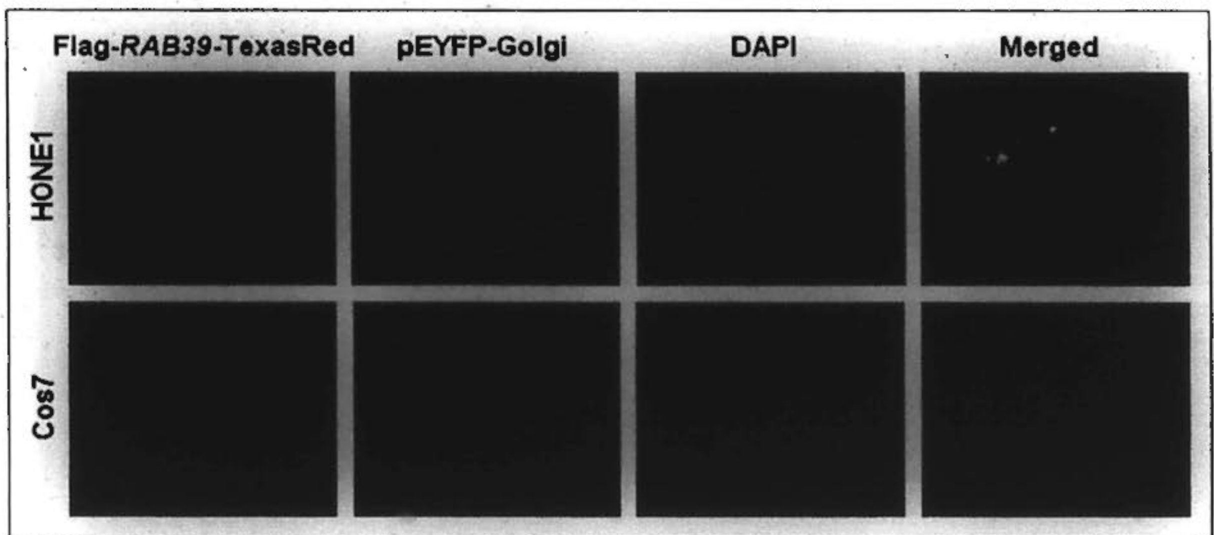

Figure 4-8. Subcellular localization of RAB39 protein. Subcellular localization of small GTPase RAB39 was shown by indirect immunostaining in both HONE1 and Cos7 cells. Left panels were the Texas Red fluorescence of Flag-tagged RAB39 protein; middle-left panels were the fluorescence of the fused protein of a Golgi specific protein and YFP; middle-right panels were the DAPI-stained cell nuclei; right panels were the merged images.

4.9 *RAB39* encodes a RAB GTPase co-localized with Golgi in cytoplasm

As specific localization of proteins often implicated their distinct functions, subcellular localization of the novel RAB GTPase encoded by *RAB39* was examined first via an online predicting server for eukaryote protein subcellular localization, pTARGET (http://bioapps.rit.albany.edu/pTARGET/). Accordingly, RAB39 protein was predicted to be localized in Golgi with a confidence of 75.1 %. To further confirm its localization, a pEYFP-Golgi vector expressing a fused protein of the yellow fluorescent protein (YFP) and a Golgi-specific protein, human beta 1, 4-glactosyltransferase, was used for the observation of Golgi. Expression vector of Flag-tagged RAB39 together with pEYFP-Golgi were co-transfected into HONE1 and Cos7 cells and then subjected to indirect immunostaining with anti-Flag antibody. In consistent with its predicted localization, *RAB39* encoded a cytoplasmic protein co-localized with Golgi in both HONE1 and Cos7 cells (Fig. 4-8).

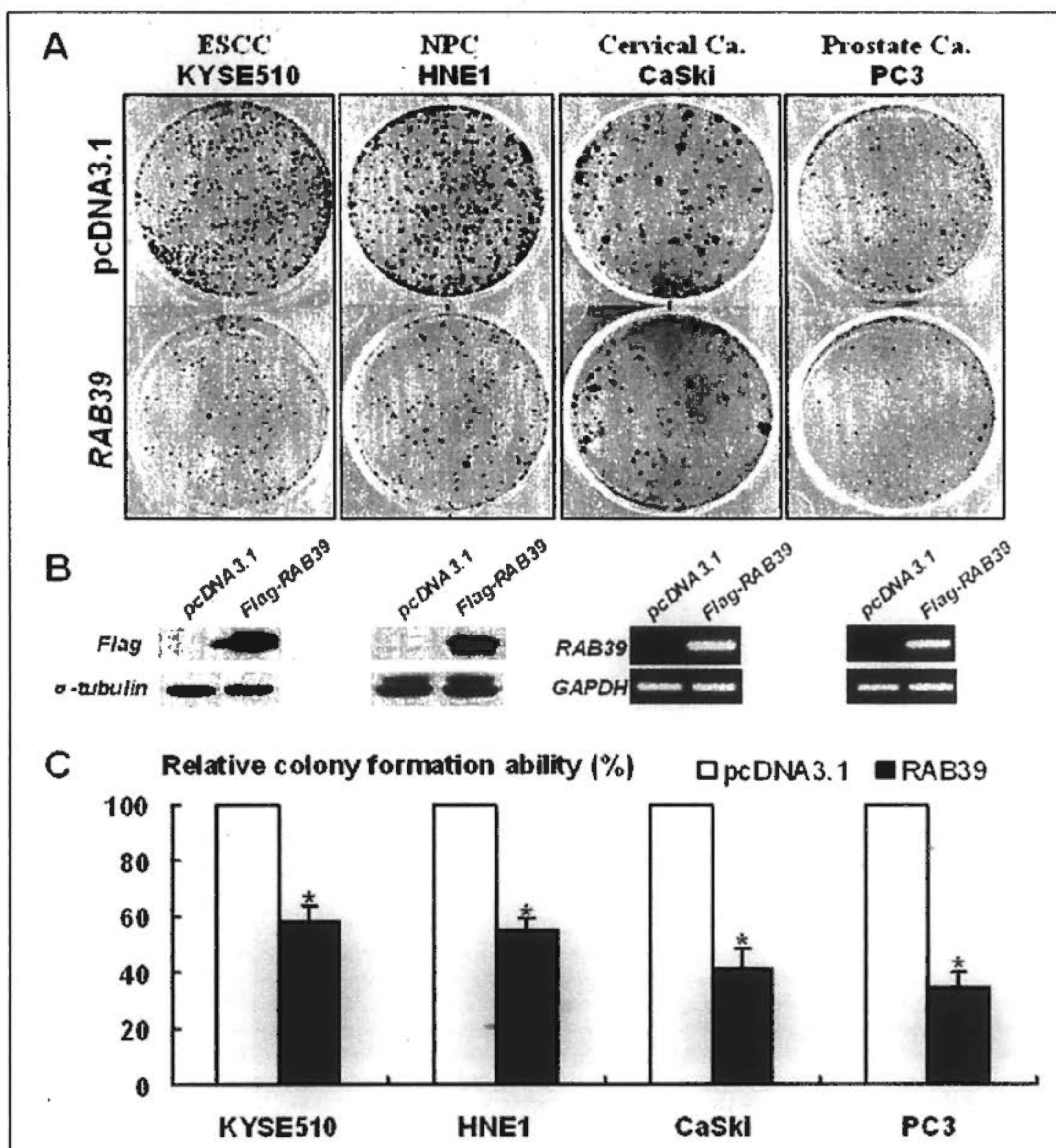

Figure 4-9, Effects of ectopic *RAB39* expression on cancer cell clonogenicity. A) Inhibition of colony formation in monolayer culture by the restoration of *RAB39* in KYSE510, HNE1, CaSki and PC3 cells; B) Western blot or semi-quantitative RT-PCR confirmed the re-expression of *RAB39* after transfection; C) Quantitative analysis of colony formation assay. The numbers of G418-resistant colonies in vector-transfected cell lines were set to 100%. Three independent experiments were carried out in triplicate. Asterisk indicates statistically significant difference (*p<0.05).

4.10 Ectopic *RAB39* expression inhibits colony formation ability of multiple caner cell lines

RAB39 was frequently downregulated/silenced by promoter methylation in multiple cancer cell lines and primary tumors, but not in immortalized cell lines and

normal epithelial tissues, strongly implying that *RAB39* had potential anti-tumorigenic abilities. To directly assess its tumor suppressive functions in cancer, the influence of ectopic *RAB39* expression on cell clonogenicity was examined for a panel of cancer cell lines with silenced and methylated *RAB39*, including ESCC cell line KYSE510, NPC cell line HNE1, cervical cancer cell line CaSki and prostate cancer cell line PC3. pcDNA3.1(+)-Flag-*RAB39* expression vector was transiently transfected into above cells for colony formation assay in monolayer cultures. Results demonstrated that re-expression of *RAB39* significantly inhibited the colony formation abilities of the silenced cell lines, indicating that *RAB39* acted as a potential functional TSG in these cancer cells (Fig 4-9).

4.11 Discussion

ESCC is one of the most common malignancies in Hong Kong and some regions of Mainland China. Both its diagnostic and therapeutic strategies are awaiting substantial improvements due to its late diagnosis until advanced-stage disease and poor response to chemotherapy (Sarah and David, 2008). Our lab is interested in studying both the genetic and epigenetic disruptions of this malignancy, with the aim of seeking novel biomarkers and therapeutic targets. Using high resolution aCGH, we previously identified an 11q22.3 copy number loss locus in multiple ESCC cell lines. As reviewed in chapter one, LOH and deletions involving 11q22 have been reported in multiple other cancers, suggesting the possible presence of candidate TSGs in this chromosomal region (Ullmann et al., 2001; Davis et al.,

1996).

Based on the fact that inactivation of an individual TSG generally was not restricted to one type of cancer, but rather simultaneously present in multiple types of malignancies (Ying et al., 2006), cell lines of NPC, another major cancer type in which the lab was interested, was also included in the initial expression screening together with several ESCC cell lines. By screening the mRNA-level expression status of eight genes within this locus using RT-PCR, *RAB39* was found to demonstrate tumor-specific downregulation/silencing in the majority of ESCC and NPC cell lines studied, thus this gene was selected for further analysis. Following studies confirmed its frequent downregulation/silencing in multiple others cancer cell lines, indicating that the transcriptional silencing of *RAB39* might be an important contributing factor in either the initiation or the progression of cancers.

As reviewed earlier, promoter methylation is a prevalent mechanism by which TSGs are inactivated in cancers (Feinberg and Tycko, 2004). Since there was a typical CGI spanning the promoter and exon1 of *RAB39*, it was possible that the downregulation of *RAB39* in cancer cell lines might be mediated by promoter methylation. As expected, promoter hypermethylation of *RAB39* was found in multiple cancer cell lines, which was inversely correlated with its mRNA-level expression in most cell lines studied. Moreover, *RAB39* mRNA level could be significantly restored by the pharmacological treatment with 5-Aza-2'-deoxycytidine in silenced cell lines, indicating that DNA methylation directly led to its downregulation/silencing. Finally, colony formation assay demonstrated that ectopic

expression of *RAB39* could efficiently inhibit the clonogenicity of multiple tumor cell lines. Collectively, it's possible that *RAB39* could be a novel epigenetically silenced candidate TSG in cancers.

RAB39 encodes a member of the human RAB small GTPase family, members of which function as molecular switches in both the exocytic and endocytic pathways, and regulate vesicular transport in eukaryotes. In human, there were more than 60 RAB GTPases identified so far, and some of them were highly conserved during evolution, implicating the importance of RAB GTPase in eukaryotic cell biology (Stenmark and Olkkonen, 2001). Although mutations of several RAB coding genes have been implicated in a number of human inherited diseases, the roles of most RAB GTPase in cancer have not been well established as some members in the Ras and Rho small GTPase families, such as Ras, RhoA and Rac1 (Chia and Tang, 2009). However, emerging evidence has suggested frequent deregulation of some RABs in cancers, including the upregulation of *RAB1*, *RAB5A*, *RAB23* and *RAB25* in some malignancies, and the downregulation of *RAB32*, *RAB37*, and *RAB45* in others, as reviewed in chapter one. Among these cancer-related RABs, *RAB25* was the most extensively studied one which was functionally characterized as an activator for both cell growth and aggressiveness in ovarian and breast cancers, although it was insufficient to cause malignant transformation on its own (Cheng et al., 2004; Caswell et al., 2007).

The detailed mechanisms underlying the involvement of RABs in cancer are still largely unknown. However, there are several possible avenues by which RABs

might influence cancer development, either in a tumorigenic/metastasis promoting way, or instead, in a tumor suppressive way. On one hand, overexpression of some RABs can influence cell proliferation by altering the vesicular transportation of growth factor receptors and related signaling pathways (Chia and Tang, 2009). On the other hand, some other RABs have recently been shown to alter cell survival via influencing the expression of cell nutrient receptors (Edinger et al., 2003).

Thus far, like most other human RAB family members, the exact physiological role of *RAB39* in vesicular transport is still not elucidated, neither is its role in cancer. To look for clues in understanding its function, the subcellular localization of RAB39 GTPase was analyzed. It's revealed that it was co-localized with Golgi, like its *Drosphila* orthorlog (Sinka et al., 2008). The precise targeting of RABs to membranes of distinct organelles determined the localization of their downstream effectors, and thus was essential for their function. Golgi associated RABs such as RAB8 and RAB11 played important roles in the vesicular traffic between Golgi and plama membrane and the secrection of proteins (Sato et al., 2008; Knodler et al., 2010), processes in which RAB39 might also be involed in. Most recently, a study showed that RAB39 could bind to the cytokine-processing caspase, caspase-1, which was essential for the proteolytical cleavage and activation of Interleukin-1β (IL-1β) precursor during inflammation regulation. RAB39 GTPase acted as a trafficking adaptor in the caspase-1-dependent secretion of Interleukin-1β (IL-1β) and was inducible by pro-inflammatory stimulus, strengthening its potential role in the signaling of the innate immune system (Becker et al., 2009). Interestingly, in

addition to its essential role in IL-1β secretion, caspase-1 could also function as a pro-apoptotic molecule in some circumstances, although it was not regarded as one of the primary pro-apoptotic caspases. For instance, it has been reported to be downregulated in ovarian cancer cell lines and its overexpression could induce obvious apoptosis (Feng et al., 2005). Thus it was intriguing to explore that whether RAB39, as a capase-1 binding protein, was also involved in caspase-1 mediated apoptosis regulation, although our preliminary experiments suggested that ectopic expression of *RAB39* could not induce apoptosis in silenced cancer cell lines on its own.

