Mechanistic Study of the Effect of CDH1 Promoter Hypermethylation on Drug Resistance and Related Gene Expression in Multidrug Resistant Human Hepatocellular Carcinoma R-HepG2 Cells

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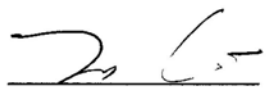

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ABSTRACT

"Epigenetic" refers to a heritable change in the gene expression pattern that is not mediated by any alterations in the primary nucleotide sequence of a gene in the genome. This change involves methylation of DNA in the gene promoter regions, modification of histone residues and chromatin remodeling. Among them, methylation of DNA promoter region is an essential step in epigenetic gene silencing and is known to be closely related to carcinogenesis and cancer progression.

The aim of this study was to explore whether any methylation of DNA promoters mechanism is involved in drug resistance of a doxorubicin-induced human multidrug resistant hepatocellular carcinoma sub-linage R-HepG2 which was established from the doxorubicin sensitive HepG2 cell line in our laboratory. In this project, it was observed that the DNA promoter methylations of ESR1, Rassf2A, CDH1 and MDR1 in R-HepG2 were higher than those in HepG2 cells respectively by methylation specific polymerase chain reaction method. Bisulfite sequencing showed that the total 32 CpGs of CDH1 promoter region in R-HepG2 cells were hypermethylated while they were hypomethylated in HepG2 cells. CDH1 is the encoding gene of E-cadherin. The promoter hypermethylation induced CDH1

silencing in R-HepG2 cells was confirmed by reverse transcription polymerase chain reaction and Western blotting that CDH1 transcription and E-cadherin expression were maintained in HepG2 cells but both were lost in R-HepG2 cells. RT-PCR of 10 multidrug resistant related genes revealed that transcription of MDR1 was obviously increased in R-HepG2 cells, transcription of MRP1 and MRP5 were slightly increased in R-HepG2 cells, transcription of MRP6 and BCRP were slightly decreased in R-HepG2 cells comparing to those in the parental HepG2 cells. This result suggests that up-regulation of P-glycoprotein expression which is the protein product of MDR1 may be one of the major causes of multidrug resistance in R-HepG2 cells. Transient transfection of CDH1 cDNA increased the CDH1 transcription and E-cadherin expression in R-HepG2 cells. I also found that the CDH1 transfected R-HepG2-CDH1 cells showed increased amount of doxorubicin uptake, increased apoptotic population of cells exposed to doxorubicin, suppressed cell migration, and decreased P-glycoprotein expression comparing to those in R-HepG2 cells. It was also found that the transcription levels of SNAI2, TWIST1, ASNA1 and FYN were obviously higher in R-HepG2 cells than those in HepG2 cells. The transcription of FYN and TWIST1 were obviously decreased in CDH1 cDNA transfected R-HepG2-CDH1 cells which displayed a negative correlation with the transcription level of CDH1 and these results imply a suppressive role of CDH1 in

regulating these genes which were involved in cancer metastasis and multidrug resistance.

Our preliminary study on effect of treatments of some potential anti-cancer drug candidates, namely Pheophorbide A (Pa), Pa combining with photodynamic therapy, Polyphyllin D (designated as HK-18), and its derivative designated as HK-27 on human breast cancer cell lines MCF-7 and MDA-MB-231 showed that the promoter methylation of CDH1 was decreased in response to treatments of Pa, HK-18, and HK-27 in MDA-MB-231 cells.

摘 要

“表遺傳”是指基於非基因序列改變所引致的基因表達水平的變化。 這些變化包括基因啓動子區 DNA 甲基化，組蛋白修飾以及染色質重塑。其中，DNA 啓動子甲基化是基因的表遺傳失活中與癌症發生和發展有密切的聯係。

本研究目的是以本研究室前期研究中以阿黴素敏感人類肝癌細胞株 HepG2 構建的多葯耐葯亞株 R-HepG2 為基礎，探索可能導致腫瘤耐藥性產生的啓動子甲基化的機制。 本研究用甲基化特異性 PCR 方法發現，在 R-HepG2 細胞株中基因 ESR1，Rassf2A，CDH1 和 MDR1 的啓動子甲基化程度均高於 HepG2。亞硫酸氫鈉測序法顯示 R-HepG2 細胞株 CDH1 基因啓動子區 32 個 CpG 位點呈高度甲基化狀態而在 HepG2 細胞株中呈低甲基化狀態。CDH1 是 E-鈣黏附素的編碼基因，其轉錄和 E-鈣黏附素的表達在 HepG2 細胞株中有活性，而在 R-HepG2 細胞株中均表現失活，這證實是 CDH1 啓動子區高度甲基化導致了 CDH1 基因在 R-HepG2 細胞株中的失活。對 10 种多葯耐藥性相關基因的 RT-PCR 結果顯示，與親本 HepG2 細胞株相比較，在 R-HepG2 細胞株中 MDR1 轉錄水平有明顯上升，MRP1 和 MPRP5 轉錄有輕微上升，MRP6 和 BCRP 轉錄有輕微下降。表明基因 MDR1 的蛋白產物 P-糖蛋白的表達上升是 R-HepG2 細胞株對阿黴素等腫瘤化療藥物產生耐藥性的主要因素之一。對 R-HepG2 細胞進行 CDH1 基因 cDNA 瞬時轉染實驗發現 CDH1 轉錄和 E-鈣黏附素表達均上升。與 RHepG2 細胞相比較這一轉染細胞 R-HepG2-CDH1 表現出增強的阿黴素攝取量，在阿黴素處理的細胞中凋亡細胞增多，細胞遷移

能力受抑制，以及 P-糖蛋白表達水平下降。研究還發現基因 SNAI2，TWIST1，ASNA1 和 FYN 在 RHepG2 細胞株中轉錄水平明顯高於 HepG2 細胞株。而在 CDH1 基因 cDNA 轉染的 R-HepG2-CDH1 細胞中 FYN 和 TWIST1 轉錄水平有明顯下降，顯示出與 CDH1 基因的表達水平呈負相關，從而提示 CDH1 對這些癌症轉移和多葯耐葯性相關的基因有負調控作用。

本研究也研究了候選癌症藥物脫鎂葉綠素 A （Pa），Pa 與光動力治療的結合，polyphyllin D（簡稱 HK-18）和它的衍生物簡稱 HK-27 在人類乳癌細胞株 MCF-7 和 MDA-MB-231 中對基因啓動子甲基化機制的影響，並發現 CDH1 啓動子甲基化在 Pa，HK-18 和 HK27 分別處理的 MDA-MB-231 細胞株中有所下降。

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Abbreviations

°C	Degree Celsius
µg	Microgram
µl	Microliter
µM	Micromolar
%	Percentage
5aza-dC	5-aza-2'-deoxycytidine
ABC	ATP binding cassette
ABCB4	ATP-binding cassette subfamily B member 4
ASNA1	Arsenite-stimulated ATPase
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid assay
BS	Bisulfite sequencing
BSA	Bovine serum albumin
BWR	BCA working reagent
CDH1	Cadherin 1
CDKN2A	Cyclin-dependent kinase inhibitor 2A
CDKN2B	Cyclin-dependent kinase inhibitor 2B
cDNA	Complementary DNA

CPT	Camptothecin
CTGF	Connective tissue growth factor
Dacogen	5-aza-2'-deoxycytidine
DMSO	Dimethylsulfoxide
DNMT	DNA methyltransferase
E-cadherin	Epithelial cadherin
ECM	Extracellular matrix
EGFR	Epidermal growth factor receptor
EMT	Epithelial-mesenchymal transition
ESR1	Estrogen receptor 1
EST	Expressed sequence tag
FBS	Fetal bovine serum
FYN	FYN tyrosine kinase proto-oncogene
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
HCC	Hepatocellular carcinoma
HDAC	Histone deacetylase
hMLH1	MutL,E.Coli, homolog of, 1;MLH1
hMSH2	MutS,E.Coli, homolog of, 2; MSH2
IC50	Half maximal inhibitory concentration

ID1	Inhibitor of DNA binding 1
JUP	Junction plakoglobin
M/V	Mass by volume
MDR1	ATP-binding cassette subfamily B member 1
MDR	Multidrug resistance
MGMT	Methylguanine-DNA methyltransferase
MRP	Multidrug resistance-associated protein
MSP	Methylation specific PCR
MSRE-PCR	Methylation sensitive restriction endonuclease PCR
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NINJ1	Nerve injury-induced protein 1
NSCLC	Non-small cell lung carcinoma
P	P value
Pa	Pheophorbide A
PBS	Phosphate Buffered Saline
PCR	Polymerase chain reaction
PD	Polyphyllin D
PDT	Photodynamic therapy

PEG	Polyethylene glycol
PGY1	P-glycoprotein
PI	Propidium iodide
PMSF	Phenylmethyl-sulphonyl Fluoride
PS	Penicillin-streptomycin
Rassf1A	RAS association domain family protein 1
Rassf2A	RAS association domain family protein 2
RNase-A	Ribonuclease A
ROS	Reactive oxygen species
RT-PCR	Reverse transcription PCR
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SFK	Src family kinases
SIP1	SMN-interacting protein 1
SNAI1	Human homolog of Drosophila Snail 1
SNAI2	Human homolog of Drosophila Snail 2
TCM	Traditional Chinese medicine
TE	Tris-EDTA
TGF-β	Transforming growth factor-β
THBS1	Thrombospondin 1

Tm	Annealing temperature
TWIST1	Human homolog of Drosophila Twist 1
U	Units
V/V	Volume by volume
Vidaza	5-aza-cytidine

Chapter 1
General Introduction

1.1 Cancer epigenetics and promoter CpG methylation

Cancer is a leading cause of death worldwide. According to the fact sheet No. 297 of WHO report in 2009, cancer accounted for 7.4 million deaths (around 13% of all deaths) in 2004; the main types of cancer leading to overall mortality were lung, stomach, liver, colon, and breast cancer. It was also estimated that there would be 12 million deaths in 2030 due to the continuous rising of death from cancer worldwide. As the result of a multi-step process of cellular functional disorder which might develop differently among individuals, all cancers acquired features that enabled them to grow without control. Crucial steps in transformation of normal cells into malignant cells are the ability of cells to be self-sufficient in growth signals and to be insensitive to growth-inhibitory signals (Hanahan and Weinberg, 2000). Various genes regulating cell growth and differentiation were altered in the process of cancer transformation. The genetic changes could occur at many levels from point mutation in a critical gene to the entire gain or loss of chromosomes. The two main types of genes involved in carcinogenesis were oncogenes and tumor suppressor genes. The aberrant activation and over-expression of oncogenes that should be negative in normal cells promoted the malignant phenotype of cancer cells while the repression of functional tumor suppressor genes failed in inhibiting cell division and promoted cell survival. Changes in multiple genes were required to successfully transform from a normal cell in to a cancer cell. Knudson's "two hits" theory in carcinogenesis was widely accepted to explain the molecular events in cancer formation. He

proposed that there should be two recessive mutations in the two alleles of one tumor suppressor gene during carcinogenesis and the second event could be caused by intragenic mutation, whole gene deletion, and chromosomal loss by non-disjunction or somatic recombination (Knudson, 2001). During the past two decades, the heritability of a new level of traits had become a hot spot in cancer research. Epigenetics, the inheritance of information on the basis of gene expression levels, referred to mitotically heritable change in gene expression that did not involve any change of the DNA sequence (Jones and Laird, 1999). Different chemical modifications of DNA and histones had been found to have profound impact on gene expression and to be faithfully copied through mitosis (Kristensen, Nielsen and Hansen, 2009). DNA methyltransferase and histone modification enzymes such as the histone deacetylase (HDAC) were believed to operate the silence of gene expression in the epigenetic mechanisms. DNA methylation is a critical mechanism for the suppression of gene expression. Cytosine residues were methylated at the carbon-5 position within the palindromic sequence CpG. The adding of methyl groups did not affect base pairing but could influence protein-DNA interactions by interacting with histone modification and chromatin remodeling mechanisms to recruit proteins which would change the chromatin structure in a densely packed transcriptional inactive state. On the contrary, CpG demethylation and other transcriptional activating modifications will recruit proteins that relax the chromatin to make it accessible for the transcription machinery. Among these three mechanisms of epigenetics, DNA methylation was the essential mechanism because of the

permanence of DNA methylation and its apparent dominance over histone modifications, once established (Cameron *et al.*, 1999). DNA methylation was an epigenetic mark that is involved in control mechanism of many biological processes. Methylation patterns of the genome were established during embryogenesis and could be maintained for many cell generations catalyzed by DNA methyltransferases (DNMTs) (Szyf, 2004). CpG dinucleotides were gathered in clusters called CpG islands, there might be as many as 30,000 CpG islands in the genome and 50-60% of these were found within the promoter region of genes (Gardiner-Garden and Frommer, 1987). It is becoming clear that DNA methylation serves as a memory device with the help of maintenance methyltransferase after its establishment during embryogenesis (Naveh-Many and Cedar, 1981). DNA methylation patterns were inherited in cells but were also frequently "remodeled" in cancer cells in a way that directly contributes to tumor suppressor gene inactivation and genomic instability (Jones and Gonzalgo, 1997). Tumor suppressor gene methylation was a well recognized mechanism of carcinogenesis in many types of cancer. As shown in Figure 1.1, CpG islands were often located within the promoter region and the first exon of genes, the promoter CpG islands region of a tumor suppressor gene was hypomethylated in normal cells and the mRNA transcription of the gene was active; while in cancer cells, the promoter region was hyper methylated and the transcription was silenced. Several agents targeting the DNA methylation mechanism had been discovered during the past years including the FDA approved DNMT inhibitors 5-aza-cytidine (Vidaza) and its deoxy analogue 5-aza-2'-deoxycytidine (Dacogen)

for the treatment of myelodysplastic syndrome and other leukaemias (Yoo and Jones, 2006).

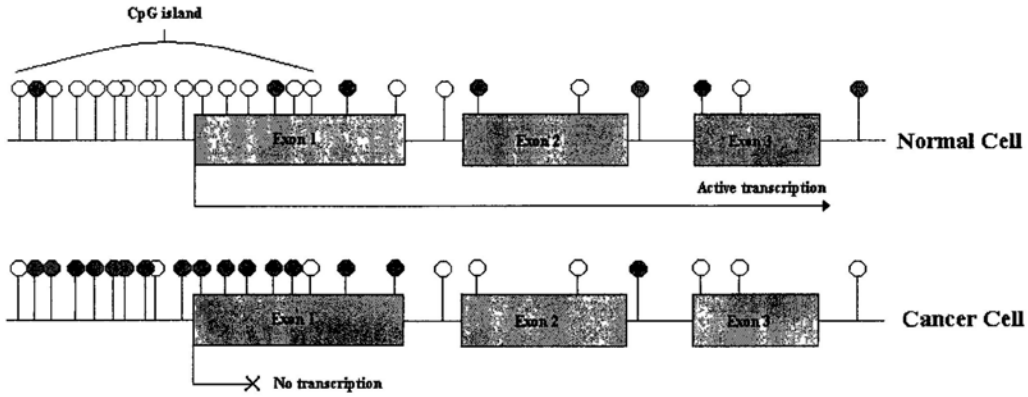


Figure 1.1 Promoter methylation and gene transcription of tumor suppressor gene in normal and cancer cells. The CpG sites of CpG island in the promoter region of normal cells were generally unmethylated (white circles) and the gene transcription was active; the CpG sites of CpG island in the promoter region of cancer cell were hypermethylated (dark grey circles) and the gene transcription was silenced.

1.2 Chemotherapeutics and multidrug resistance

During the past six decades lots of anti cancer agents were discovered including the following well-known drugs: Taxol (Paclitaxel), the first member of the Taxanes that promoted microtubule assembly which was originally found in the Pacific Yew tree; Camptothecin (CPT), a topoisomerase I inhibitor which was derived from the Chinese Camptotheca tree; Cisplatin, a platinum-based compound; and Doxorubicin, a member of the Anthracyclines which was known to treat a wide range of cancers targeting at the topoisomerase II. Most of the chemotherapeutical strategies target at the rapid dividing cells which may also harm normal cells with a fast dividing rate, such as the cells in the bone marrow, digestive tract and hair follicles. Besides killing cancerous cells, these chemotherapeutics may induce some of the common side effects (Jayson and Harris, 2006). Besides all these risks, the development of multidrug resistance (MDR) has become a major problem in chemotherapy since the cancerous cells with decreased drug sensitivity would become tolerant to the chemotherapeutics thus result in treatment failure. Many solid tumors were initially sensitive to chemotherapy but would recur or progress with treatment failure of conventional cytotoxic chemotherapy. However, the knowledge about the molecular characteristics of tumors after conventional treatment failure or the underlying mechanisms that drove the acquisition of drug resistance was limited (Agarwal and Kaye, 2003). Laboratory studies had identified a wide variety of biochemical pathways and hundreds of genes that could potentially influence the response to

chemotherapy in cancer cells which could involve decreased drug uptake, increased drug efflux, increased detoxifying system, increased repair of DNA damage by chemotherapy or activated anti-apoptotic factors, as well as a dysfunctional extracellular matrix (ECM). As a highly heterogeneous disease, cancers shared the ability of being resistant to chemotherapy but the development of drug resistance might involve different genes and molecules. The innate resistant state exhibit various characteristics, namely, poorly dividing tumor cells as deduced from observed accumulation of cells in mid-G1 phase and decreased DNA replication processivity, increased DNA repair associated with cell cycle delay in late S and G2 phases preventing occurrence of mitotic catastrophe and cell apoptosis, increased drug efflux potential by ATP binding cassette (ABC) transporters which played essential roles in the determination of drug pharmacokinetics, and dysfunctional ECM with decreased renewal ability of ECM and basement membrane components, most likely resulting in decreased stimulation of angiogenesis (Graudens *et al.*, 2006). The first factor that was identified as mediator of MDR was the well-known membrane-embedded drug efflux pump MDR1/P-glycoprotein/ABCB1. It was also the first identified member of the ABC transporters. This protein family was consisted of 48 members and divided by their phylogenetic characteristics into seven subfamilies from ABCA to ABCG (Dean *et al.*, 2001). At least 18 of them were described to be associated with drug transport (Table1.1) The expression patterns of these genes might be responsible for inter-individual differences in drug sensitivity. Up-regulation of DNA repair pathways would be responsible for the resistance to

DNA targeting anticancer drugs including alkylating agents and platinum containing compounds. Regulating factors including lots of participating proteins of the reversion repair, base excision repair, nucleotide excision repair, mismatch repair and double-strand break repair pathways were important causes of cancer resistance to DNA targeting drugs (Kaina and Christmann, 2002; Lage, 2008). As the main type of programmed cell death, apoptosis was the major threat to cancer cells exposed to various chemotherapeutics. The pro-apoptotic and anti-apoptotic Bcl-2 family proteins and their regulatory effects on the expression of Bcl-2 were revealed as a fundamental biological mechanism of tumor suppression (Viktorsson *et al.*, 2005). Some of these proteins were reported to be related to chemotherapy response (Johnstone *et al.*, 1999; Kojima *et al.*, 1998; Saito *et al.*, 2004).