It is noteworthy that a number of RAB members have recently been found frequently methylated in various tumors, including *RAB32* in colon cancer, *RAB37* in lung cancer and *RAB45* in melanoma (Maat et al., 2008; Shibata et al., 2006; Wu et al., 2009). More importantly, hypermethylation of *RAB45* was associated with decreased survival in melanoma, indicating the potential prospects of using these methylated RABs as cancer biomarkers (Maat et al., 2008). To our knowledge, we are the first to identify that a novel RAB GTPase, *RAB39* is frequently methylated in multiple cancer cell lines as well as in primary tumors. Regarding the dramatic difference of *RAB39* methylation status between primary ESCC/NPC and corresponding normal tissues, it's intriguing to investigate the role of *RAB39* methylation as a biomarker in both ESCC and NPC in future.

To summarize, the current study identified an 11q22.3 small RAB GTPase gene *RAB39* as an epigenetically silenced candidate TSG in ESCC and multiple other

cancers. Re-introducing *RAB39* into cancer cells could dramatically suppress cancer colony growth, with the underlying mechanisms remained to be elucidated. Given the emerging roles of some overexpressed RAB GTPase in cancer proliferation and invasion as reported, it is of great value to elucidate the potential anti-tumorigenic roles of those RABs inactivated in cancers, including *RAB39*, in order to reach a comprehensive understanding in the relation of RAB GTPase family and cancer development.

Limitations of this study: Genes downregulated in mRNA-level was selected as promising candidates in this study, however, protein levels of candidate genes in cancer cell lines and primary tumors were not studied. This study should beome more convincing if the candidates could be selected based on protein level expression of genes. For the candidate TSG *RAB39* identified here, limited functional role of it in carcinogenesis was characterized. More functional experiments should be carried out to confirm the role *RAB39* plays in essential cancer-related biological processes, such as proliferation and cell movement. In addition, the sample size of primary tumors used in methylation study is not large enough in order to reach a convincing conclusion on whether *RAB39* methylation could be used as diagnosis markers.

Chapter 5, Identification of *WDRX* as a novel epigenetically silenced TSG candidate at 15q21.3

5.1 Three 15q copy number loss loci revealed by aCGH in ESCC cell lines

1) A **15q14** copy number loss locus represented by a BAC clone bA261P5 was detected in 6/10 ESCC cell lines, including EC1, EC18, EC109, HKESC3, KYSE520 and SLMT1 according to previous aCGH data. Bioinformatics searching in the Ensembl Genome Browser mapped bA261P5 to 15q14 spanning ~170 kb, between BAC clones bA70A1 and bK2033D15 (Fig. 5-1A). Nine genes in and around bA261P5 were selected as candidates for future analysis (Table 5.1).

2) A **15q21.1** copy number loss locus represented by a BAC clone bA315O8 was detected in 4/10 ESCC cell lines, including EC1, EC18, HKESC3 and SLMT1. bA315O8 was mapped to 15q21.1 spanning ~202 kb, between bA519G16 and bA90J19 (Fig. 5-1B). Sixteen genes in and around bA315O8 were selected for further analysis (Table 5.1).

3) A **15q21.3** copy number loss locus represented by a BAC clone bA215J7 was detected in 3/10 ESCC cell lines, EC1, HKESC3 and SLMT1. bA215J7 is mapped to 15q21.3 spanning ~165 kb, between bA232J12 and bA178D12 (Fig. 5-1C). Totally sixteen genes in and around bA215J7 were selected for expression profiling (Table 5.1).

5.2 Expression profiling of genes located in three 15q putative TSG loci

The number of candidate genes was first narrowed down by identifying the

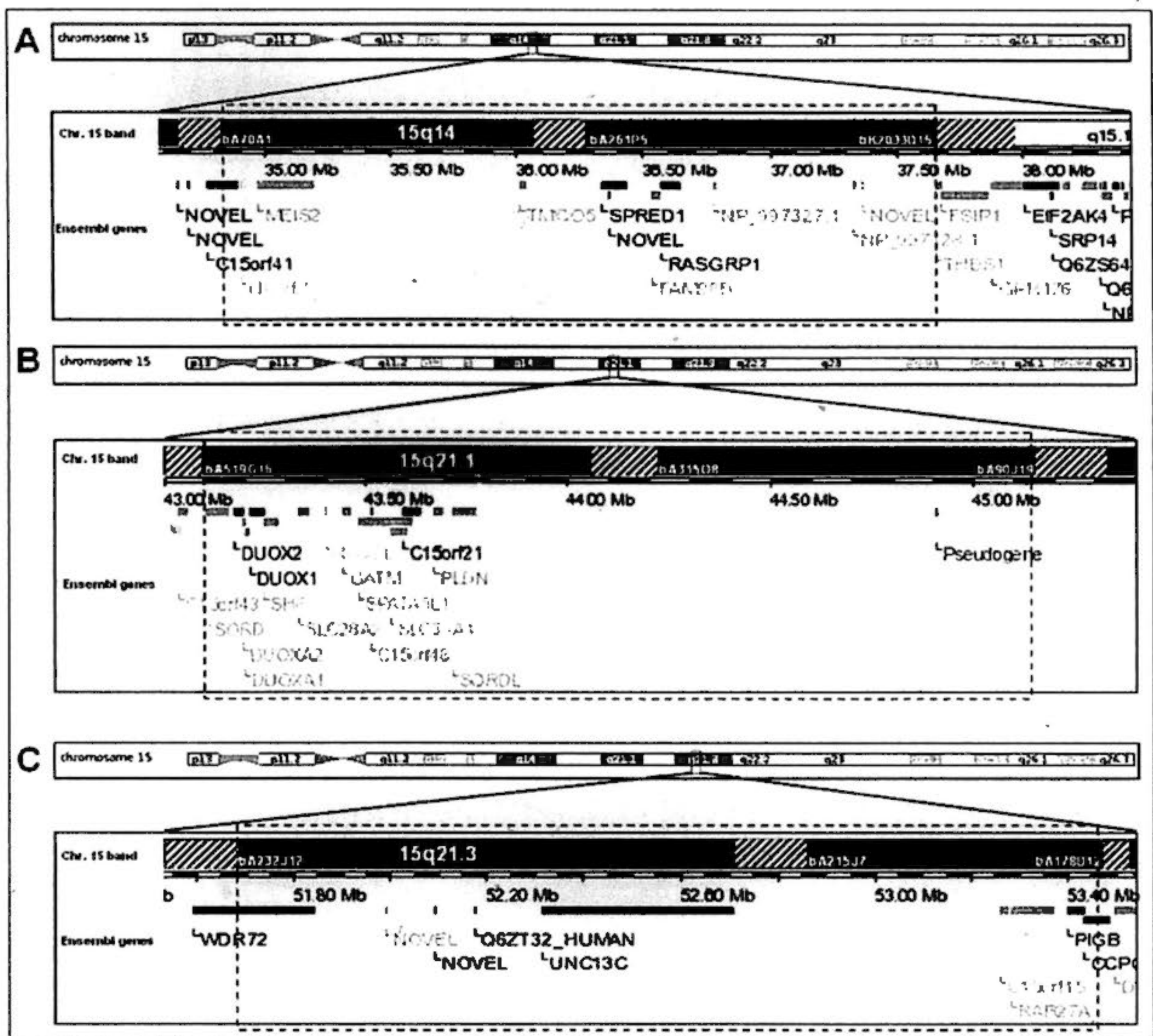

Figure 5-1. Diagram showing the locations of three 15q copy number loss loci. The minimal deletion regions (boxed by dashed lines) were defined by BAC clones, which were indicated by shadowed bars. Upper panel: Structure of chromosome 15 and the location of each copy number loss locus (rectangle) were shown. Lower panel: The candidate genes residing in and around each copy number loss locus were shown. Partially adopted from the Ensembl database (www.ensembl.org/index.html).

genes which were frequently downregulated or silenced in cancer cell lines, but not in normal tissues nor in immortalized epithelial cell lines. RT-PCR was performed with gene specific RT-PCR primers for 9 genes in 15q14, 16 genes in 15q21.1 and 16 genes in 15q21.3, in 8 ESCC cell lines, 3 NPC cell lines and normal tissues of esophagus, larynx, trachea and testis, with *GAPDH* as an internal control. As the result shown in Fig 5-2, 15q14 genes *TMCO5* and *FLJ35695*, 15q21.1 genes

Table 5-1. Information of the candidate genes in three 15q candidate TSG loci.

Region	Gene	Domain/motif	Protein Function	CGI
15q14	*LOC728257*	N/A	Unknown	Yes
	MEIS2	Homeobox	TF	Yes
	TMCO5	TM	Unknown	Yes
	LOC728288	N/A	Unknown	Yes
	LOC644701	N/A	Unknown	Yes
	SPERD1	WASP homology region 1	Cell communication; signal transduction	Yes
	FAM98B	N/A	Unknown	Yes
	RASGRP1	Ras GEF; EF	Guanyl-nucleotide exchange factor	Yes
	FLJ35695	N/A	Unknown	Yes
15q21.1	*LOC197135*	N/A	Unknown	Yes
	B2M	Immunoglobulin C-Type	MHC class I receptor activity	Yes
	RNF36	Ring	Apoptotic protease activator	Yes
	LOC653381	N/A	Unknown	Yes
	SORD	N/A	Catalytic activity; metabolism, energy pathways	Yes
	DUOX2	TM	NADPH oxidase; metabolism, energy pathways	Yes
	DUOXA1	TM	Necessary for proper localization and maturation of dual oxidase 1	Yes
	DUOX1	TM; EF	NADPH oxidase	Yes
	SLC28A	TM	Auxiliary transport protein activity	Yes
	LOC645263	N/A	Unknown	Yes
	GATM	N/A	Amidinotransferase activity,	Yes
	SPATA5L1	AAA	Unknown	Yes
	C15ORF48	TM	Low or completely missing expression in ESCC	Yes
	SLC30A4	TM	Involved in transporting zinc out of the cytoplasm	Yes
	PLDN	CC	Intracellular vesicle trafficking; Cell growth and/or maintenance	Yes
	SQRDL	N/A	Oxidoreductase activity	Yes
15q21.3	*KIAA1370*	N/A	Unknown	Yes
	WDRX	WD40 domain	Unknown	Yes
	UNC13C	N/A	Unknown	Yes
	C15ORF15	Metallochaperone-like	Unknown	Yes
	RAB27A	Rab	GTPase activity; immune response; protein localization	Yes
	PIGB	N/A	Mannosyltransferase activity	Yes
	CCPG1	TM; CC	Unknown	Yes
	DYX1C1	Tetratricopeptide repeats	Unknown	Yes
	PYGO1	PHD zinc finger	Transcription regulator activity	Yes
	PRTG	Fibronectin type 3 domain	Unknown	Yes
	NEDD4	HECTc	Ubiquitin-specific protease	Yes
	RFXDC2	N/A	TF	Yes
	TEX9	CC	Unknown	Yes
	LOC645877	N/A	Unknown	Yes
	LOC145783	N/A	Unknown	Yes
	TCF12	Helix loop helix; CC	TF	Yes

TM, transmembrane domain; CC, Coiled Coil motif; AAA, ATPases associated with a variety of cellular activities domain; EF, EF-hand, calcium binding motif; HECTc, Domain Homologous to E6-AP Carboxyl Terminus with.

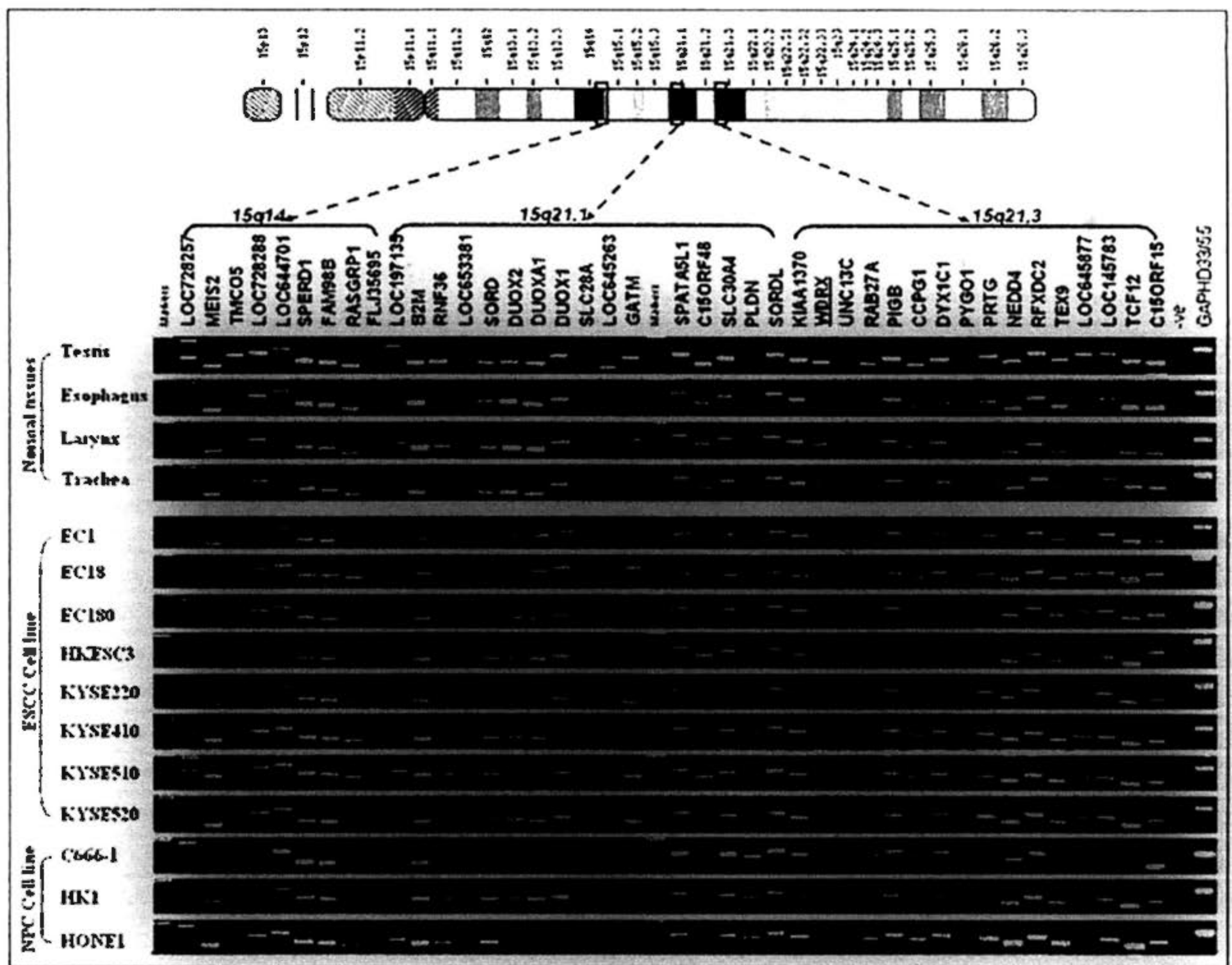

Figure 5-2. mRNA-level expression profiling of 41 candidate genes in three 15q putative TSG loci in a panel of ESCC and NPC cell lines. Upper panel: Schematic diagram showing the copy number loss loci (boxed); Lower panel: Gel electrophoresis photos showing the mRNA expression level of 41 genes in four normal tissues, 8 ESCC cell lines and 3 NPC cell lines. *WDRX*, underlined, was selected as a candidate for further study. *GAPDH* was used as an internal control.