ABC-transporter		Drugs	Physiological substrates	References
HUGO nomenclature	Common names			
ABCA2	ABC2	estramustine, mitoxantrone	steroids	[76, 207–209]
ABCA3	ABC3	doxorubicin	surfactant production	[77]
ABCB1	MDR1-P-gp, P-170, P-gp, MDR1, PGY1	"classical" MDR spectrum (Table 1)	phospholipids, neutral and cationic organic compounds	[3, 5, 10, 12, 210]
ABCB2	TAP1	mitoxantrone, Epipodophyllotoxins	peptides	[3, 78, 79]
ABCB3	TAP2	mitoxantrone, Epipodophyllotoxins	peptides	[3, 78, 79]
ABCB4	MDR3-P-gp, MDR3, PGY3	paclitaxel, Vinca alkaloids	phosphatidylcholine	[2, 80]
ABCB5		doxorubicin, camptothecin, 5-fluorouracil	pigment transport ?	[88, 89, 93]
ABCB6	MTABC3	cisplatin, camptothecin	mitochondrial porphyrin uptake	[83, 84]
ABCB11	BSEP, SPGP, ABC16, PGY4	Paclitaxel	bile salts	[5, 81, 82]
ABCC1	MRP, MRP1	anthracyclines, Vinca alkaloids, Epipodophyllotoxins, methotrexate	glutathione-, and other conjugates, organic anions, leukotrienes	[32–34, 211]
ABCC2	MRP2, cMOAT	platin-drugs, anthracyclines, Vinca alkaloids, Epipodophyllotoxins, camptothecins, methotrexate	glutathione-, and other conjugates, organic anions, leukotriene C_4	[33, 37, 38, 212, 213]
ABCC3	MRP3, MOAT-D, MLP2	Vinca alkaloids, Epipodophyllotoxins, methotrexate, cisplatin	glucuronides, bile salts, peptides	[41, 214–216]
ABCC4	MRP4, MOAT-B	nucleotide analogues, methotrexate	organic anions	[41, 217–219]
ABCC5	MRP5, MOAT-C	nucleotide analogues	organic anions, cyclic nucleotides	[41, 220, 221]
ABCC6	MRP6	anthracyclines, Epipodophyllotoxins, cisplatin	glutathione conjugates	[5, 52]
ABCC10	MRP7	taxanes, Vinca alkaloids	organic anions, cyclic nucleotides, bile salts, leukotriene C_4	[48, 56]
ABCC11	MRP8	5-fluorouracil	organic anions, cyclic nucleotides, leukotriene C_4	[48, 49]
ABCG2	BCRP, MXR, ABCP	mitoxantrone, anthracyclines, camptothecins, topotecan	prazosin	[58–61, 222]

DNA repair mechanism	Corresponding DNA repair pathways	Participating proteins
Reversion repair	Single-step repair by MGMT	MGMT
	Repair by AlkB homologous	ABH1; ABH2; ABH3
Base excision repair (BER)	Short patch repair (SPR)	Glycosylases I, II; Polβ; XRCC1; PARP-1; Lig III
	Long patch repair (LPR)	Glycosylases I, II; Polβ; RF-C; FEN1; Pol δ; Pol ε; PCNA
Nucleotide excision repair (NER)	Global genomic repair (GGR)	DDB1; DDB2; RPA; HR23B; ERCC1; XPA; XPB (ERCC3); XPC; XPD (ERCC2); XPE; XPF; XPG; GTFH1; GTFH2; GTFH3; GTFH4; CDK7; CCNH; MNAT1; Pol δ; Pol ε; PCNA; Lig I
	Transcription-coupled repair (TCR)	GTFH1; GTFH2; GTFH3; GTFH4; CDK7; CCNH; MNAT1; XPB (ERCC3); XPD (ERCC2); FFIIS; CSA; CSB; XPF; XPG; Pol δ; Pol ε; Lig I
Mismatch repair (MMR)	Mismatch repair (MMR)	hMSH2; hMSH6; hMLH1; hPMS2; Pol δ; Exo I; Lig
Double-strand break repair (DSB)	Homologous recombination (HR)	MRE11; NBS1; Rad50; Rad51; Rad51B; Rad51C; RAd51D; Rad52; RPA; XRCC2; XRCC3
	Non-homologous end-joining (NHEJ)	Ku70; Ku80 (XRCC5); DNA-PKCs; FEN1; MRE11; NBS1; Rad50; XRCC4; XRCC7; Artemis; Lig IV

Table 1.1 ABC transporters and DNA repair mechanisms related to drug transport. (Lage, 2008)

Thus it seems that the drive of multidrug resistance required expression changes of multiple genes from multiple mechanisms rather than a single gene or pathway. It was quite clear that the expression of multiple genes did change following chemotherapy, but if not gene mutations, what caused these changes? Besides the mechanisms of RNA interference, protein modifications which might modulate gene expression at different levels, epigenetic changes could also be a crucial driving force behind the acquisition of drug resistance. Epigenetic control of gene activity was a common mechanism in the eukaryotic genome. The epigenetic control mechanism was based on CpG methylation of DNA, histone modifications and chromatin remodeling. The H3 and H4 histones had long tails protruding from the nucleosome which could be covalently modified at multiple sites. These modifications included acetylation, phosphorylation, methylation, ubiquitination, sumoylation, etc. (Jenuwein and Allis, 2001), combinations of these modifications could interact with DNA methylation and chromatin remodeling molecules thus regulating gene expression and chromatin condensation. Among these three different levels of epigenetic control of gene activity, DNA CpG methylation was the only mechanism that could be inherited by cells through mitosis so that the epigenetic information of protein expression profiles could be passed down to daughter cells making CpG methylation the most critical mechanism of the epigenetic mechanisms. For many genes such as hMLH1, BRCA1 and CDH1, aberrant methylation of CpG islands in promoter region was a far more frequent mechanism of gene inactivation in sporadic tumors than gene mutation or deletion (Glasspool *et al.*, 2006). Gene inactivation by

DNA methylation could occur at a much higher rate than inactivation of the gene by mutation (Bhattacharyya *et al.*, 1994), so if inactivation of genes was an important cause of drug resistance, the inactivation of genes caused by promoter methylation might be responsible for a much larger part of the clinical drug resistance cancer patient cases than mutations. There was growing evidence that CpG island methylation of genes with a known direct involvement in drug responses had a potential role in predicting clinical outcome following chemo therapy. To study the methylation patterns of genes in order to categorize patients with cancers occurring at same locus and to identify which patients might benefit from particular chemotherapeutic regimes of biological therapies are in urgent need.

1.3 Objectives

In the previous study of our lab, a doxorubicin-induced multidrug resistant sub-linage of human hepatocellular carcinoma (HCC) HepG2 cells has been established, namely R-HepG2 cells (Chan *et al.*, 2000). In this study, I used this multidrug resistant model R-HepG2 cell line and its parental doxorubicin-sensitive HepG2 cell line to study the patterns of promoter DNA methylation in multiple tumor related genes which might be altered during the development of multidrug resistance. I further studied the mechanism of the metastasis related CDH1 gene on the relationship between its loss through promoter DNA methylation and the multidrug resistant phenotype of the R-HepG2 cells and searched for possible molecules of different pathways which might be involved and collaborated with CDH1 to influence the drug sensitivity of the cells. At last, I tried to setup a method to search for anti-cancer drug candidates targeting the promoter DNA methylation mechanism for better targeted therapy in cancer treatment. Our study provided new molecular evidence for the research on cancer multidrug resistance and helped interpreting the complexity of mechanisms behind this phenomenon. Therapeutics targeting the DNA methylation mechanism might not only be useful in regular cancer treatment, but also had their potentials in targeted reversion of methylation in specific genes such as CDH1 to re-sensitize the multidrug resistant cancer cells to chemotherapy. The activation of these genes might also contribute in preventing cancer invasion and metastasis.

Chapter 2

Identification of promoter methylation patterns of tumor related genes in HepG2 and R-HepG2 cells

2.1 INTRODUCTION

Development and progression of cancer is associated with the accumulation of complex genetic and epigenetic aberrations. Aberrant methylation at CpG dinucleotides in the promoter regions is the most robust of chromatin modifications as a causal factor in transcriptional inactivation (Best and Carey, 2009). Change in promoter CpG islands' methylation pattern of tumor related genes is an important epigenetic alteration in carcinogenesis. Comparing to normal tissues, cancers frequently display lower levels of genomic 5-methylcytosine (Feinberg and Vogelstein, 1983), but it was found that higher levels of 5-methylcytosine overlap many promoters (Esteller *et al.*, 2001; Dai *et al.*, 2001). While disparate types of cancer may display hypermethylation of the same promoters, there are significant differences in the methylation patterns between tumor types (Costello *et al.*, 2000). Several tumor suppressor genes have been found epigenetically silenced in cancers through aberrant promoter hypermethylation such as hMLH1 (Herman *et al.*, 1998) –involved in mismatch repair, BRCA1 (Esteller *et al.*, 2000) –double strand breaks repairer, p73 (Corn *et al.*, 1999) –p53 related tumor suppressor, TIMP-3 (Bachman *et al.*, 1999) –invasion inhibitor, and Rassf1A (Dammann *et al.*, 2001) –a ras oncogene related tumor suppressor.

In this chapter, the promoter methylation patterns of several selected genes were examined by different approaches to compare their difference between a pair of chemotherapeutics sensitive and resistant cell lines. Two human hepatocellular

carcinoma (HCC) cell lines HepG2 and its doxorubicin-induced multidrug resistant sub-linage RHepG2 were used to compare the methylation patterns of selected tumor related genes. Six tumor related genes (CDKN2B, CDKN2A, MGMT, MDR1, CDH1, hMLH1) were selected for the Methylation Sensitive Restriction Endonuclease-PCR (MSRE-PCR) assay. Eight tumor related genes (CDKN2A, MDR1, CDH1, ESR1, hMLH1, hMSH2, Rassf1A, Rassf2A) were selected fot the Methylation Specific PCR (MSP) assay. For further study, bisulfite sequencing (BS) was applied to reveal 32 CpG dinucleotides' methylation patterns in the promoter region of CDH1 gene. All these tumor related genes selected are reported to have methylation change in their promoter regions in carcinogenesis. Among these genes, CDKN2B was also known as p15, the encoding gene of cyclin-dependent kinase 4 inhibitor B, it could prevent the activation of CDK kinases by cyclin D thus causing cell cycle G0/G1 phase arrest (Hannon and Beach, 1994). CDKN2A is also known as $p16^{INK4A}$, the encoding gene of cyclin dependent kinase inhibitor 2A, and its regulatory roles on CDK4 kinase and p53 could halt the cell cycle G1 progression (Liggett and Sidransky, 1998). MGMT is the encoding gene of methylguanine-DNA methyltransferase, and its promoter methylation is related to the responsiveness of the tumors to alkylating agents (Esteller *et al.*, 2000; Fry *et al.*, 2008). MDR1 (also named as ATP-binding cassette, subfamily B, member 1, ABCB1) is the encoding gene of a multidrug resistant protein P-glycoprotein (PGY1) which serves as a transmemberane pump transporting a variety of drugs out of the cell (Wang *et al.*, 2006). CDH1 is the encoding gene of epithelial cadherin (E-cadherin), a specific

calcium ion-dependent cell adhesion molecule, and its functions include the suppression of tumor invasion, migration and differentiation (Ji *et al.*, 1997; Jeanes *et al.*, 2008). ESR1 (Estrogen Receptor 1) plays a role in the progression of human breast cancer from hormone dependence into independence and it is related to the suppression of liver cancer (Zhai *et al.*, 2006; Liu *et al.*, 2008; Gaudet *et al.* 2009). hMLH1 is a gene involved in DNA mismatch repair, mutations and promoter methylation caused abolishment of this gene which is related to carcinogenesis of several types of cancer (Herman *et al.*, 1998; Wang *et al.*, 2003). hMSH2 is another DNA mismatch repairer gene, and its inactivation by methylation contributes to the carcinogenesis sometimes together with the inactivation of hMLH1 (Wang *et al.*, 2003). Rassf1A and Rassf2A are two members of the Ras Association Domain Family Proteins. They are both tumor suppressor genes involved in ras pathways as effecter proteins of the large ras family. These two genes were found to be inactivated through promoter methylation in various cancers (Vos *et al.*, 2003).

2.2 MATERIALS AND METHODS

2.2.1 Cell lines and cell culture

Human breast cancer cell line MDA-MB-231(ATCC, #HTB-26) is an estrogen receptor negative adenocarcinoma cell line derived from metastatic site pleural effusion. Human breast adenocarcinoma cell line MCF-7 (ATCC, #HTB-22) is an estrogen receptor positive adenocarcinoma cell line derived from metastatic site pleural effusion. HepG2 is a human hepatocellular carcinoma (HCC) cell line. These three cell lines were purchased from American Type Culture Collection (ATCC). R-HepG2 cell line is a doxorubicin-induced multidrug resistant sub-linage of HepG2 cell line. It was established in our lab from HepG2 cells by adding of increasing concentrations of doxorubicin in the culture medium for several generations as described (Chan *et al.*, 2000). All cell lines are adherent cells.

RPMI-1640 culture medium (Gibco Invitrogen. #23400-21) was used to culture these cells. The RPMI-1640 powder was dissolved in deionized water and buffered with sodium bicarbonate (Sigma-Aldrich Co. #S6297）. The pH of the medium was adjusted to 7.2 before filtration.

This medium was filtrated using a presterilized 0.22μm Steritop（Millipore Co. #SCGPT05RE）filter and stored at 4℃. This medium was supplemented with 10% sterile Fetal Bovine Serum (FBS）(Gibco BRL Life Technologies Inc. #10270）and 1% antibiotics Penicillin-Streptomycin (PS) (100×PS, Gibco BRL Life Technologies

Inc. #10378) before use. 0.25% Trypsin-EDTA (Gibco Invitrogen. #25200) were kept in 50ml aliquots at -20℃.

All the cells were cultured in 75 cm^2 or 150 cm^2 tissue culture flasks in humidified incubator at 37℃ supplying 5% CO_2. The cell lines were subcultured every 3-4 days when approximately 3/4 of the flask bottom area was covered with cells. Cells in exponential growth phase were harvested for different assays. For subculture, the medium was discarded and the cells washed by 37℃ PBS (pH7.4) twice. The cells were then incubated with 1-2 ml of trypsin-EDTA solution at 37℃ for 3-5 minutes. RPMI-1640 medium was added to the cell suspension to stop trypsinization. The cell suspension was then centrifuged at 1000×g for 3 minutes and a suitable amount of cells were seeded into a new culture flask with fresh complete medium. The cell lines were also kept in liquid nitrogen for long time storage. The storage medium contains 50% FBS, 10% DMSO and 40% RPMI-1640.

2.2.2 Eukaryotic genomic DNA extraction

QIAGEN Blood & Cell Culture DNA Kit with Genomic-tip 20/G (Cat No. 13323) was used for the preparation of genomic DNA from cultivated cells. The preparation protocol has a little modification based on the QIAGEN Genomic DNA Handbook. The preparation of all buffer used can be found in QIAGEN Genomic DNA Handbook (QIAGEN, 2001)

2×10^6 cells were cultivated and harvested from 90mm petri-dish. 1ml trypsin-EDTA was added to the cell monolayer and waited until the cells detach, the suspension was then transferred to a 10ml Falcon tube on ice. The culture dish was washed with 2ml cold PBS (pH 7.4), this liquid was then transfered to the tube and centrifuged at 4℃, 1500xg for 5 minutes and the supernatant discarded. The pellet was suspended in 4ml cold PBS, and then centrifuged at 4℃, 1500xg for 5 minutes, after the supernatant was discarded, the cells were then suspended in 0.5ml PBS.

Buffer C1 and distilled water were equilibrated to 4℃, and other buffers to room temperature. 0.5ml buffer C1 and 1.5ml distilled water were added to the cell suspension and mixed by inverting the tube, and then incubate on ice for 10 minutes. After centrifuging at 4℃, 1300xg for 15 minutes, the supernatant was discarded. 0.25ml buffer C1 and 0.75ml distilled water were added to the pellet, and the pellet was suspended by vortex, after centrifuging at 4℃, 1300xg for 10 minutes, the supernatant was discarded. 1ml RNaseA supplemented buffer G2 was added and vortexed for 1min. 25µl 20mg/ml proteinase K (USB, #76230Y) was added and

incubated at 50℃ for 1-2 hours.

A QIAGEN Genomic-tip 20/G was equilibrated with 2ml buffer QBT. The sample was vortexed for 10 seconds and applied to the column and allowed to empty by gravity flow. The column was washed with 1ml buffer QC for 3 times then the genomic DNA was eluted with 2ml buffer QF, the eluate was collected and the column was allowed to drain by gravity flow. The DNA was precipitated by adding 2ml isopropanol into the eluate. After mixing well and centrifuging at 4℃, 3000xg for 15 minutes, the supernatant was then discarded. The pellet was washed with 1ml cold 70% ethanol and centrifuged at 4℃, 3000xg for 5 minutes. The pellet was dried and dissolved with 100μl ddH_2O at 56℃. The DNA yield was determined by absorbance at 260nm and the DNA purity was determined by calculating the ratio of absorbance at 260nm to absorbance at 280nm (NanoDrop® Spectrophotometer, ND-1000, Thermo Fisher Scientific Inc.). Pure DNA has an A260/A280 ratio of 1.7-1.9. The prepared DNA samples were stored at -20℃.

2.2.3 Methylation sensitive restriction endonuclease-PCR (MSRE-PCR)

The basic theory of this MSRE-PCR assay is the same recognition site 5'-CCGG-3'of two restriction endonucleases *HpaII* (New England BioLabs, #R0171L) and *MspI* (New England BioLabs, #R0106L). When the internal C in the sequence CCGG is methylated, *MspI* can cleave the sequence while *HpaII* cannot. So the sequence containing 1 or more than 1 sequence CCGG with a unmethylated internal C will be digested by both *HpaII* and *MspI*, but the sequence containing 1 or more than 1 CCGG sequence with methylated internal Cs will be digested by *MspI* only. *MspI* serves as a control that the amounts of input DNA, endonuclease and digestion time are appropriate (Sharrard *et al.*, 1992). Another basis of this technology is the fact that promoter CpGs are usually methylated or unmethylated together to control the conformation of the promoter region to avoid/accept transcription factors' interactions. By designing primers flanking the CCGG sequence, it is easy to use PCR to examine the methylation status of the CpG dinucleotides in CCGG sequences. The details were shown in Figure 2.1. Primers for MSRE-PCR are shown in Table 2.1.

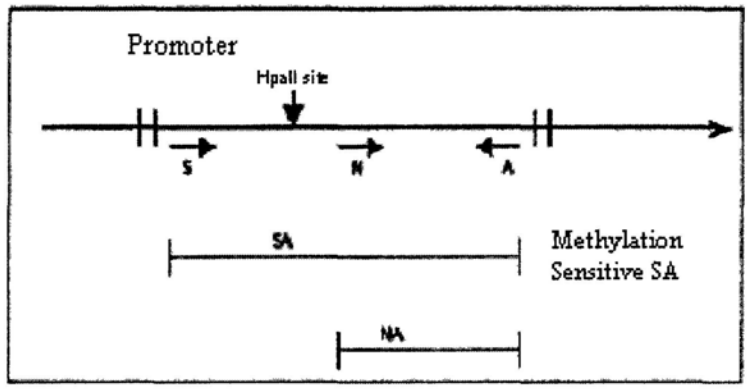


Figure 2.1 MSRE-PCR primer design and analysis for selected gene. The primers S, N, A and their directions were shown as arrow heads on the left. After *HpaII* digestion, PCR product NA would be produced regardless of the methylation/unmethylation of the CpG in the *HpaII* recognition site but product SA would be produced only when this site was methylated.

Primer name	Primer S (sense), 5'->3'	Primer N (neutral), 5'->3'	Primer A (antisense), 5'->3'	Size, NA/SA bp	Annealing temperature, °C
CDKN2B	CGAGGCGGGGCAGTGAG	CTAGGAGACCTGGGCTCAGC	TCAAGAACCAGCGGGCG	126/234	50
CDKN2A	GAAGAAAGAGGAGGGGCTG	GGTCGGGTAGAGGAGGTGC	GCGCTACCTGATCCAATTC	170/340	50
MGMT	TCTTCCTGTCTCAGCCTTCC	GCATAGGTGCTGAGTTGAATC	AATACTGTATCCCGATTCTTCTC	253/418	50
CDH1	GGCAATACAGGGAGACACAGC	GGCTCAAGCGGTCCTCTGG	GCACGGTTCTGATTCCACTG	155/360	50
MDR1	TGAAGTCCTCTGGCAAGTCC	-	ATTCTCCCTCCCGGTTCC	206	50
hMLH1	GGAGAGGAGGAGCCTGAGAAG	GAACCAATAGGAAGAGCCGGAC	AGATGCTCAACGGAAGTGCCT	255/392	50

Table 2.1 Primers for MSRE-PCR Primers for amplification of methylation specific PCR and methylation non-specific PCR were shown in 5'-3' direction, together with the product size and annealing temperature of PCR.