LOC653381, *SLC28A* and *LOC645263*, as well as 15q21.3 genes *UNC13C* and *PYGO1*, were downregulated or silenced in multiple ESCC and NPC cell lines. However, none of these genes was highly expressed in normal esophagus, larynx, or trachea tissues. Thus they were not considered as potential TSG candidates for further study. On the contrary, 15q21.1 gene *GATM* and 15q21.3 gene *WDRX* were highly expressed in normal tissues while dramatically downregulated or silenced in multiple ESCC and NPC cell lines, thus both could be promising candidates.

However, literature reviewing indicated that *GATM* was an imprinted gene in both human and mouse which was involved in creatine biosynthesis, a physiological process with no obvious association with carcinogenesis (Sandell et al., 2003; Zhou et al., 2007b). Therefore, *GATM* was also excluded from further analysis. The other candidate, *WDRX*, a novel gene without known function, encodes a WD40-repeat containing (WDR) protein. WDR proteins comprise a large protein family in eukaryotes and perform diverse functions including cytoskeletal organization, vesicular fusion, signal transduction, transcription regulation, cell cycle control and apoptosis (Li and Roberts, 2001). Importantly, several WDR genes have been found to cause human inherited diseases when mutated (Tullio-Pelet et al., 2000; LoNigro et al., 1997; Henning et al., 1995; Bassi et al., 1999). Most recent evidence suggested that a number of WD40-repeat containing proteins such as *FBW7* and *WDR6* were involved in carcinogenesis (Welcker and Clurman, 2008; Xie et al., 2007). Thus *WDRX* was selected as a 15q candidate gene for further study.

5.3 *WDRX* at 15q21.3 is downregulated in multiple cancer cell lines

WDRX consists of 20 exons with a single open reading frame and encodes a protein of 1102 amino acids containing seven WD40-repeat domains (Fig. 5-3A and B). In contrast to its low mRNA-level expression in the heart, spleen, breast and brain tissues, *WDRX* was highly expressed in some human adult and fetal tissues, including larynx, trachea, lung, cervix and testis, while moderately expressed in liver, pancreas, stomach, esophagus, rectum, ovary and prostate (Fig. 5-3C). The

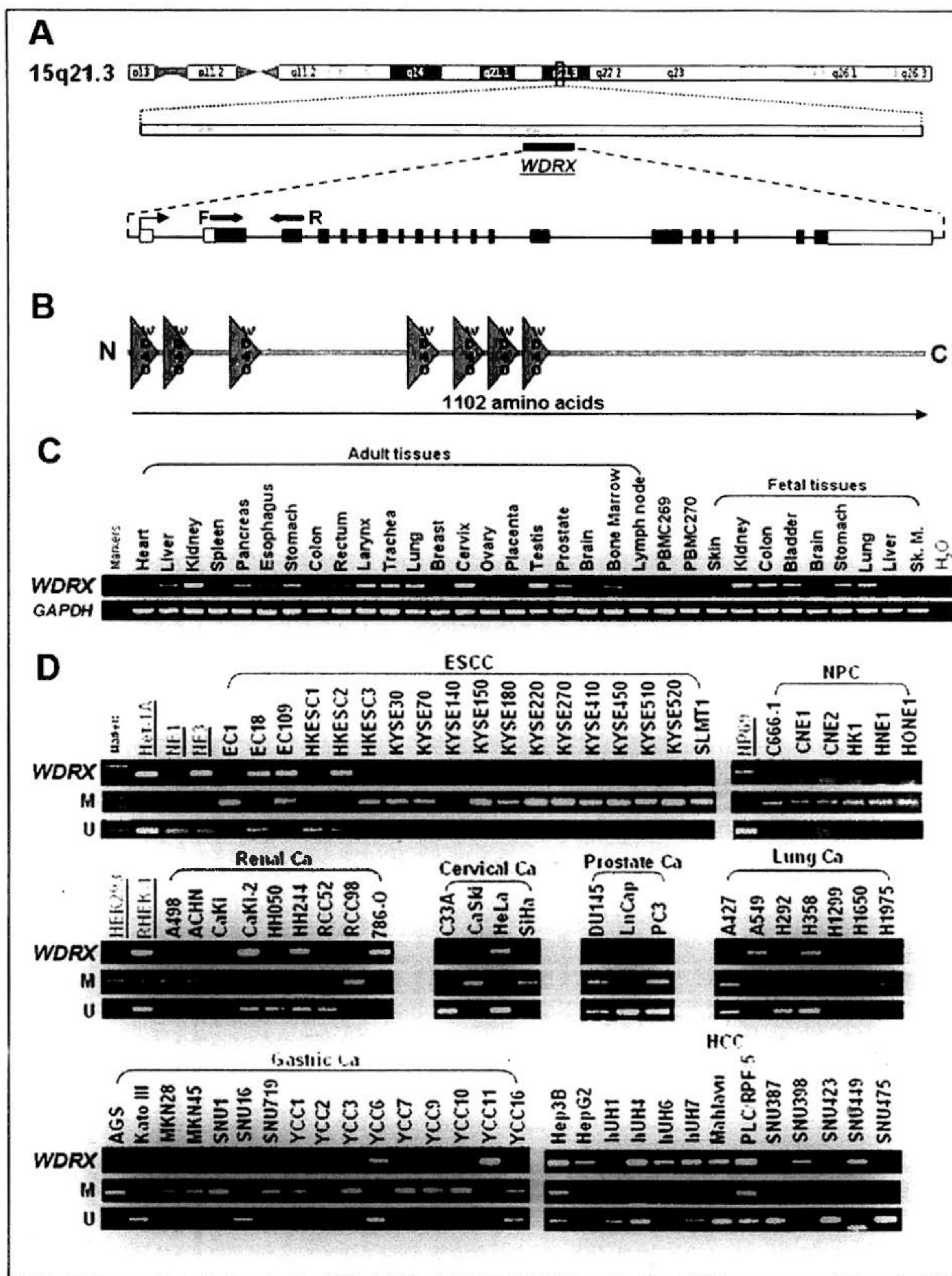

Figure 5-3. Structure of *WDRX* transcript and its expression/methylation patterns in normal and malignant samples. A) Schematic diagram showing the relative position of *WDRX* in 15q as well as the structure of its transcript. The forward and reverse RT-PCR primers were indicated with arrows; B) Schematic structure of the 1102a.a. WDRX protein showing the seven WD40-repeat domains (triangles). N, n-terminal; C, c-terminal; C) Semi-quantitative RT-PCR results showing the expression pattern of *WDRX* in normal human adult and fetal tissues, with *GAPDH* as an internal control. Sk. M., skeletal muscle. D) RT-PCR and MSP

results showing that *WDRX* was frequently downregulated/methylated in multiple cancer cell lines. The methylation status of *WDRX* promoter was analyzed using MSP. Promoter methylation was detected in cancer cell lines in which *WDRX* expression was reduced or silenced, but not in most immortalized epithelial cell lines (underlined) studied.

expression profile of *WDRX* in a large collection of carcinoma cell lines and some immortalized epithelial cell lines was further determined using semi-quantitative RT-PCR. In contrast to its normal expression in adult tissues of esophagus, cervix, kidney, liver, lung, prostate, and stomach, *WDRX* was frequently downregulated or silenced in cancer cell lines arising from above tissues (Fig. 5-3D). In addition, *WDRX* expression was also detected in immortalized esophageal (Het-1A and NE3), nasopharyngeal (NP69) and Renal (HEK293 and RHEK-1) epithelial cell lines (Fig. 5-3D). Thus, a tumor-specific downregulation pattern was observed for *WDRX*.

5.4 Identification of a new transcriptional start site for *WDRX*

5' RACE assay revealed the actual transcriptional start site (TSS) of *WDRX* in esophageal tissue, which was located 49 nucleotides preceding the *WDRX* TSS in NCBI database (Fig. 5-4). Thus 49 nucleotides were added to *WDRX* exon1 in its 5' direction, without changing the numbering of *WDRX* exons, nor greatly influencing the position of the putative *WDRX* promoter.

5.5 Confirmation of the transcription initiating ability of *WDRX* promoter

In order to assess whether the putative promoter region upstream of the *WDRX* TSS could functionally initiate transcription, the -390-+235 genomic region was

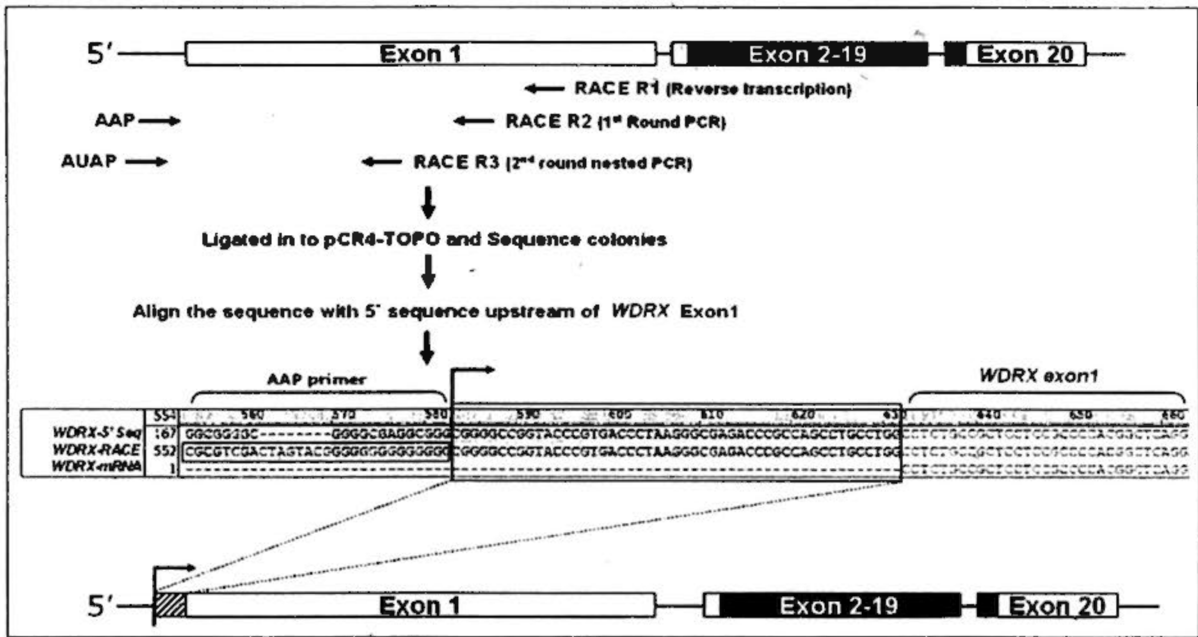

Figure 5-4. General procedures and result of 5' RACE assay for *WDRX*. Middle panel: The AAP primer and 49 nucleotides added to *WDRX* exon1 were boxed. The new TSS was indicated by curved arrow; Lower panel: Schematic diagram showing new TSS as well as the nucleotides (dashed shadow) added to exon1.

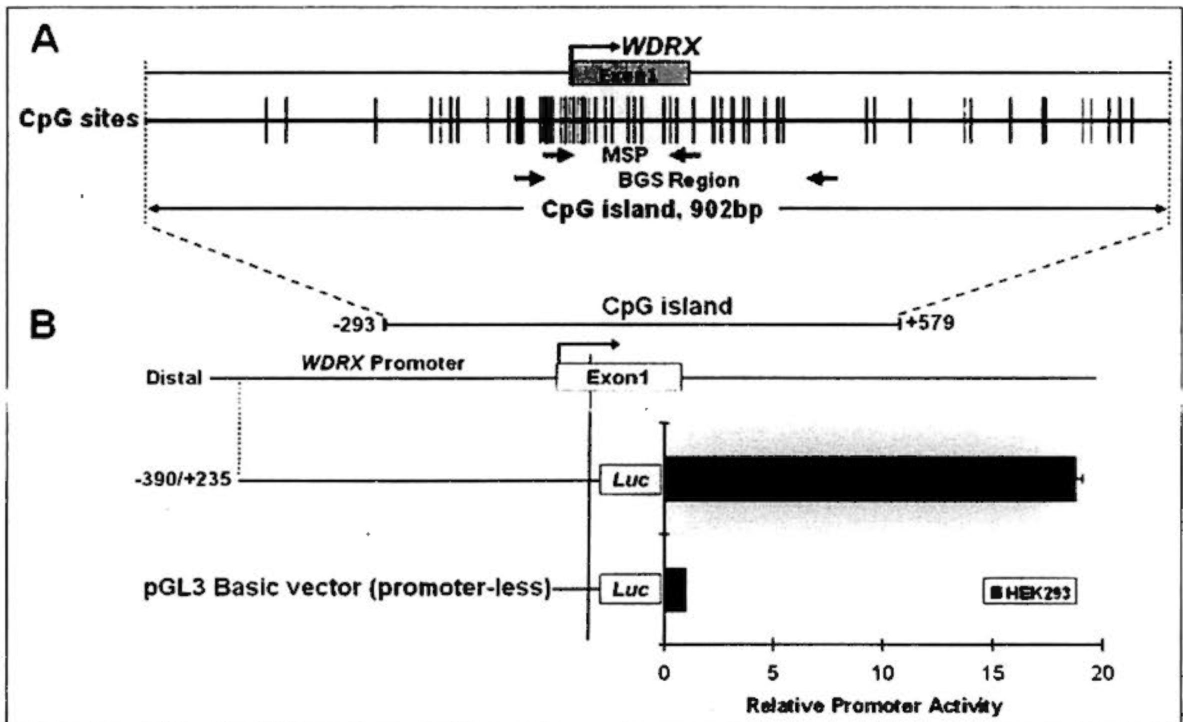

Figure 5-5. The structure and relative position of *WDRX* promoter CGI, and results for *WDRX* promoter-luciferase reporter assays. A) A 902bp CGI spanning *WDRX* exon1 and promoter. Relative positions of MSP and BGS primers, and the region analyzed by BGS were indicated by arrows; B) Luciferase activity of a *WDRX* promoter construct, relative to the empty vector, was shown. Data were means and standard deviations of three independent assays. The promoter fragment was proved to be able to drive the transcription of the luciferase reporter gene in HEK293 cells.

cloned and ligated into pGL3-Basic vector. Transcription initiating activity of this fragment was tested by transiently transfecting the construct into HEK293 cells, and results showed that this promoter fragment could lead to a dramatic increase of luciferase activity in HEK293 cells, when compared to control vector (Fig. 5-5B), indicating that the putative *WDRX* promoter was able to initiate transcription *in vitro*.