MSRE digestion – 2μg genomic DNA from each cell line was digested by 20 Units (U) of *HpaII* or *MspI* in a total reaction system volume of 30ul at 37℃ for 16 hours. 10U of these endonucleases were applied to the reaction systems when initiating, another 10U of these endonucleases were applied to the reaction.

Digested DNA Purification – After digestion, the digested genomic DNA was purified by QIAquick Gel Extraction Kit (QIAGEN, #28706) following the instructions in QIAquick Gel Extraction Kit Handbook. 100μl buffer QG was applied to the digestion solution and mixed well. 130μl isopropanol was added to the sample and mix. A QIAquick spin column was placed in a 2ml collection tube and the mixed sample was applied to the column, centrifuged at room temperature at 15000×g for 1minute. Flow-through was discarded and 0.75ml buffer PE was added to the column and centrifuged for 1 minute, then the flow-through was discarded. It was further centrifuged at room temperature and 15000×g for 1minute. The column was placed into a clean 1.5 ml microcentrifuge tube and the DNA was eluted by adding 30ul buffer EB to the center of the QIAquick membrane in the column. The column was kept still for 1 min and then centrifuged at room temperature with 15000×g for 1minute. This purified DNA was used immediately in the following PCR step or stored at -20℃ in aliquots.

PCR – PCR was carried out with Model #9902 Veriti™ 96-well Thermal Cycler (Applied Biosystems) and Platinum® Taq High Fidelity DNA Polymerase (Invitrogen,

#11304-11). The 25μl reaction system was composed of 1× High Fidelity PCR Buffer, 0.2mM dNTP mixture (Invitrogen, #479307), 2mM $MgSO_4$, 0.2μM each primer, 50ng purified digested genomic DNA and 1.0 unit of Platinum® Taq High Fidelity DNA Polymerase. The thermal cycling program was: 94℃ 2 minutes; touchdown cycles: 94℃ 30 seconds, 62℃-50℃ 30 seconds (-0.5℃/cycle) –for 25 cycles, 72℃ 45 seconds; normal cycles: 94℃ 30 seconds, 50℃ 30 seconds, 72℃ 45 seconds, -for 10 cycles; 72℃ 10 minutes.

PCR products (10μl/sample) were analyzed on 2% ethidium bromide agarose gel electrophoresis in 1×Apex TBE buffer (Genesee Scientific, #20-131) at 100 V for 30 minutes. The UltraPure™ Agarose (Invitrogen, #16500-100) was used for all agarose gel electrophoresis analysis with Ethidium Bromide Solution (Pharmacia Biotech, #17-1328-01) at a working concentration of 0.2μg/ml gel. GeneRuler™ 100bp DNA ladder (Fermentas, #SM0243) and λ DNA-*HindIII*/φX-*HaeIII* (Finnzymes, #F303SD) was used as marker for all agarose gel electrophoresis in our research.

2.2.4 Bisulfite modification of eukaryotic genomic DNA

Bisulfite sequencing is a methodology by using bisulfite treated DNA to determine its methylation pattern. Treatment of DNA by bisulfite converts cytosine residues into uracil, but the 5-methylcytosine remains unaffected. Bisulfite treatment introduces specific changes to the DNA sequence which reveals all the methylated cytosine in a segment of DNA. Analyses can be performed basing on this converted DNA sample to show the methylation pattern and the change in methylation for various purposes. The details were shown in Figure 2.2

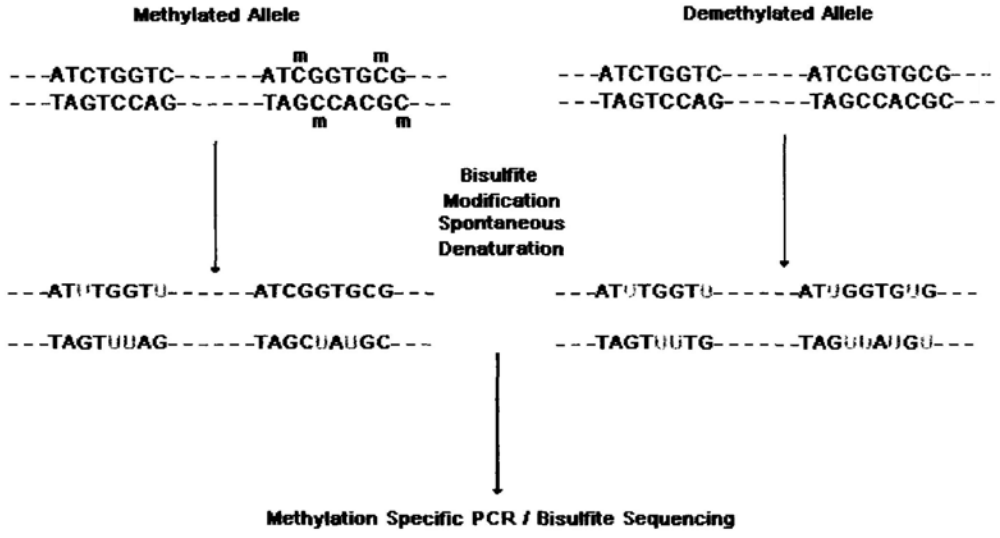


Figure 2.2 Basic theory of bisulfite modification. The alterations of bisulfite modification appear in methylated and demethylated DNA segments. The 5-methylcytosines are shown in red while the uracils are shown in blue. The 5-methylcytosines in the methylated allele are resistant to conversion. After the bisulfite treatment, unique sequence is generated depending on how the methylation pattern is distributed in the genome.

In our study, eukaryotic genomic DNA was prepared as described in the last section. CpGenome™ DNA Modification Kit (CHEMICON®, #S7820) was used for DNA bisulfite modification. The protocol of bisulfite modification was based on the user's manual of the kit, but several modifications were made during optimization. This assay has two main steps and all reagents were freshly prepared and used immediately following preparation. Step 1: 3M NaOH: 1g NaOH was added into 8.3ml dH_2O to make a solution. Reagent I (for 1 reaction): 0.277g Modification Reagent I was dissolved in 571μl dH_2O and the pH was adjusted to 5.0 with 16μl 3M NaOH. 1μg genomic DNA was dissolved in dH_2O to a total volume of 100μl, 7μl 3M NaOH and 2μl Reagent IV were added into the solution and mixed by inverting the tube for several times. The solution was incubated at 37℃ for 15 minutes. 550μl freshly prepared Reagent I was added to the solution and vortexed, this reaction solution was incubated in water bath at 50℃ for 12 hours. Step 2: Reagent II: 1μl of β-mercaptoethanol (Sigma-Alderich, #M7154) was added to 20ml of dH_2O. 750μl of this solution was added to 1.35g of Modification Reagent II for each sample to be modified. Reagent V: 900μl ethanol, 93.4μl dH_2O, and 6.6μl 3M NaOH were mixed to a final concentration of 20mM NaOH and 90% ethanol. Modification Reagent III was vigorously vortexed to make a suspension. 5μl Modification Reagent III was added into the reaction solution. 750μl Reagent II was added into the solution and mixed briefly. The solution was incubated at room temperature for 10 minutes, then centrifuged at 5000×g for 10 seconds. The supernatant was discarded and the pellet washed by vortexing for 10 seconds with 1ml 70% ethanol for 3 times, then followed

by centrifugation at 5000×g for 10 seconds after vortexing each time to collect pellet. They were centrifuged at 15000×g for 2 minutes after washing step and the remaining supernatant was removed with a pipette tip. 50μl Reagent V was added to the pellet, vortexed briefly, incubated at room temperature for 5 minutes and centrifuged at 5000×g for 10 seconds. 1ml 90% ethanol was added and then vortexed briefly to suspend the pellet and this step was repeated once. The supernatant was discarded and the tube was allowed to dry for 20 minutes at room temperature. 25μl TE buffer was added to the pellet and vortexed, and then the sample was incubated for 15 minutes at 56℃ to elute DNA. The product was used as template for following assays immediately after preparation because this chemical "damaged" single strand DNA is not stable for long time storage.

2.2.5 Methylation specific PCR (MSP)

MSP is a methylation analysis uses carefully designed methylated-specific primers and unmethylated-specific primers for the detection of methylated/demethylated DNA sequence thus distinguish the methylation pattern of its priming sequence. After bisulfite modification, all cytosine residues in the DNA were converted into uracil except those with a methyl group. The targeting sequence of methylated and demethylated sequences would have distinct sequences like CG...CG...CG versus UG...UG...UG. So these specific primers could only bind to its specific counterpart. After cycles of PCR, the signal could be amplified to be detectable by agarose gel electrophoresis. As shown in Figure. 2.3, 8 genes were selected for MSP in this part. CpGenome^{TM} Universal Methylated DNA (Chemicon, #S7821) was used as methylation positive control (methylation^{+}), which is enzymatically (*SsssI*) methylated human male genomic DNA. CpGenome^{TM} Universal Unmethylated DNA Set (Chemicon, #S7822) was used as methylation negative control (methylation^{-})for MSP in this research, which has two samples, namely the Universal Unmethylated DNA Vial A which is human genomic DNA, Unmethylated DNA Vial B which is genomic DNA from a human fetal cell line. The primers of selected genes used in MSP are shown in Table 2.2.

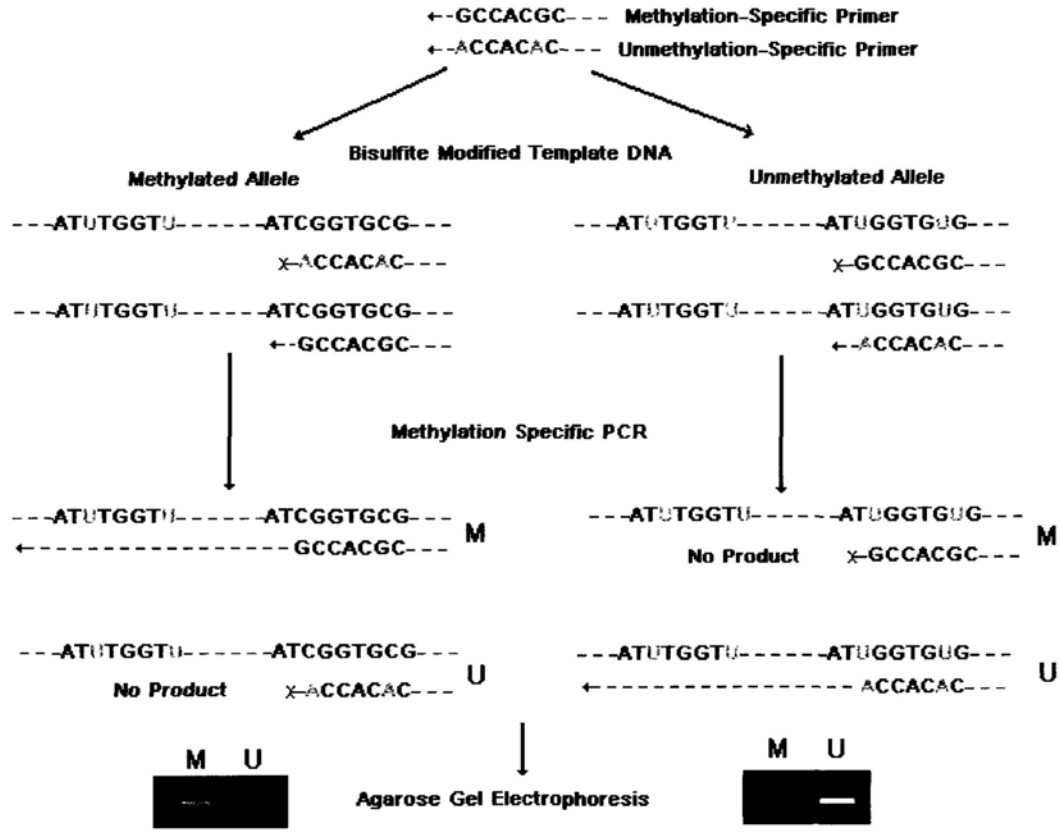


Figure 2.3 Basic theory of methylation specific PCR. This is a very sensitive method to detect a methylated region using methylation/unmethylation-specific primers on bisulfite-converted genomic DNA. These primers will only anneal to sequences that are methylated/unmethylated because only those 5-methylcytosines in the methylated sequence can resist the conversion by bisulfite. So the methylation status of the priming region can be determined by electrophoresis after MSP.

Primer set	Sense primer, 5'->3'	Antisense primer, 5'->3'	Size, bp	Annealing temperature (Tm), °C
MDR1-M	CTCTCTAAACCCGCGAACGAT	TTGGGGGTTTGGTAGCGC	115	60
MDR1-U	ACTCTCTAAACCCACAAACAAT	GTTGGGGGTTTGGTAGTGT	117	58
ESR1-M	TTTGGGATTGTATTTGTTTTCGTC	AACAAAATACAAACCGTATCCCCG	192	59
ESR1-U	TTTTGGGATTGTATTTGTTTTTGTTG	AAACAAAATACAAACCATATCCCCA	192	59
CDH1-M	TTAGGTTAGAGGGTTATCGCGT	TAACTAAAAATTCACCTACCGAC	116	57
CDH1-U	TAATTTTAGGTTAGAGGGTTATTGT	CACAACCAATCAACAACACA	97	53
CDKN2A-M	TTATTAGAGGGTGGGGCGGATCGC	GACCCCGAACCGCGACCGTAA	150	65
CDKN2A-U	TTATTAGAGGGTGGGGTGGATTGT	CAACCCCAAACCACAACCATAA	151	60
Rassf1A-M	GTTGGTATTCGTTGGGCGC	GCACCACGTATACGTAACG	160	56
Rassf1A-U	GGTTGTATTTGGTTGGAGCC	CTACAAACCTTTACACACAACA	180	56
Rassf2A-M	GTTCGTCGTCGTTTTTTAGGCG	AAAAACCAACGACCCCCGCG	150	58
Rassf2A-U	AGTTTGTTGTTGTTTTTTAGGTGG	AAAAAACCAACAACCCCCACA	150	54
hMLH1-M	ACGTAGACGTTTTATTAGGGTCGC	CCTCATCGTAACTACCCGCG	118	58
hMLH1-U	TTTTGATGTAGATGTTTTATTAGGGTTGT	ACCACCTCATCATAACTACCCACA	124	60
hMSH2-M	TCGTGGTCGGACGTCGTTC	CAACGTCTCCTTCGACTACACCG	133	60
hMSH2-U	GGTTGTTGTGGTTGGATGTTGTTT	CAACTACAACATCTCCTTCAACTACAC CA	144	60

Table 2.2. Primers for MSP Primers for amplification of methylation-specific PCR and demethylation-specific PCR were shown in 5'-3' direction, together with the product size and annealing temperature of PCR.

PCR – PCR was carried out with Model #9902 Veriti™ 96-well Thermal Cycler (Applied Biosystems), HotStarTaq® Plus DNA Polymerase and HotStarTaq® Plus Master Mix Kit (QIAGEN, #203605). The 20μl reaction system was composed of 1× Q Solution, 1× CoralLoad PCR Buffer, 0.2mM dNTP mixture (Invitrogen, #479307), 0.2μM each primer, 40ng bisulfite modified genomic DNA and 0.5 unit of HotStarTaq® Plus DNA Polymerase. The thermal cycling program was: 95℃ 2 minutes; touchdown cycles: 95℃ 30 seconds, 66℃~52℃ 30 seconds (-1℃/cycle) –for 15 cycles, 72℃ 30 seconds; normal cycles: 95℃ 30 seconds, Tm-2℃ 30 seconds, 72℃ 30 seconds, -for 30 cycles; 72℃ 4 minutes. The PCR products were analyzed on 2% agarose gel electrophoresis.

2.2.6 Bisulfite sequencing (BS) of CDH1 promoter

Bisulfite sequencing is a method to determine the methylation pattern of all CpG dinucleotides in a prime flanked region. Bisulfite-specific primers are designed to be flanking but not involving the methylation site of interest. After bisulfite modification and PCR using bisulfite-specific primers, the PCR products were cloned into T vector prior to sequencing for adequate sensitivity. The sequencing result of each colony shows the methylation pattern of one DNA allele in a cell.

As a demethylation agent, 10μM DNMT inhibitor 5aza-2'-deoxycytidine (5aza-dC; Sigma-Aldrich, A3656) was added to R-HepG2 cells and incubated for 72 hours before harvesting. 5aza-dC was changed every 24 hours during incubation. 5aza-dC was dissolved in PBS at 10mM and stored at -80℃ in aliquots

For bisulfite sequencing of CDH1, the primers were:

CDH1-BS-S: 5'-TAGTAATTTTAGGTTAGAGGGTTAA-3',

CDH1-BS-A: 5'-AAATACCTACAACAACAACAACAAC-3'.

The 387-bp PCR product covers 32 CpG sites are shown in Figure 2.4. (Leung *et al.*, 2006)

PCR – PCR was carried out with Model #9902 Veriti^{TM} 96-well Thermal Cycler (Applied Biosystems), HotStarTaq® Plus DNA Polymerase and HotStarTaq® Plus Master Mix Kit (QIAGEN, #203605). The 20μl reaction system was composed of 1× Q Solution, 1× CoralLoad PCR Buffer, 0.2mM dNTP mixture (Invitrogen, #479307), 0.2μM each primer, 40ng bisulfite modified genomic DNA and 0.5 unit of HotStarTaq® Plus DNA Polymerase. The thermal cycling program was: 95℃

2minutes; touchdown cycles: 95℃ 30 seconds, 61℃~52℃ 30 seconds (-1℃ /cycle) –for 10 cycles, 72℃ 45 seconds; normal cycles: 95℃ 30 seconds, 50℃ 30 seconds, 72℃ 45 seconds, -for 30 cycles; 72℃ 4 minutes.

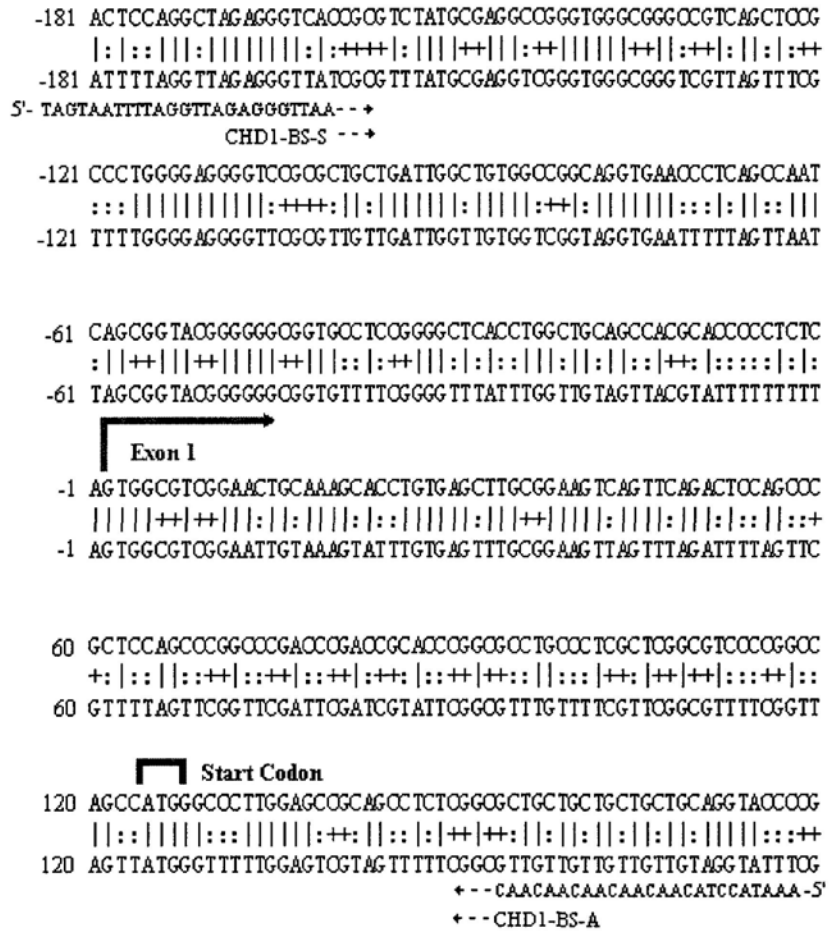


Figure 2.4 Strucure of the CDH1 promoter region. The 387bp CDH1 promoter region covers 32 CpG dinucleotides from -186bp to +176bp flanking the CDH1 transcription start codon shown in the figure. The upper row shows the sequence before bisulfite modification, the lower row shows the sequence after bisulfite modification with all CpG dinucleotides unconverted by bisulfite. These CpGs are marked with "++", the cytosines converted are marked with ":", the unconverted bases are marked with "|". The starting G of exon one was counted as "1" and its upstream A is shown as "-1". This figure was generated by MethPrimer (Li and Dahiya, 2002) with modifications.