5.6 Methylation status of *WDRX* promoter in tumor cell lines

5.6.1 Promoter CGI analysis for *WDRX*

Based on the data from the 5'RACE assay and promoter luciferase assay, methylation analysis was carried out to elucidate the role of promoter methylation in the tumor-specific downregulation of *WDRX*. According to analysis in CpG island searcher (http://cpgislands.usc.edu/), there existed a typical CGI spanning the putative promoter region and exon1 of *WDRX* from -293-+579 (Fig. 5-5A), making it possible to study the relation between the transcriptional downregulation of *WDRX* and its promoter methylation.

5.6.2 MSP reveals that *WDRX* is methylated in multiple tumor cell lines

MSP was performed to study the methylation status of *WDRX* promoter CGI in different cancer cell lines. Several pairs of MSP primers targeting the methylated (M) or unmethylated (U) alleles of *WDRX* promoter CGI were designed. The optimal M or U primer pairs with best amplification efficiency and specificity were used for MSP analysis. In addition, the selected M and U primer pairs were excluded from

Table 5-2. Summary of the frequencies of *WDRX* promoter methylation in carcinoma cell lines and primary tumors.

Samples	**Promoter methylation (%)**
Carcinoma cell lines	
Nasopharyngeal	6/6 (100%)
Esophageal	14/18 (78%)
Gastric	14/16 (88%)
Lung	2/7 (29%)
Hepatocellular	2/13 (15%)
Cervical	2/4
Prostate	3/3
Immortalized normal epithelial cell lines	
NP69, HET-1A, NE1, NE3	0/4
Normal Esophageal Tissues	1/7 (14%)
Normal Nasopharyngeal Tissues	2/5 (40%)
Primary Tumor Samples	
ESCC	8/20 (40%)
NPC	35/37 (95%)

amplifying unbisultifted DNA. Results showed that *WDRX* was methylated in downregulated tumor cell lines including NPC, ESCC, gastric, lung and cervical carcinomas, but not in most immortalized normal epithelial cell lines including NP69, Het1A, NE1 and NE3, indicating that the promoter CGI methylation of *WDRX* was tumor-specific (Fig. 5-3D, Page 101). Frequencies of *WDRX* promoter methylation in above tumor cell lines were summarized in Table 5-2.

5.6.3 BGS confirms the tumor-specific methylation of *WDRX* promoter CGI

BGS was conducted to analyze the detailed methylation status of 34 individual CpG sites within the *WDRX* promoter CGI and to validate the MSP results. As expected, BGS results were consistent with those of MSP, indicating that dense methylation of

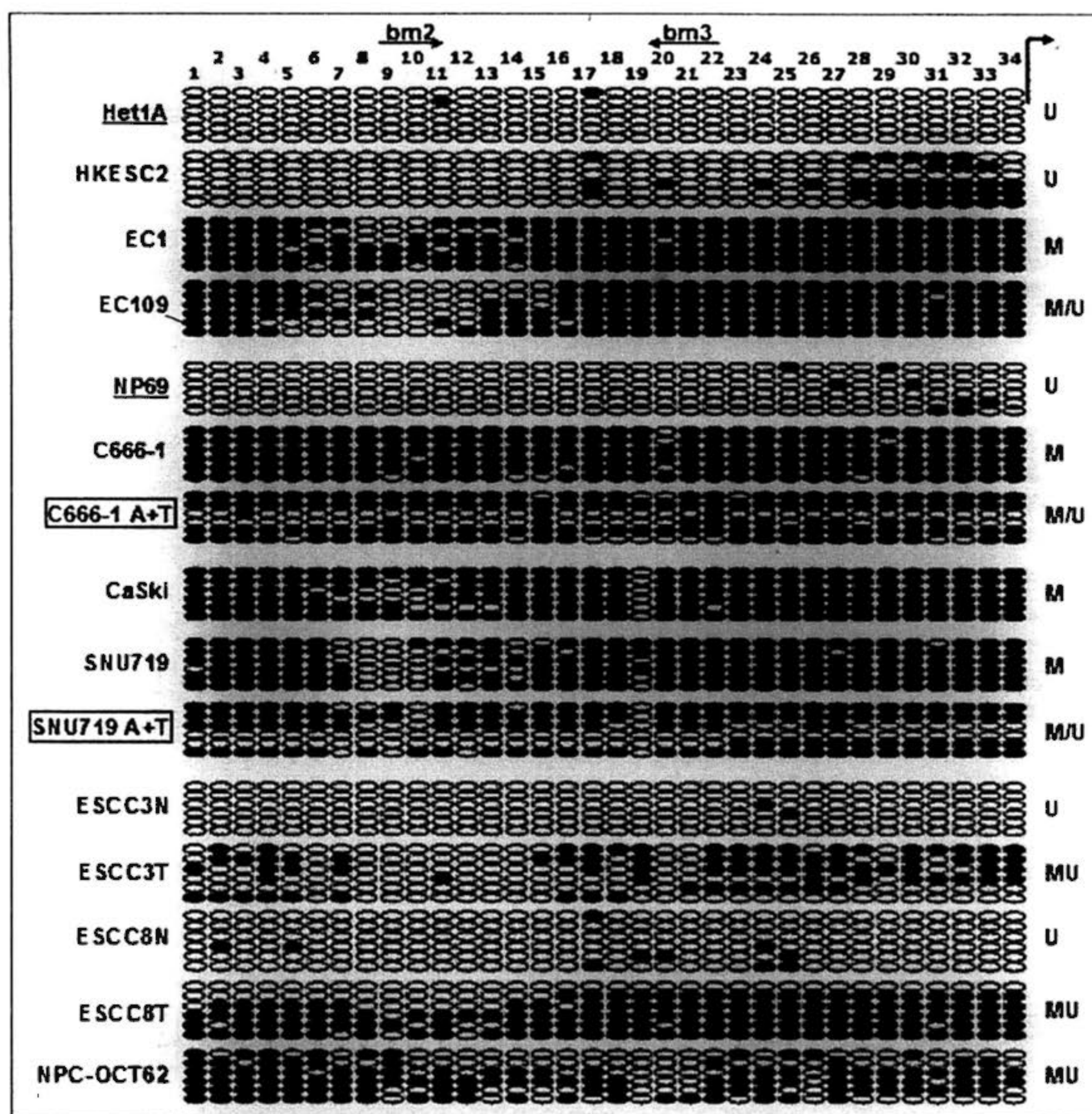

Figure 5-6. Methylation status study of *WDRX* promoter CGI using BGS. The methylation status of 34 CpG sites in *WDRX* promoter CGI was analyzed in representative immortalized cell lines (underlined), cancer cell lines of different origins, cell lines treated by Aza and TSA (boxed), NPC primary tumor (OCT62) as well as samples from primary ESCC malignant (T) and surgical marginal (N) esophageal tissues. The right panel indicated the *WDRX* methylation status revealed by MSP. Relative positions of MSP primers and TSS were indicated by curved arrows. One row of circles indicated an allele of *WDRX* promoter CGI. Open circle, unmethylated CpG; Filled circle, methylated CpG.

the included CpGs was only observed in the methylated cancer cell lines revealed in MSP, including ESCC (EC1, EC109), NPC (C666-1), gastric (SNU719) and cervical (CaSki) cancers, but not in unmethylated non-tumor cell lines Het1A and NP69, nor in unmethylated ESCC cell line HKESC2 (Fig. 5-6).

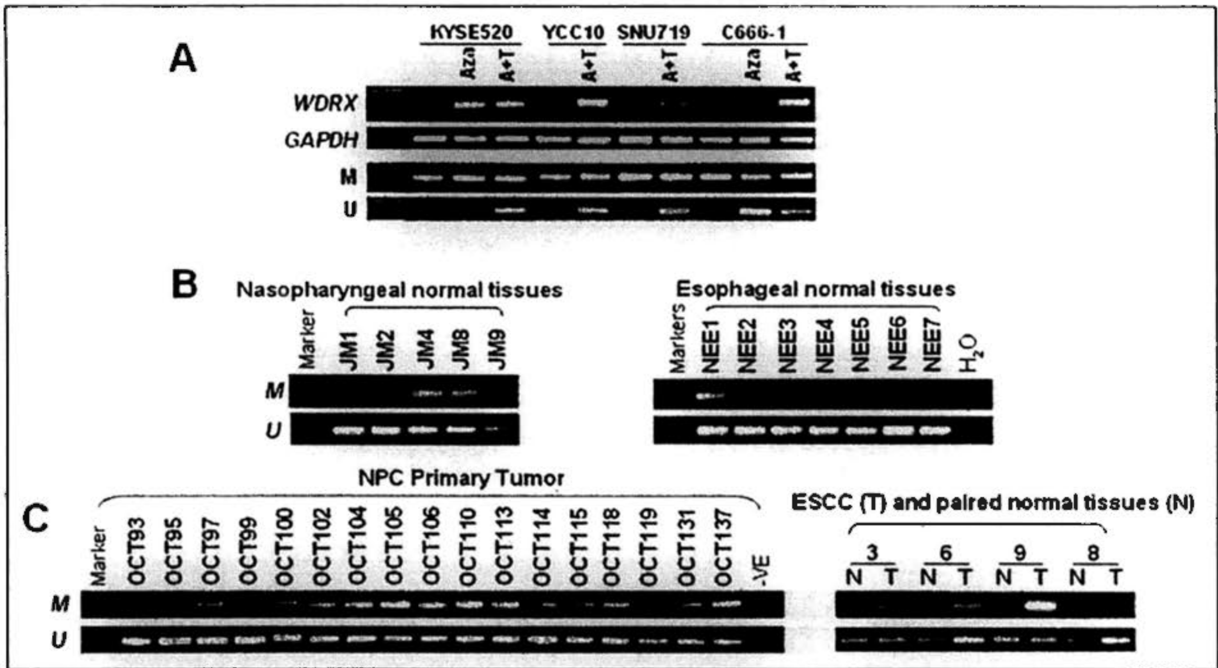

Figure 5-7. *WDRX* reactivation upon pharmacological demethylation treatment and its methylation patterns in primary tumors. A) Pharmacological treatment induced the demethylation of *WDRX* CGI and restoration of its mRNA-level expression, as analyzed by MSP and RT-PCR. A, Aza; T, TSA; M, methylated; U, unmethylated; B,C) Representative methylation study of *WDRX* in normal tissues, primary NPC, as well as paired ESCC malignant (T) and surgical marginal (N) tissues, as analyzed by MSP. M, methylated; U, unmethylated.

5.7 Restoration of *WDRX* expression by pharmacological demethylation

To examine whether promoter methylation directly mediated the silencing of *WDRX*, cancer cell lines C666-1, KYSE520, SNU719 and YCC10 were treated with DNMT inhibitor Aza combined with or without HDAC inhibitor TSA. After treatment, *WDRX* expression levels were dramatically increased in all cell lines examined, together with obvious increase in unmethylated alleles of the *WDRX* promoter CGI, as revealed by MSP (Fig. 5-7A) and further confirmed by BGS (Fig. 5-6). As predicted, combined treatment of Aza and TSA led to even higher *WDRX* expression levels. These results demonstrated that promoter methylation directly contributed to the downregulation of *WDRX* in cancer cell lines.

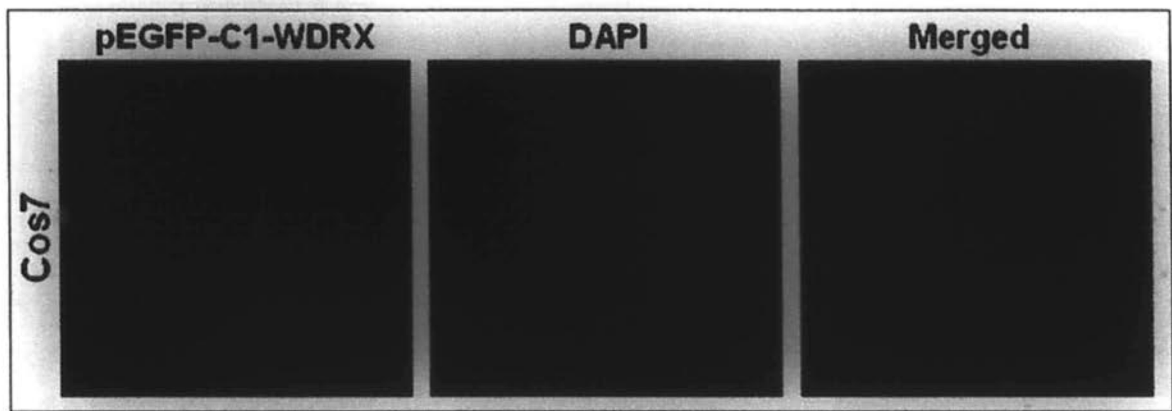

Figure 5-8. Subcellular localization of WDRX in Cos7 cells. Left panel showed the green fluorescence of GFP-WDRX fusion protein; middle panel showed the DAPI-stained cell nuclei; right panel showed the merged image.