The PCR products were analyzed on 2% agarose gel electrophoresis. The PCR product bands were then excised from the gel and purified by QIAquick Gel Extraction Kit (QIAGEN, #28706) following the instructions in QIAquick Gel Extraction Kit Handbook, same as the MSRE-PCR DNA purification part. The only extra step is in the first step. After excising the fragment with the PCR product band from the gel, 300μl buffer QG was added to the fragment and incubated at 50℃ for 10 minutes to dissolve the gel.

Ligation: The purified PCR product was cloned into TA cloning vector by the Original TA Cloning® Kit (Invitrogen, #C45-0046). In the 10μl ligation system, 10ng PCR product was mixed with sterile dH_2O, 1× ligation buffer, 50ng pCR® 2.1 vector and 4U T4 ligase. This ligation system was incubated at 14℃for 16 hours.

For the bacterial culture for transformation，E.coli strain JM109 in a 15% (v/v) glycerol stock（laboratory stock） was used as a host for all vector transformation. LB Broth (USB, #75852) and LB Agar（USB, #75851)were used for bacterial culture. These media were supplemented with 100μg/ml ampicillin (Sigma-Aldrich, #A0166). The ampicillin powder was diluted by deionized water to 100mg/ml stock solution and stored at-20℃.

Competent Cells: Competent JM109 cells were prepared following Cohen's Calcium Chloride method with some modifications. A single colony was picked from

a plate freshly grown for 16 hours at 37℃ and transferred into 100ml of LB broth in a 1 liter flask. The culture was incubated for 3 hours at 37℃ in a rotary shaker at 300cycles/minute. The cells were transferred into 50ml falcon tubes and centrifuged at 4℃, 3000×g for 10 minutes. The medium was decanted from the cell pellets and the pellet was resuspended in 10ml sterile 0.1M $CaCl_2$ and stored on ice for 30 minutes. After centrifuging at 4℃, 3000×g for 10 minutes, the supernatant was decanted and pellet was resuspended in 2ml sterile 0.1M $CaCl_2$ for each 50ml of original culture. This competent cells were then stored at -80℃ in aliquots.

Transformation: 100ul suspension of competent cells was transferred to the ligation system; the contents were mixed by swirling the tube gently. The tube was kept still on ice for 30 minutes. The tube was then incubated at 42℃ for 90 seconds then on ice for 5 minutes. 800μl SOC was added to the mixture and the culture was incubated at 37℃ for 1 hour. After centrifuging at 13000×g for 1 minute, the supernatant was discarded. 200μ SOC and 1μl 100mg/ml ampicillin were added to resuspend the pellets and the transformed cells were gently spreaded over the surface of the agar-ampicillin plate. The plate was left at 37℃ until the liquid was absorbed. The plate was inverted and incubation was continued at 37℃. Colonies should appear in 12-16 hours to a diameter of 2-3mm.

10 colonies were picked from each DNA sample after the incubation. The colonies were incubated in 2ml LB broth with 100mg/ml ampicillin at 37℃ for 16

hours in a rotary shaker at 200 cycles/minute. The plasmids DNA were prepared by Mini-M^{TM} Plasmid DNA Extraction System (VIOGENE, #GF1002). 1ml cells was transferred to a 1.5 ml tube and centrifuged at 12000×g for 1 minute. 250μl buffer MX1 was added to resuspend the cell pellet; 250μl buffer MX2 was added and gently mixed to lyse the cells until the lysate becomes clear; 350μl buffer MX3 was added to neutralize the lysate. The sample was centrifuged at 12000×g for 10 minutes and the supernatant was applied to a Mini-M^{TM} column in a collection tube. The sample was centrifuged at 12000×g for 1 minute, and the flow through was discarded. The column was washed with 0.5ml buffer WF by centrifuging for 1 minute, and the flow through was discarded. The column was washed with 0.7ml buffer WS by centrifuging for 1 minute, and the flow through was discarded. The column was centrifuged at 12000×g for 3 minutes to remove residual ethanol. The plasmid DNA was eluted with 50μl buffer EB by standing the column for 2 minutes and centrifuged at 12000×g for 2 minutes. The plasmid was stored at -20℃.

The plasmids were sent to Tech Dragon LTD for sequencing using M13 Forward and/or M13 Reverse Primers (Tech Dragon LTD). The sequencing data were observed by Chromas 1.45 software (programmed by Conor McCarthy). The strategy of BS is shown in Figure 2.5.

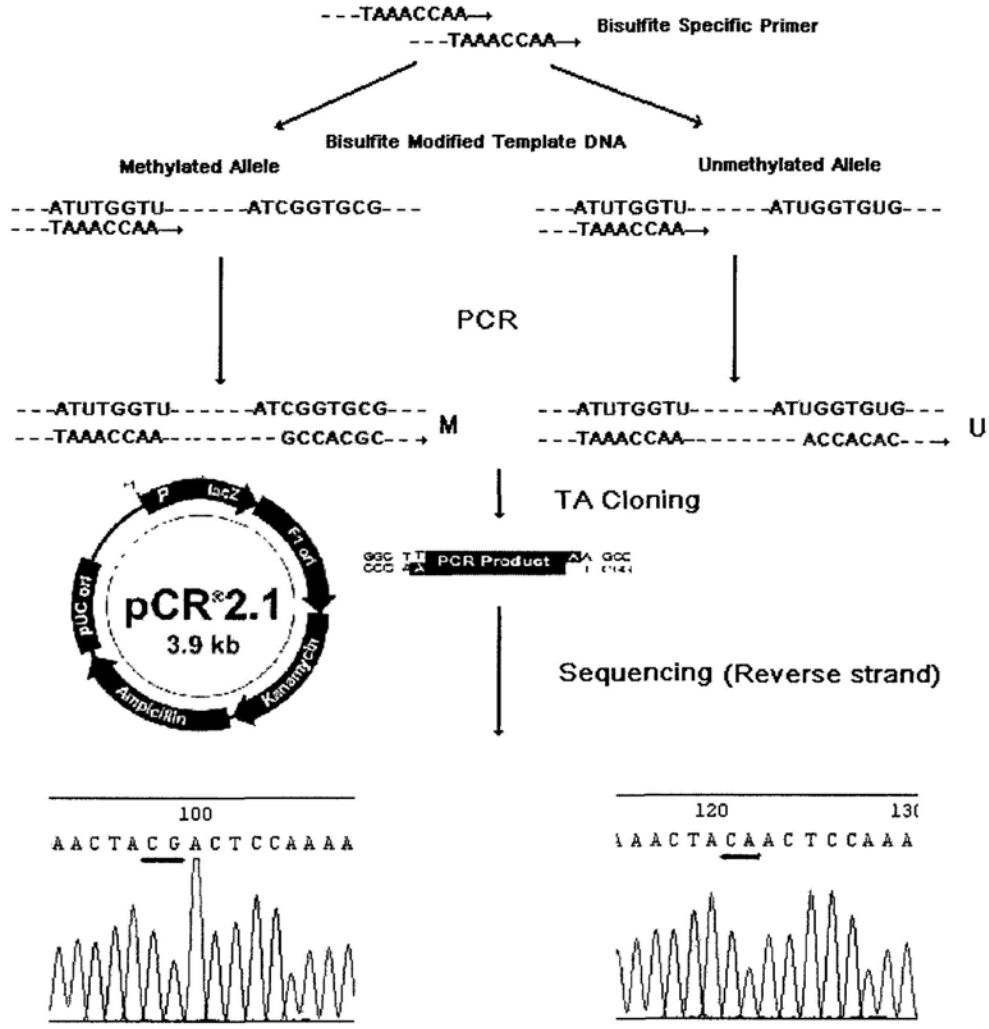


Figure 2.5 Basic theory of bisulfite sequencing. Following bisulfite modification, PCR will be carried out using bisulfite-specific primers that do not discriminate between methylated and unmethylated sequences. The PCR products were cloned by TA cloning. Colonies were selected for sequencing, and the methylation patterns of the site of interest were analyzed.

2.3 RESULTS

2.3.1 Promoter methylation status of tumor related genes in HepG2 and R-HepG2 cell lines by MSRE-PCR

To examine promoter region methylation of 6 selected tumor related genes (CDKN2B, CDKN2A, MGMT, MDR1, CDH1, hMLH1), I adapted the easy and fast MSRE-PCR for the promoter CpG islands pattern analysis of these genes.

Difference in methylation pattern between HepG2 and R-HepG2 cells in these selected genes was examined. PCR was performed with undigested DNA sample to see whether all the methylation sensitive SA bands and methylation insensitive NA bands could be amplified (Figure 2.6).