5.8 *WDRX* is frequently methylated in primary ESCC and NPC

Next, in order to reveal the direct involvement of *WDRX* promoter methylation in the of human primary tumor, *WDRX* promoter methylation was analyzed in a panel of primary ESCC and NPC, together with normal epithelial tissues as controls. Whereas 8/20 (40%) primary ESCC showed promoter methylation of *WDRX*, only 1/20 (5%) paired surgical marginal esophageal tissues and 1/7 (14%) normal esophageal tissues from healthy individuals had *WDRX* methylation (Fig. 5-7B and C). In addition, *WDRX* promoter methylation was detected in 35/37 (95%) primary NPC from Asian Chinese, while with a significantly lower methylation frequency (2/5, 40%) in nasopharyngeal tissues from healthy individuals (Fig. 5-7B and C).

5.9 WDRX is localized in cytoplasm

To investigate the subcellular localization of WDRX, a pEGFP-C1-WDRX plasmid was constructed by ligating the *WDRX* ORF into pEGFP-C1 vector, which could successfully encode a fused GFP-WDRX protein as verified by western blot

(Fig. 5-10A, Page 111). The pEGFP-C1-WDRX vector was transiently transfected into Cos7 cells to detect the subcellular localization of WDRX. As shown in Fig. 5-8, *WDRX* encoded a WDR protein which was primarily localized in cytoplasm.

5.10 Ectopic *WDRX* expression inhibits clonogenicity in multiple tumor cell lines

The frequent downregulation and methylation of *WDRX* in multiple cancer cell lines and primary tumors, but not in immortalized epithelial cell lines and normal tissues, led to the assumption that *WDRX* might be an epigenetically silenced candidate tumor suppressor. To study the potential anti-tumorigenic role of *WDRX*, the effect of ectopic *WDRX* expression on the growth of tumor cells was investigated using colony formation assay. Mammalian expression vector pcDNA3.1(-)-*WDRX* was transiently transfected into ESCC, NPC and cervical cancer cell lines (KYSE150, KYSE510, HNE1 and CaSki) which had completely silenced and methylated *WDRX*. The colony formation efficiency of each transfected cancer cell line was evaluated by monolayer culture under the selection pressure of G418. Results showed that ectopic expression of *WDRX* significantly reduced the clonogenicity in studied cancer cell lines, when compared with cells transfected by empty vector only ($p<0.05$; Fig. 5-9). These data suggested that *WDRX* indeed possessed growth inhibitory activities in cancer cells.

5.11 *WDRX* inhibits WNT/β-catenin signaling in KYSE150 cells

In order to reveal the molecular pathways that *WDRX* might regulate in ESCC, the influence of its re-expression on the luciferase activity of a serie of pathway

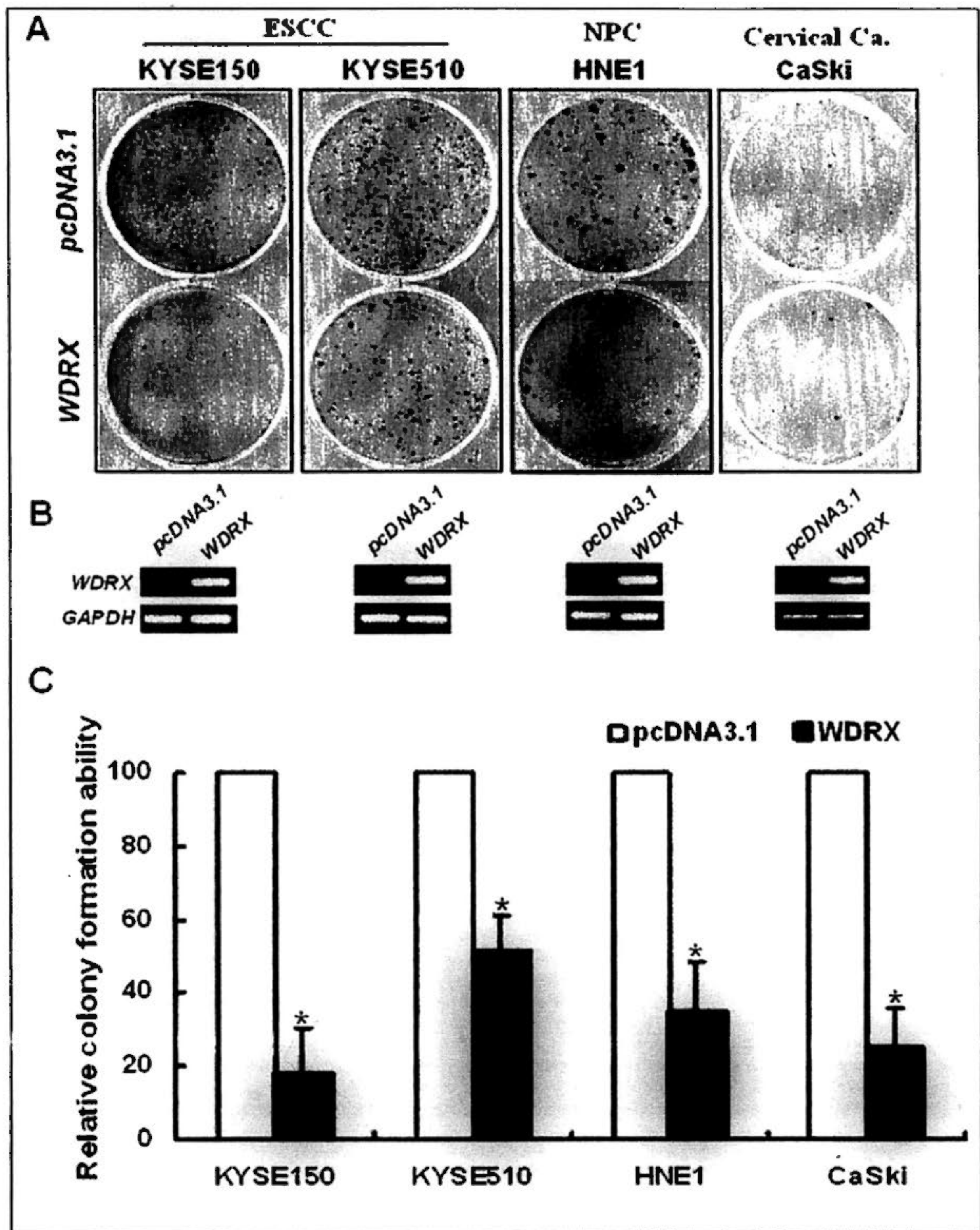

Figure 5-9. Effects of ectopic *WDRX* expression on cancer cell clonogenicity. A) Inhibition of colony formation in monolayer culture by the restoration of *WDRX* in KYSE150, KYSE510, HNE1 and CaSki cells; B) Semi-quantitative RT-PCR shows the *WDRX* re-expression after transfection; C) Quantitative analysis of colony formation. The numbers of G418-resistant colonies in vector-transfected cell lines are set to 100%. Three independent experiments are carried out in triplicate. Asterisk indicates statistically significant difference (*$p<0.05$).

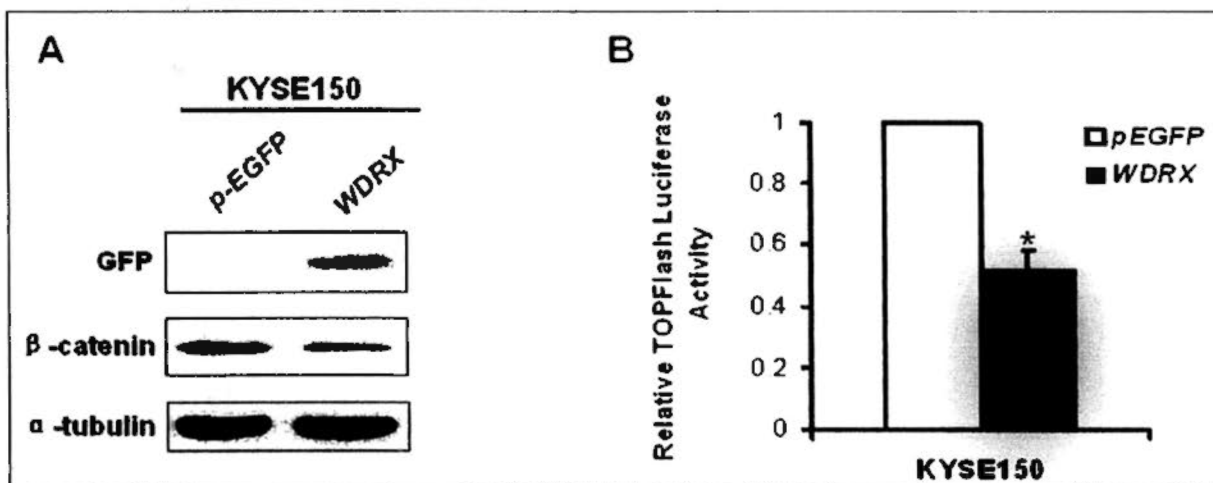

Figure 5-10. *WDRX* antagonizes WNT/β-catenin signaling in KYSE150 cells. (A), Ectopic expression of *WDRX* reduced β-catenin protein level in KYSE150. The ectopic expression of *WDRX* was confirmed by the presence of a 150.4 kDa GFP-WDRX fused protein; (B) *WDRX* expression resulted in the downregulation of the luciferase activity of TOPFlash reporter (*, $p<0.05$).

reporter constructs, such as SRE, AP-1 and TOPFlash, was assessed in a silenced ESCC cell line KYSE150. As a result, ectopic expression of *WDRX* significantly inhibited the transcriptional activity of the TOPFlash reporter (Fig. 5-10A). TOPFlash contains four multimerized T-cell factor/lymphoid enhancer factor (TCF/LEF) binding sites and its transcriptional activity could reflect the activity of WNT/β-catenin signaling (Korinek et al., 1997). To verify the possible role of *WDRX* in repressing WNT/β-catenin signaling as indicated in the TOPFlash reporter assay, its influence on the intracellular β-catenin protein level was examined in KYSE150 cells. The results demonstrated that *WDRX* re-expression led to significantly reduced protein level of β-catenin, as compared with vector control (Fig. 5-10B). Collectively, these data indicated that *WDRX* could negatively modulate the WNT/β-catenin signaling in ESCC cells.

5.12 Discussion

Based on previous aCGH data in ESCC cell lines, mRNA expression profiling for 41 genes in three 15q copy number loss loci was carried out to search for novel TSG candidates. A 15q21.3 gene *WDRX* was revealed to be downregulated among most ESCC and NPC cell lines, while highly or moderately expressed in corresponding normal tissues and immortalized cell lines. In addition, it was also frequently downregulated in multiple other cancer cell lines derived cervical, renal, lung and gastric cancers. By MSP and BGS, *WDRX* promoter CGI was found to be frequently methylated in multiple caner cell lines, with its promoter methylation well correlated with its reduced expression in ESCC and NPC cells. Further more, pharmacological demethylation could induce the translational re-expression of *WDRX*, indicating that promoter methylation directly mediated its transcriptional silencing in cancer cells. More importantly, ectopic expression of *WDRX* could significantly suppress the clonogenicity of multiple cancer cell lines, indicating that it could play a critical role during cancer development as a potential TSG candidate. In addition, ectopic *WDRX* expression led to the inactivation of TCF/β-catenin/--dependent transcription as revealed by luciferase reporter assays, and the reduction of intracellular β-catenin protein level in KYSE150 cells, suggesting that *WDRX* could function as a tumor suppressor at least partially through antagonizing aberrant WNT/β-catenin signaling.

WDRX is a novel member of the human WDR protein family and contains seven WD40-repeat domains.. The characteristic WD40-repeats in WDR protein was

first characterized in the β subunit of the large G protein, which also contained seven WD40-repeats like *WDRX* (Clemen et al., 2008). It is believed that several WD40-repeats together could form a circularized beta-propeller structure, which might act as docking platforms to facilitate protein interaction and the formation of various transient protein complexes. Although mulitple WDRs have been associated with diverse biological processes, the functional role of *WDRX* remains elusive so far. However, it has recently been proposed to associate with some human diseases(Paterson et al.; El-Sayed et al., 2009).

By sequence similarity analysis, the closest human paralog of *WDRX* is revealed to be *WDR7*, followed by *KTNB1*, *SMU1* and *WDR5* (according to http://www.genome.jp/kegg/ssdb/). *WDR7* is involved in vesicle mobilization and Ca^{2+}-dependent exocytosis of neurotransmitter at synapses, as the β subunit of rabconnectin-3 which could bind with Rab3A GDP/GTP exchange protein (Coleman and Bykhovskaia, 2009); *KTNB1* is one of the subunits in the heterodimer protein katanin which functions to sever and disassemble microtubules; *SMU1* appears to be involved in DNA replication and the maintenance of chromosome integrity (Sugaya et al., 2005); while *WDR5* is a core subunit of human MLL and SET1 histone H3K4 methyltransferase complex (Trievel and Shilatifard, 2009). Evidence indicated that all four *WDRX* paralogs above acted as components of protein complexes in order to exert their specific biological roles, possibly via their WD40-repeat domains, implying that *WDRX* might also participate in the formation of some unknown functional protein complexes with its seven WD40-repeats.

Recently, the emerging role of WDR proteins in the ubiquitin proteosome pathway has been highlighted. For example, a novel human WDR protein, L2DTL, was found to be able to bind both CUL4-DDB1 E3 ubiquitin ligase complex and one of its targets, CDT1 (chromatin licensing and DNA replication factor 1). CDT1 contributed to tumorigenicity by causing genomic instability and could be degraded by CUL4-DDB1 complex in response to DNA damage. The WD40-repeat domains in L2DTL were found to be necessary to facilitate the CDT1 proteolysis by CUL4-DDB1 (Higa et al., 2006a). Moreover, loss of L2DTL, but not the other WDR proteins, could directly prevent the degradation of CDT1, suggesting that L2DTL might act as the molecular adaptor mediating the specific substrate recognition of CUL4-DDB1 E3 ligase. In addition to L2DTL, WDR proteins DDB2, WDR26, TLE1-3, WDR82, GRWD1, SMU1 and a group of histone methylation regulators including WDR5, EED, and RBBP, were all identified to interact with CUL4-DDB1 complex *in vivo* (Higa et al., 2006b).

Interestingly, besides the CUL4-DDB1 E3 ligase, WDR proteins could also act as substrate-adaptors for another type of E3 ligase, the SCF (complex of SKP1, CUL1 and F-box protein)-type ubiquitin ligase. For instance, FBW7 (F-box and WD repeat domain-containing 7), one of the substrate recognition components in SCF ligase, could specifically recognize several important proto-oncogene proteins including c-Myc, cyclin E, Notch and JUN, and mediate their proteolysis via proteosome, thus *FBW7* functions as an important tumor suppressor which is frequently disrupted in various malignancies (Welcker and Clurman, 2008). While its

F-box domain directly mediates the interaction of FBW7 with SKP1, its WD40-repeats form an eight-bladed barrel shaped beta propeller structure with phosphodegron binding pockets, which is responsible for its binding with the phosphorylated substrates (Hao et al., 2007). Mutations in the WD40-repeats are predicted to impair the substrate binding ability of FBW7. In addition, it's demonstrated that mutations in the highly conserved arginines in its WD40-repeats can interfere with the capability of wild type FBW7 to degrade substrates such as cyclin E and c-Myc, strengthening the essential role that the WD40-repeats play in the substrate-specific recognition of FBW7 (Akhoondi et al., 2007).

Taken these facts and our functional findings that *WDRX* could diminish the intracellular β-catenin protein in ESCC cells into account, it becomes quite intriguing to analyze the possibility that *WDRX* might bind with some E3 ubiquitin ligase, and in turn function as a substrate-recognizing adaptor in the ubiquitin-proteasome proteolytic degradation of β-catenin or other oncogenic molecules. In this way it might play an anti-tumorigenic role in cancer development, mimicking the degradation of cyclin E and c-Myc by SKP1-CUL1-FBW7 and the degradation of CDT1 by CUL4-DDB1-L2DTL. Importantly, it has been reported that β-Trcp (*FBW1*), another SKP1 associated F-box and WD40-repeats containing protein, could specifically recognize phosphorylated β-catenin and mediate its degradation via the ubiquitination apparatus in *Xenopus*, which provided strong supports for above hypothesis.

It's known that WNT/β-catenin signaling is closely related with the

development of human cancer, in addition to its vital roles during development. Activation of this signaling leads to stabilization and nuclear translocation of β-catenin, which, in turn, forms a transcription activation complex with members of TCF/LEF family to stimulate the expression of β-catenin target genes, many of which are involved in tumorigenesis through regulating cell growth, proliferation and death (Polakis, 2000). Genetic and epigenetic disruptions in multiple essential molecules in the WNT network could result in deregulated activation of this signaling which was associated with tumorigenesis in multiple tumors, such as colorectal cancer (Ying and Tao, 2009; Bienz and Clevers, 2000). Actually, aberrant activation of WNT/β-catenin signaling has also been frequently detected in ESCC (Kimura et al., 1999; Salahshor et al., 2008; Ninomiya et al., 2000). For example, previous study demonstrated the significant accumulation of soluble β-catenin and its nuclear translocation in ESCC tissues but not in normal esophageal tissues (Kimura et al., 1999). Likewise, another report showed deregulated WNT/β-catenin signaling with aberrant accumulation of cytosolic and nuclear β-catenin in an ESCC tissues array study (Salahshor et al., 2008). Furthermore, it's proposed that cleavage of E-cadherin by metalloproteinases dissociated a-catenin, β-catenin and cadherins from cytoskeleton to the cytosol and thus increased the cytosolic pool of β-catenin in ESCC cells (Marambaud et al., 2002). If the hypothesis that *WDRX* could mediate the degradation of β-catenin via ubiquitin-proteasome pathway could be validated, the epigenetic silencing of *WDRX* might represent one of the molecular mechanisms for the activation of WNT/β-catenin signaling in ESCC ant thus contribute to the development of this malignancy.

In short, a 15q21.3 WDR protein coding gene *WDRX* was identified as a candidate TSG frequently inactivated by promoter methylation in ESCC and NPC. Ectopic expression of *WDRX* in cancer cells without its expression could dramatically suppress cancer cell colony growth and inhibit the WNT/β-catenin signaling, indicating a potential role as a functional TSG for this gene. Further study is required to explore the detailed mechanism underlying its regulation in WNT/β-catenin pathway as well as the exact roles it played in cancers.

Limitations of this study: The 15q study has the same limitations as mentioned for the 11q one, including the identification strategy, the inadequate sample size for methylation study, and the limited functional study. In addition, though it's identified that *WDRX* could antagonize the WNT/β-catenin pathway, the underlying mechanism was not fully elucidated and its influence on the downstream biological effects of WNT signaling, such as proliferation and cell cycle regulation, were not studied either.

Chapter 6, *ADAMTS8* is an epigenetically silenced TSG candidate negatively modulating ERK pathway in NPC and ESCC

6.1 Previous identification of *ADAMTS8* as an 11q25 candidate TSG silenced by promoter methylation in NPC and multiple other cancers

Based on previous aCGH in a panel of NPC cell lines, a locus with frequent copy number loss spanning about 6 Mb in 11q24.2 and 11q25 was identified between BAC clones bA168K9 and bA419F8. After profiling the mRNA-level expression and methylation status of genes located within this locus, *ADAMTS8*, a member of the human ADAMTS metalloprotease family (reviewed in chapter one), was identified to be frequently downregulated in NPC and ESCC cell lines studied. *ADAMTS8* was found to reside between bA264E20 and pAC1064E20, two BAC clones within the 6 Mb copy loss locus (Fig 6-1A), just next to another ADAMTS member *ADAMTS15*.

According to previous research work on this gene in our lab (most data were not shown here), *ADAMTS8* was broadly expressed in normal human tissues and immortalized epithelial cell lines, but was frequently downregulated due to promoter methylation in multiple cancer cell lines, including nasopharyngeal, esophageal, gastric, colorectal, and cervical cancers. With a typical CpG island spanning the putative promoter and exon1 of *ADAMTS8* (Fig 6-1B), MSP and BGS were carried out which indicated that promoter methylation of *ADAMTS8* was well correlated with its transcriptional inactivation in NPC and other cancer cell lines (Fig 6-1D). Moreover, both pharmacological demethylation and genetic demethylation (DNMT

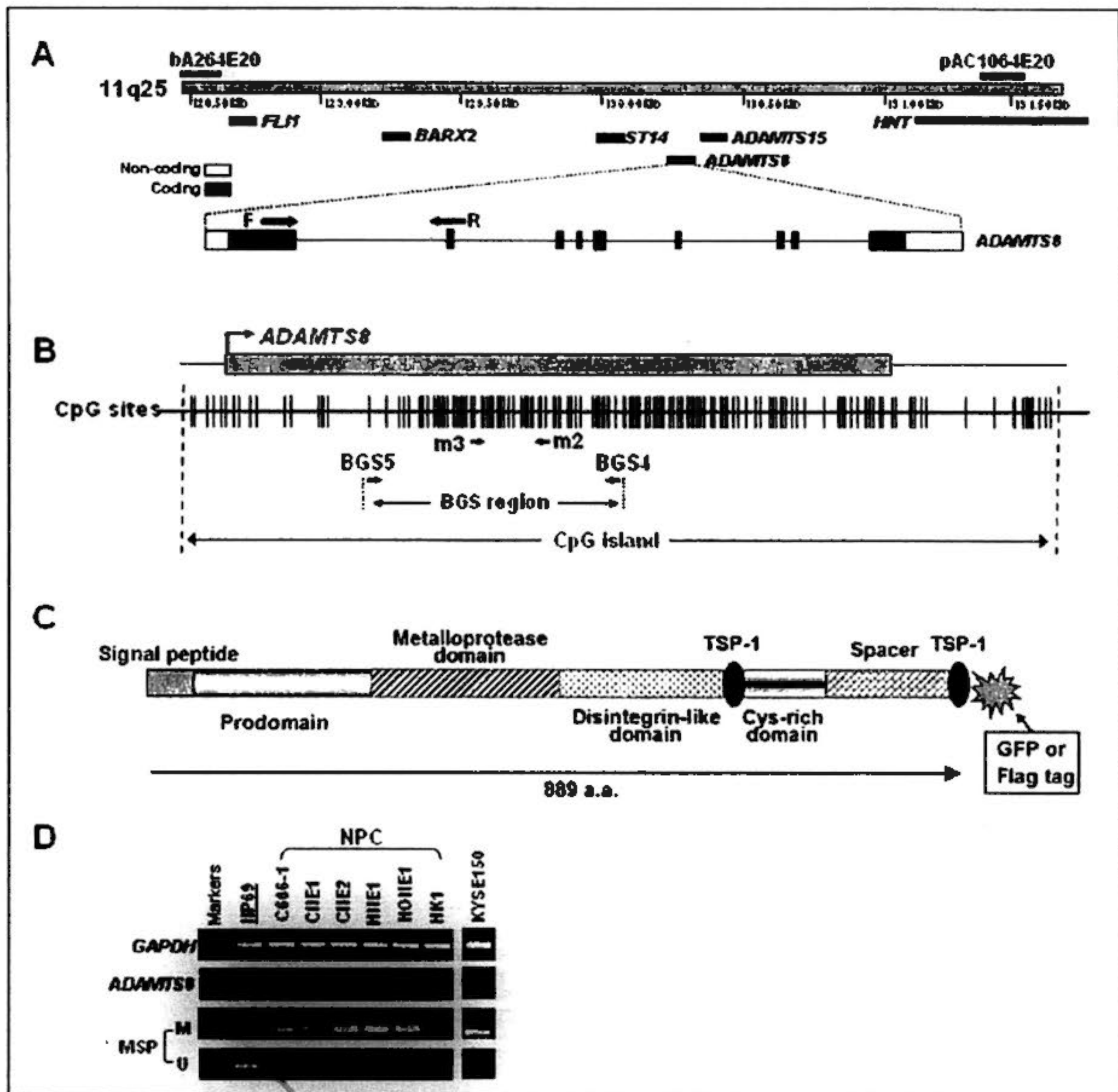

Figure 6-1. ***ADAMTS8*** **is an epigenetically silenced candidate TSG in 11q25.** A) Schematic diagram showing the relative position of *ADAMTS8* in 11q25 as well as the structure of its transcript. The forward and reverse RT-PCR primers were indicated with arrows. The positions of genes located adjacent to *ADAMTS8* inducing *FLI1*, *BARX2*, *ST14*, *ADAMTS15* and *HNT* were also shown; B) Structure of the CGI spanning *ADAMTS8* exon1 and promoter. Respective positions of MSP and BGS primers, and the region analyzed by BGS were indicated by arrows; C) Structure of ADAMTS8 protein showing the major functional domains including the prodomain, the metalloprotease domain, the disintegrin-like domain and two TSP-1 domains. The position of the FLAG-tag and fused GFP was also indicated; D) Representative RT-PCR and MSP results showing that *ADAMTS8* was downregulated/methylated in NPC cell lines as well as an ESCC line KYSE150, but not in immortalized nasopharyngeal epithelial cell line NP69 (underlined), with *GAPDH* as an internal control. M, methylated; U, unmethylated. Adopted from previous work by Dr. Yajun Wang in current lab.

knockout) dramatically restored the mRNA expression of *ADAMTS8* in silenced cell lines, indicating that DNA methylation directly mediated its transcriptional inactivation. In addition, promoter methylation of *ADAMTS8* was frequently detected in primary NPC samples, but seldom in normal nasopharyngeal epithelial tissues, suggesting that *ADAMTS8* methylation played a role in the development of primary NPC.

Most recently, two ADAMTS family members, *ADAMTS12* and *ADAMTS15*, were demonstrated as negative modulators for ERK pathway in cancers. Moreover, somatic mutations have been implicated for *ADAMTS15* in human colorectal cancer, strengthening the emerging role of this metalloprotease family in cancer (Viloria et al., 2009; El Hour et al., 2010). Given our previous identification of *ADAMTS8* as an epigenetically silenced candidate TSG, and the recent research progress on ADAMTS members, it was worthwhile to carry out functional studies on this gene to elucidate its potential anti-tumorigenic roles via modulating ERK pathway.

6.2 Subcellular localization of ADAMTS8

Using pTARGET (http://bioapps.rit.albany.edu/pTARGET/), ADAMTS8 was predicted to be a secreted protein with 100% confidence, which is similar to other ADAMTS members, such as ADAMTS1 which was associated with the extracellular matrix (ECM) after secretion (Kuno and Matsushima, 1998). To directly examine its subcellular localization, a pEGFP-N1-*ADAMTS8* expression vector was constructed and transiently transfected into Cos7 cells for the expression of ADAMTS8 fused

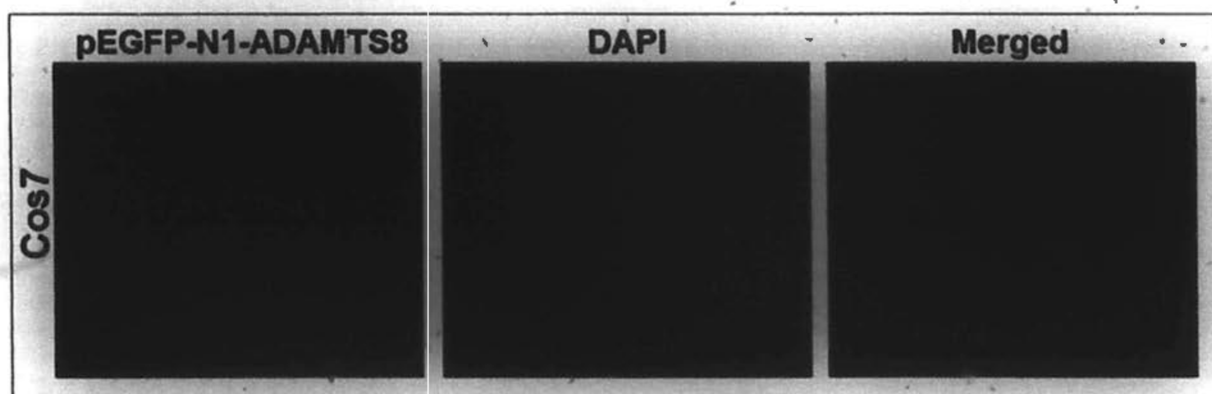

Figure 6-2. Sub-cellular localization of ADAMTS8 in Cos7 cells. Left panel was the FITC green fluorescence of ADAMTS8-GFP fusion protein; middle panel was the DAPI-stained cell nuclei; right panel was the merged image.

with C-terminal GFP (Fig. 6-1C). As shown in the fluorescence picture (Fig. 6-2), ADAMTS8 was distributed throughout the cytoplasm, most likely due to its overexpression, but with a typical staining pattern of multiple green granules scattered outside the cell membrane, resembling the subcellular localization of ADAMTS1, implying that the ADAMTS8 protease might also attach with the ECM after secretion like ADAMTS1.

6.3 *ADAMTS8* ectopic expression inhibits colony formation ability of NPC and ESCC cell lines

Since there was a predicted signal peptide in the N-terminal of ADMATS8 protein (Fig. 6-1C), thus an expression vector with C-terminal Flag-tagged pcDNA3.1(+)-*ADAMTS8* -Flag was constructed for functional study. In order to assess the influence of *ADAMTS8* on tumor cell clonogenicity, this expression vector was transiently transfected into a NPC cell line HONE1 and an ESCC cell line KYSE150, in which *ADAMTS8* was methylated and silenced (Fig. 6-1D), for colony

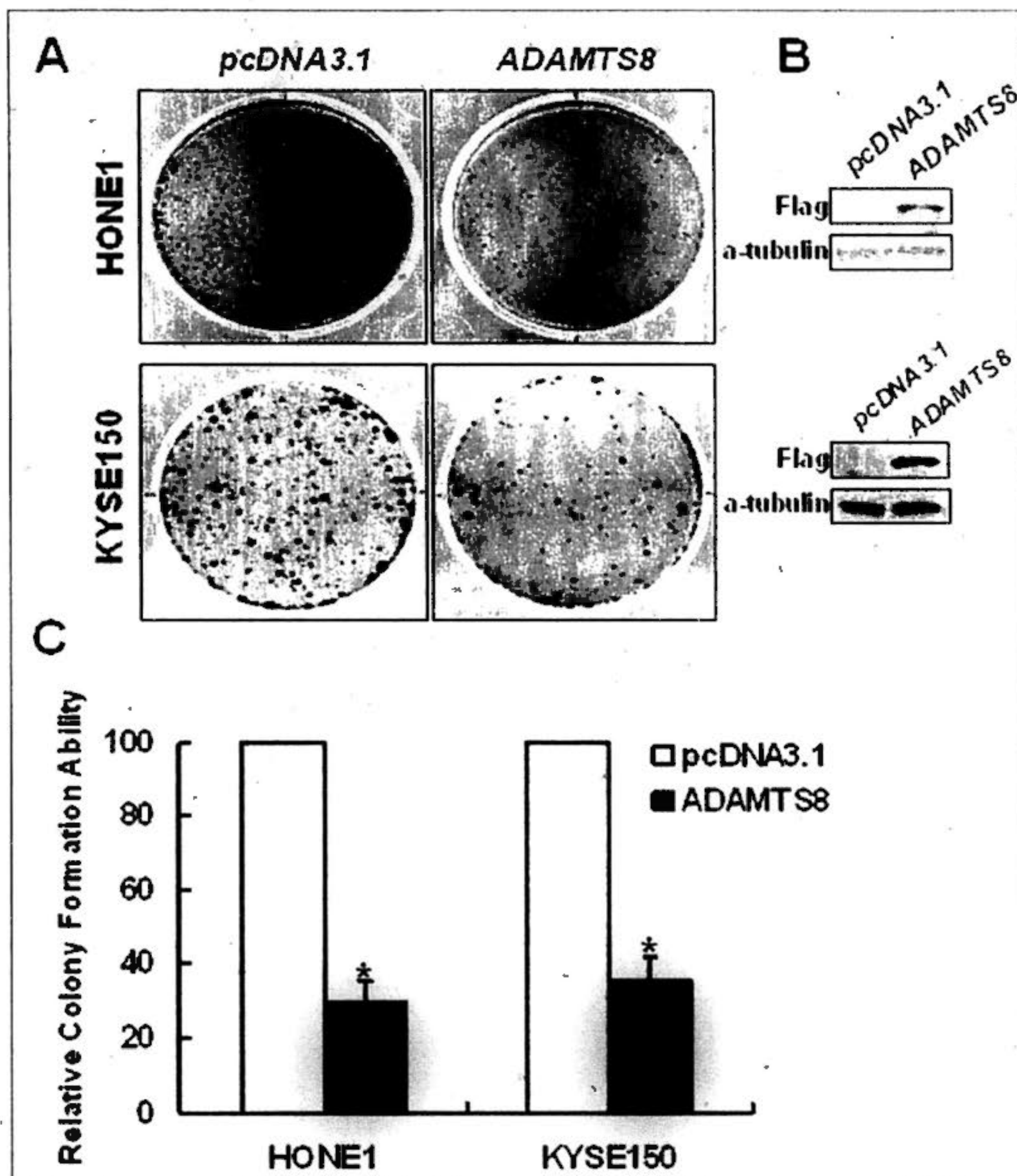

Figure 6-3. Effects of ectopic *ADAMTS8* expression on cancer cell clonogenicity. A) Inhibition of colony formation in monolayer culture by the restoration of *ADAMTS8* in NPC cell line HONE1 and ESCC cell line KYSE150; B) Western blot with the anti-Flag antibody showed the *ADAMTS8* re-expression after transfection. α-tubulin was used as an internal control; C) Quantitative analysis of colony formation. The numbers of G418-resistant colonies in vector-transfected cell lines were set to 100%. Three independent experiments were carried out in triplicate. Asterisk indicates statistically significant difference (*$p<0.05$).

formation assay in monolayer culture. Results demonstrated that ectopic *ADAMTS8* expression significantly inhibited the colony formation capabilities of both cancer cell lines, indicating that *ADAMTS8* could act as a potential functional TSG in both NPC and ESCC (Fig. 6-3).

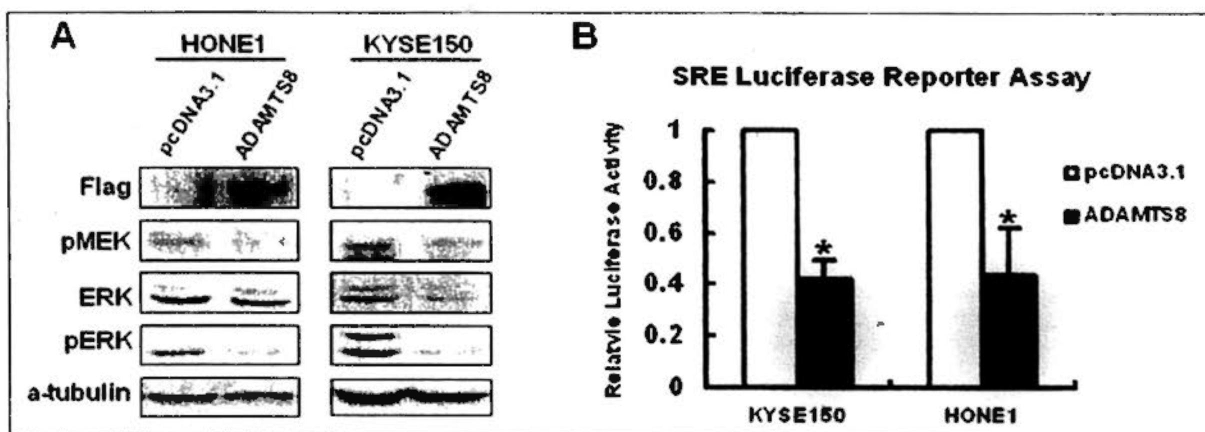

Figure 6-4. *ADAMTS8* inhibits ERK pathway in NPC and ESCC cells. (A), Ectopic expression of *ADAMTS8* reduced protein level of phosphorylated MEK and phosphorylated ERK, without influencing total ERK in both HONE1 and KYSE150 cells. The re-expression of *ADAMTS8* was confirmed by the presence of the 105.5 kDa ADAMTS8-Flag protein. α-tubulin was used as an internal control; (B) *ADAMTS8* re-expression resulted in downregulated luciferase activity of SRE reporter construct in both HONE1 and KYSE150 cells (*, $p<0.05$).

6.4 ADAMTS8 negatively modulates ERK pathway in NPC and ESCC cell lines

To study the possible roles of *ADAMTS8* in regulating ERK pathway in NPC and ESCC, protein levels of the intracellular phosphorylated ERK were examined in both HONE1 and KYSE150 cells by western blot, with or without *ADAMTS8* re-expression. Results showed that ectopic expression of *ADAMTS8* led to significantly reduced intracellular protein levels of phosphorylated ERK, without obvious influence on the protein level of total ERK (Fig. 6-4A). As predicted, *ADAMTS8* overexpression also caused the downregulation of phosphorylated protein levels of the upstream kinase MEK. Given that activated ERK signaling could induce Elk phosphorylation in cell nucleus, which then mediated the serum response element (SRE)-dependant transactivation of a series of target genes, the influence of *ADAMTS8* re-expression on the transcriptional activity of a SRE luciferase reporter was also examined. Results demonstrated that transcriptional activity of SRE reporter

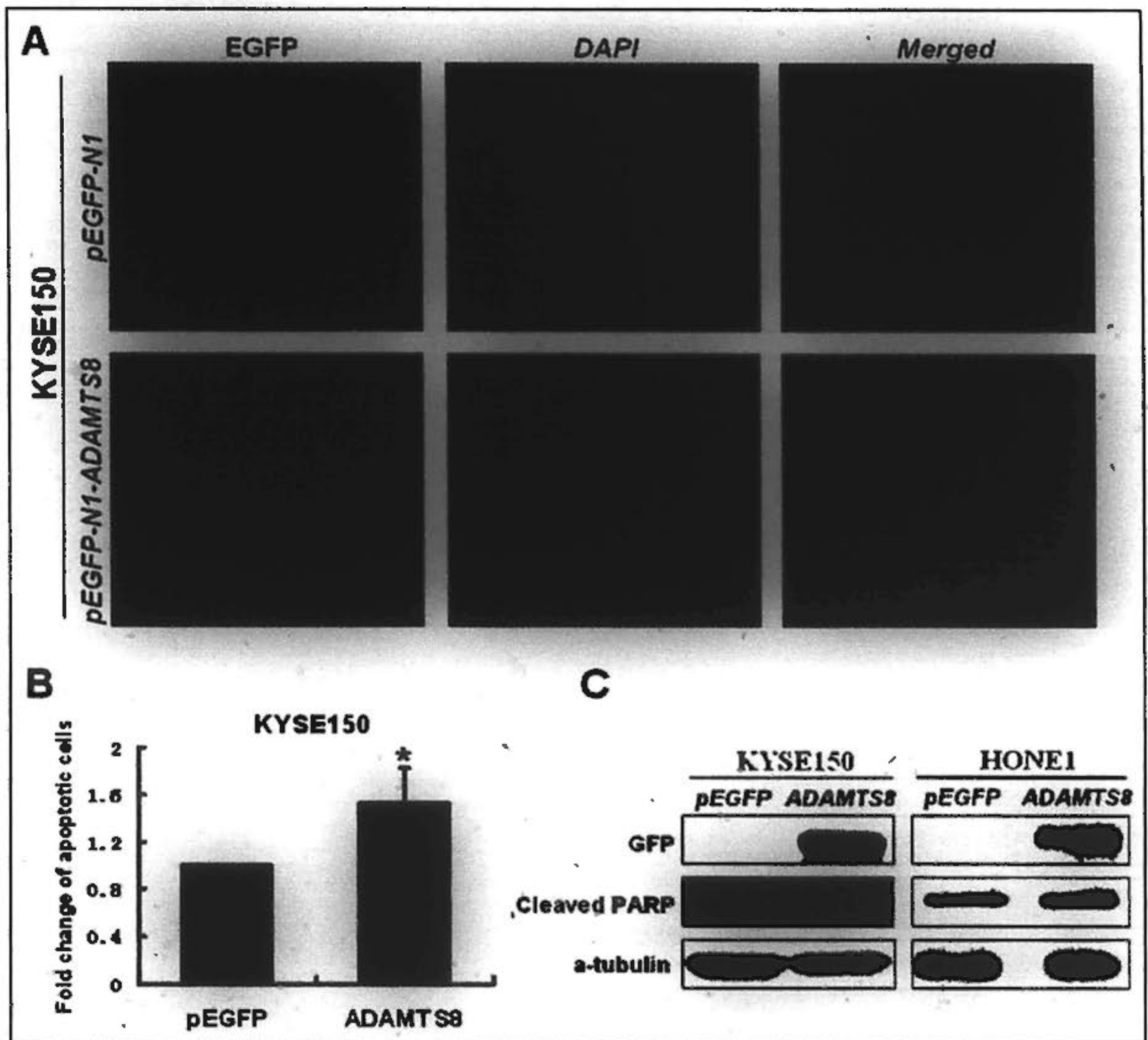

Figure 6-5. ***ADAMTS8*** **exerts pro-apoptotic activity in ESCC and NPC cell lines.** A) KYSE150 cells were transfected with pEGFP-N1-*ADAMTS8* or control vector. At 48 hours post-transfection, cells were stained with DAPI and photographed under fluorescence microscope. Red arrows indicated transfected cells (nuclei) undergoing apoptosis-associated morphological changes. B) Fold change of apoptotic cells with typical nuclear morphological changes (*, $p<0.05$). C) Western blot result showing ectopic expression of *ADAMTS8* elevated the protein level of cleaved PARP, a molecular hallmark of apoptosis, in both KYSE150 and HONE1 cells. Re-expression of *ADAMTS8* was confirmed by the presence of a 123.5 kDa ADAMTS8-GFP protein. α-tubulin was used as an internal control.

was significantly decreased in *ADAMTS8* re-expressing HONE1 and KYSE150 cells (Fig. 6-4B). Taken together, these data indicated that *ADAMTS8* could negatively modulate the ERK pathway in both NPC and ESCC cells.

6.5 ADAMTS8 exerts pro-apoptotic activity in ESCC and NPC cell lines

Using the pEGFP-N1-*ADAMTS8* vector expressing fused ADAMTS8-GFP protein, it was able to detect the influence of *ADAMTS8* restoration on cancer cell morphology. 48 hours after transfection, when compared with the empty vector transfected cells, more ADAMTS8-GFP transfected KYSE150 cells underwent significant morphological changes, with obvious cell shrinkage and rounding, as well as DNA condensation and fragmentation in nuclei (Fig. 6-5A and B), which were typical phenomena observed in cells undergoing apoptosis. Further moie, protein level of cleaved poly (ADP-ribose) polymerase (PARP), a molecular hallmark for apoptosis (Simbulan-Rosenthal et al., 1998), was significantly up-regulated in both KYSE150 and HONE1 cells upon *ADAMTS8* re-expression (Fig. 6-5C), indicating that the ADAMTS8 protease could exert pro-apoptotic activity in both ESCC and NPC.

6.6 Discussion

Mitogen-activated protein kinase (MAPK) cascades tranduce extracellular signals to multiple controlling machineries involved in various cellular biological processes. Among the six known MAPK cascades, Ras/Raf/MEK/ERK (extracellular signal-regulated kinase) cascade is the best characterized one, which is activated by multiple extracellular signals such as growth factors, mitogens, serum and cytokines (Dhillon et al., 2007). Stimulations first result in the ligand-mediated activation of receptor-linked tyrosine kinases (RTK), such as EGFR, which triggers the activation

of Ras and in turn causes the activation of the core triple Ser/Thr kinase cascade. Following its activation by Ras, Raf (MAPKKK) phosphorylates and activates MEK1/2 (MAPKK), which then phosphorylates and activates ERK1/2 (MAPK) (Johnson and Lapadat, 2002) The 43 kDa ERK1 and 41 kDa ERK2 share 83% identity and are broadly expressed in all human tissues. Activated ERK1/2 could phosphorylate and regulate various substrates including cytosol proteins (e.g. RSK, MSK and MNK), cytoskeleton proteins (e.g. paxillin) and membrane proteins (e.g. CD120a and calnexin). At the same time, phosphorylated ERK also translocates to cell nucleus and phosphorylates various transcription factors including Elk-1, c-Fos, c-Myc and STAT3 (Roux and Blenis, 2004; Raman et al., 2007). By regulating these substrates, Ras/Raf/MEK/ERK pathway is involved in the controlling of multiple biological processes and its activation dramatically promotes cell proliferation, survival, and motility. The constitutive activation of ERK pathway has been implicated in nearly one third of all human cancers, which is mostly caused by disruptions in the early signaling stages of this cascade, including the overexpression or activating mutations of RTK, elevated production of extracellular ligands, as well as Ras and Raf mutations (Dhillon et al., 2007; Raman et al., 2007).

Recently, the ADAMTS (a disintegrin and metalloproteinase domain with thrombospondin motifs) protein family has been functionally implicated in cancer development because of the identification of some members like *ADAMTS1*, *ADAMTS12* and *ADAMTS15* as negative modulators for the ERK pathway (Suga et al., 2006; Viloria et al., 2009; Llamazares et al., 2007). Besides, members such as

ADAMTS1 and *ADAMTS8* have been related to angiogenesis inhibition (Luque et al., 2003; Vazquez et al., 1999). In this study, a previously identified epigenetically silenced candidate TSG *ADAMTS8* was studied for its anti-tumorigenic effect in NPC and multiple other tumors. It was revealed that ectopic expression of *ADAMTS8* could inhibit clonogenicity of both NPC and ESCC cells, probably due to its potent ERK-antagonizing effect and pro-apoptotic activity. Evidence provided in this study which demonstrated that *ADMATS8* could significantly downregulate the protein level of phosphorylated ERK and inhibit the translationally transactivating effects of ERK pathway on its downstream targets, further strengthened the remarkable roles of ADAMTS members in the regulation of Ras/Raf/MEK/ERK signaling. In addition, a pro-apoptotic role was also revealed for *ADAMTS8* in both NPC and ESCC, which has not yet been reported for any other ADAMTS member, implicating that this protein family might also be involved in the regulation of programmed cell death during carcinogenesis. Since the activation of the Ras/Raf/MEK/ERK signaling could directly inhibit apoptosis and promote cell survival via increasing the transcription of anti-apoptotic Bcl-2 family members and inhibitor of apoptosis proteins (IAPs), and via phosphorylating apoptosis regulatory molecules including Bad, Bim, Mcl-1 and Caspase 9 (McCubrey et al., 2007), it is thus intriguing to elucidate whether the induction of apoptosis by ADAMTS8 in both cancers was caused by the inhibition of ERK pathway or by other unknown mechanisms in future.

All ADAMTS members share a complex structure characterized by the

presence of a metalloproteinase domain and a series of thrombospondin-1 (TSP-1) repeats. They are secreted to the pericellular space and interact with the ECM components via the TSP-1 motifs, which are required for the normal biological function of ADAMTS protein including both the ERK-antagonizing and angiogenesis-inhibiting effects revealed for some members (Rocks et al., 2008) For example, a genetic mutation of 24544ΔG in *ADAMTS15* detected in primary colon cancer resulted in a mutant ADAMTS15 protein without its C-terminal TSP-1 domains. Consequently, its ability of downregulating phosphorylated ERK was significantly harmed in the mutant protein, indicating that TSP-1 motifs were essential for its tumor suppressive activity (Viloria et al., 2009). Moreover, *Xenopus Adamts1* could also inhibit the phosphorylation of ERK, which was dependant on its C-terminal TSP-1 repeats, instead of its metalloprotease activity, probably via the interaction between its TSP-1 repeats and unknown cellular receptors (Suga et al., 2006). Interestingly, the anti-angiogenesis effect of *ADAMTS1* was also potentially conveyed by the direct binding of its TSP-1 domain with vascular endothelial growth factor (VEGF) (Luque et al., 2003), which further highlighted the importance of TSP-1 repeats in the versatile function of ADAMTS members. These reports led to a hypothesis that *ADAMTS8* could also repress the ERK pathway through its interaction with some unknown membrane receptors via its C-terminal TSP-1 motif.

The anti-tumorigenic roles of ADAMTS proteases have been extensively studied in recent years, several members of which showed both *in vitro* and *in vivo* tumor suppressor properties. For instance, while inhibiting ERK pathway *in vitro*,

ADAMTS15 obviously restrained tumor growth and invasion in immunodeficient mice. Importantly, multiply genetic inactivating mutations in *ADAMTS15* were detected in primary colorectal cancers, highlighting its potential role as a tumor suppressor (Viloria et al., 2009). Besides, *ADAMTS12* could significantly inhibit the growth of A549 lung cancer cells in immunodeficient mice, and protect the host from tumor progression and inhibiting angiogenesis in an *ADAMTS12* knockout mice model (Llamazares et al., 2007; El Hour et al., 2010). These studies carried out in animal models and human primary tumors strongly supported the correlation between ADAMTS protein and cancer. Moreover, this correlation is further elevated by the fact that multiple ADAMTS genes were epigenetically silenced in cancers. As previously reviewed, promoter methylation of *ADAMTS1*, *ADAMTS9*, *ADAMTS12*, and *ADAMTS18* has already been reported in NSCLC, CRC, ESCC and NPC (Lo et al., 2007; Choi et al., 2008; Moncada-Pazos et al., 2009; Jin et al., 2007b). Actually, promoter methylation of *ADAMTS8* was also previously implicated in both NSCLC and glioma (Dunn et al., 2006; Dunn et al., 2004).

To summarize, *ADAMTS8* was first identified as a downregulated gene in a copy loss locus revealed by aCGH in NPC, then characterized as a candidate TSG epigenetically silenced in multiple cancer cell lines and primary tumors, and finally demonstrated to exhibit tumor suppressive roles via inhibiting ERK pathway and promoting apoptosis. Collectively, these data provided potent evidence indicating that *ADAMTS8* was a functional tumor suppressor candidate, whose detailed anti-tumorigenic roles were worthy of further exploration in future.

Limitations of this study: Though it's revealed that *ADAMTS8* could negatively modulate ERK pathway in NPC and ESCC, the exact molecular mechanism for its regulation on this signaling is not elucidated in this study. Its influence on the level of ERK downstream molecules as well as the biological effects controlled by ERK pathway (e.g. cell proliferation and skeleton structure) should be further studied with more functional experiments. In addition, the apoptosis-inducing effect of *ADAMTS8* should be further confirmed by other experiments such as flow-cytometry; whether this effect is dependent on the ERK modulating ability of *ADAMTS8* or not is also to be studied.

Chapter 7, Conclusions and future perspectives

7.1 Conclusions

Aside from classical genetic abnormalities, epigenetic disruptions, especially promote CpG hypermethylation, also play essential roles in the initiation and progression of cancer by inactivating multiple functionally important tumor suppressor genes. Identification of novel epigenetically silenced tumor suppressors will enhance our understandings in the molecular mechanisms of tumor pathogenesis, and imply clues for the establishment of effective and accurate diagnostic markers as well as novel therapeutic strategies. Both aCGH and genome-wide methylation analysis have been proved to be effective methods for TSG identification.

In the present study, using an integrative epigenetic and genomic approach, I performed a series of candidate TSG screening and validating work within several 11q and 15q copy number loss loci detected by an ESCC aCGH. In addition, as the continuation for previous attempts of seeking candidate TSGs in an 11q25 copy number loss locus revealed by a NPC aCGH, I also studied the tumor suppressive mechanism for *ADAMTS8*, one of the previously identified 11q25 candidates. Briefly, the findings in this project were concluded as below:

1) ***RAB39* is an epigenetically silenced TSG candidate in 11q22.3**. Via profiling the mRNA expression of eight genes in an 11q22.3 copy number loss locus, *RAB39* was identified as a frequently downregulated gene in ESCC and NPC cell lines. It was further found to be also downregulated or silenced in multiple other

cancer cell lines including gastric, colorectal, and cervical cancers, while broadly expressed in most normal human tissues and multiple immortalized human epithelial cell lines. By analyzing the methylation status of *RAB39* promoter CGI using MSP and BGS, it was revealed that its transcriptional inactivation was directly caused by promoter hypermethylation, which was further verified by the dramatic reactivating effects conferred by demethylating treatment with DNMTs inhibitor in silenced cell lines. In addition, *RAB39* was found to be frequently methylated in primary ESCC, NPC and gastric cancers, but rarely in normal esophageal and nasopharyngeal epithelial tissues, indicating a tumor-specific methylation manner. Importantly, re-introduction of *RAB39* could remarkably suppress colony growth of multiple cancer cell lines, implying its role as a potential functional tumor suppressor in 11q22.3.

2) ***WDRX* is an epigenetically silenced 15q21.3 TSG candidate inhibiting WNT/β-catenin signaling.** Based on previous ESCC aCGH data, mRNA expression of 41 genes residing in three copy number loss loci within 15q14, 15q21.1 and 15q21.3 was examined in a panel of ESCC and NPC cell lines. As a result, a 15q21.3 gene *WDRX* was found to be a downregulated gene in most cancer cell lines studied. Besides, transcriptional inactivation of *WDRX* was also frequently observed in multiple other cancer cell lines including gastric, renal, cervical, prostate and lung cancers, although it was normally expressed in the corresponding normal human tissues and in multiple immortalized epithelial cell lines. Using MSP and BGS, I found that *WDRX* promoter CGI was intensely methylated in cell lines with

transcriptional silencing. In addition, pharmacological treatment significantly restored the expression of *WDRX* mRNA, indicating that CGI methylation did act as a direct cause for its inactivation in caner cells. Besides, *WDRX* was also found to be frequently methylated in primary ESCC and NPC, but with significantly lower methylation frequencies in normal esophageal and nasopharyngeal epithelial tissues. More importantly, the ectopic expression of *WDRX* could significantly suppress the clonogenicity of multiple cancer cell lines, indicating the tumor suppressive function of this novel WDR protein. Furthermore, preliminary functional analysis proved that *WDRX* could downregulate the intracellular protein level of β-catenin and repress the transcriptional activity of WNT/β-catenin signaling, which possibly represented one of the underlying mechanisms of its anti-tumorigenic capability.

3) *ADAMTS8* is a functional TSG candidate in 11q25, acting as a negative modulator for the ERK pathway and a possible apoptosis enhancer in both NPC and ESCC. Previous data in the lab revealed *ADAMTS8* as an epigenetically silenced 11q25 candidate TSG in NPC and multiple other tumors. Functional studies were thus carried out which showed that *ADAMTS8* could remarkably inhibit the clonogenicity of NPC cell line HONE1 and ESCC cell line KYSE150. Furthermore, *ADAMTS8* could downregulate the intracellular protein level of phosphorylated ERK, inhibit the transcriptionally activating capability of the ERK pathway, and act as a possible pro-apoptotic protease in NPC and ESCC cancer cell lines, underscoring its role as a functional TSG candidate.

7.2 Future research perspectives

In this project, two novel genes *RAB39* and *WDRX* in 11q and 15q, respectively, were proposed as epigenetically silenced TSG candidates and the anti-tumorigenic function of a previous identified 11q candidate TSG *ADAMTS8* was examined. However, there are still some research perspectives to be explored in future, in order to further verify the TSG candidacy of these genes and achieve in-depth understanding for the detailed roles they play in cancer development.

1) Genetic inactivation of the identified candidates. Presence of somatic inactivating mutations in primary tumors is a defining characteristic of most well-characterized TSGs (Robertson et al., 1999). Although recent experimental evidence supported epigenetic silencing to be a more common avenue in TSG inactivation compared with genetic mutations (Schuebel et al., 2007), and the identified candidate genes in the present study were subjected to frequent silencing due to promoter methylation in cancer cell lines, these facts could not undermine the necessity of characterizing mutations of these candidate genes within primary malignancies, in order to further confirm their TSG candidacy and get a more thorough understanding of their inactivation in cancer. Thus, extensive genomic sequencing of these candidates in primary tumor samples might be required in future explorations.

2) Methylation analysis in more primary tumors as well as in samples with clinical follow-up data. In the present study, the identified candidate genes showed significantly higher promoter methylation frequencies in primary NPC and ESCC

when compared with corresponding normal tissues. However, the amount of primary tumors and normal tissue samples examined was relatively limited. It would help to further verify the tumor-specific methylation pattern of these candidates, if the scale of samples could be remarkably extended. In addition, analyzing the methylation status of these candidates in samples with diagnosis or survival data would help to determine their potential as novel diagnostic or prognostic biomarkers.

3) Further characterization of the tumor suppressive functions and mechanisms for the candidates. The conclusion of identifying *RAB39* and *WDRX* as potential tumor suppressor candidates was mainly drawn on their frequent downregulation in multiple cancer cell lines, their tumor-specific methylation pattern, and their clonogenicity-inhibiting ability in multiple cancer cell lines. However, their influence on some important biological behaviors, such as cancer cell proliferation and invasion, as well as the underlying molecular mechanisms are still to be further explored, despite that there is already a clue for the anti-tumorigenic function of *WDRX*. *ADAMTS8* was found to exert its tumor suppressive function through repressing the ERK pathway in the present study, but the downstream effects of its inhibition on this pathway in cancer cells are yet to be examined both *in vitro* and *in vivo*. In addition, its pro-apoptotic role should also be further validated by other experimental techniques and the exact mechanisms for this role are also to be revealed.

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