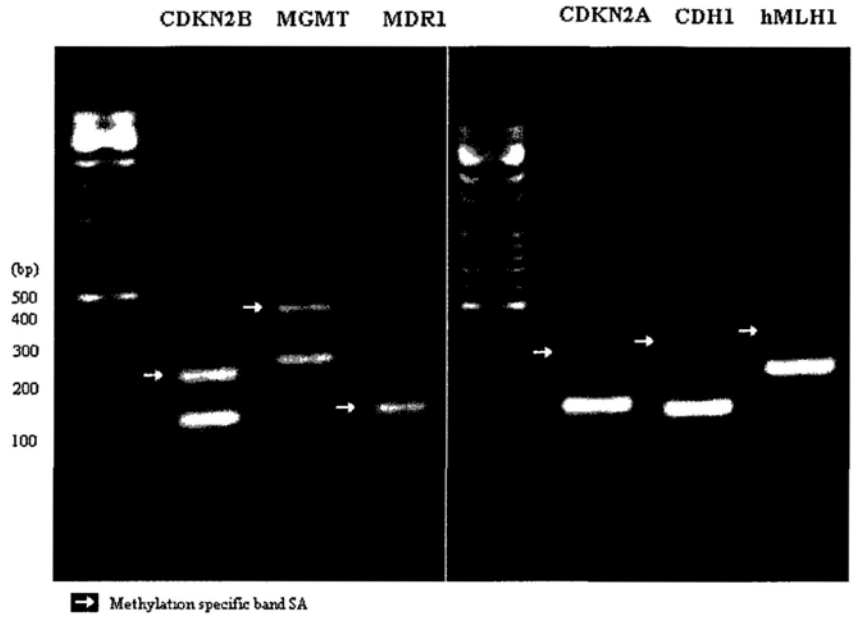


Figure 2.6 Agarose electrophoresis of MSRE-PCR with undigested DNA in the selected genes. All SA bands are marked with an arrow. Data are representative of two independent experiments.

To optimize the proper input amount of DNA, digestion enzyme and the digestion time in the digestion step, CDH1 gene in human breast cancer cell line MDA-MB-231 and MCF-7 were tested because several papers had reported the methylated CDH1 promoter in MDA-MB-231 cells and the unmethylated CDH1 promoter in MCF-7 cells using different methylation detecting systems (Graff *et al.*, 1995; Hiraguri *et al.*, 1998). The procedure of MSRE-PCR for all selected genes had been fixed as shown in the Materials & Methods part. Results were shown in Figure 2.7.

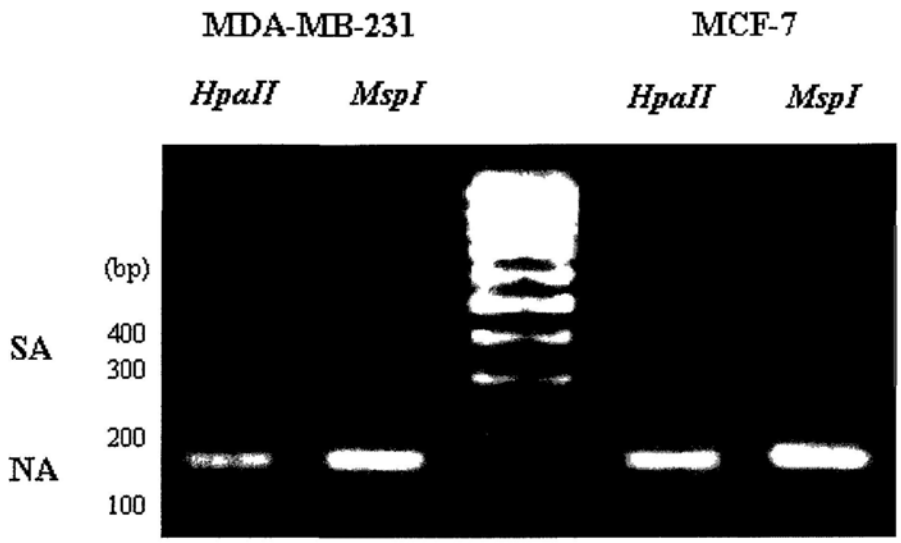


Figure 2.7 Agarose electrophoresis of MSRE-PCR of CDH1 in MDA-MB-231 and MCF-7 cell lines. 2μg genomic DNA from MDA-MB-231 and MCF-7 cell lines was digested by 20 Units (U) of *HpaII* or *MspI* in a total reaction system volume of 30ul at 37℃ for 16 hours. Only the DNA from MDA-MB-231 with a methylated CDH1 promoter could produce a methylation specific SA band after MSRE-PCR. Data are representative of two independent experiments

As shown in Figure 2.8, CDKN2B, MGMT, CDKN2A and hMLH1 promoters were found to be unmethylated both in HepG2 and R-HepG2 cells. There were only NA bands but no methylation specific SA band. These genes kept being unmethylated while the cells become multidrug resistant. The methylation of MDR1 and CDH1 promoter seemed to have increased because the *HpaII* digested R-HepG2 sample produced a stronger SA band than in HepG2 cells.

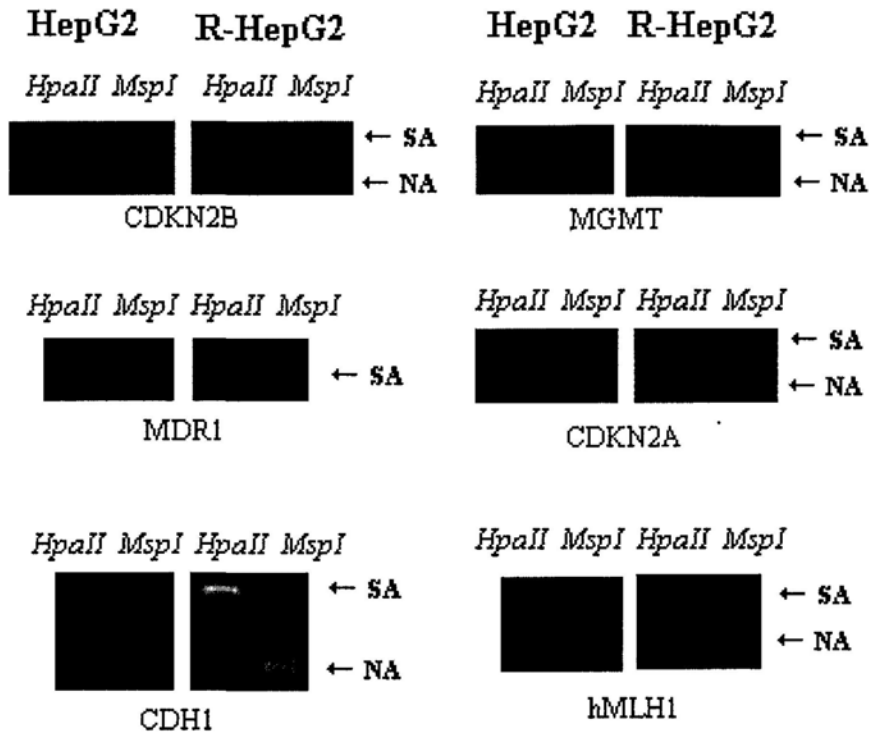


Figure 2.8. Agarose gel electrophoresis of MSRE-PCR of selected genes CDKN2B, MGMT, MDR1, CDKN2A, CDH1 and hMLH1 in HepG2/R-HepG2 cells. SA and NA were marked with arrows. Data are representative of two independent experiments

2.3.2 Methylation status of tumor related genes in control DNA by Methylation Specific PCR

To examine promoter region methylation of 8 selected tumor related genes(CDKN2A, MDR1, CDH1, ESR1, hMLH1, hMSH2, Rassf1A, Rassf2A), I adapted the more sensitive MSP for the analysis of promoter CpG islands in these genes.

Difference in methylation pattern between HepG2 and R-HepG2 cells in these selected genes was examined. MSP optimization of genes with methylation^{+}/methylation^{-} control DNA was performed. PCR was optimized and performed with methylation^{+}/methylation^{-} control DNA -CpGenome^{TM} Universal Methylated DNA and CpGenome^{TM} Universal Unmethylated DNA Set (Figure 2.9). For most of the genes, both methylation and unmethylation primers could produce unique PCR product in methylation^{+} control DNA and at least one methylation^{-} control DNA. The methylation^{+} control of hMSH2 produced a strong methylation specific PCR product and a weak unmethylation specific PCR product. The U-a methylation^{-} control of Rassf2A produced both methylation and unmethylation specific PCR products. The U-b methylation^{-} control of Rassf1A produced no PCR product.

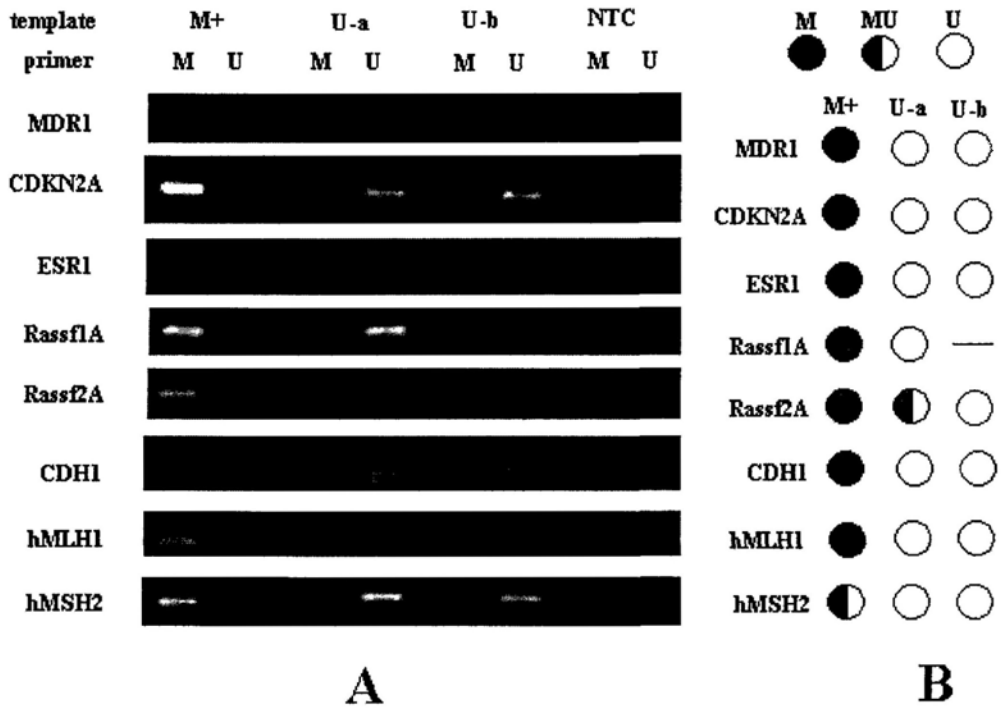


Figure 2.9 Agarose gel electrophoresis results of MSP with methylation$^+$/methylation$^-$ control DNA. (A) Methylation$^+$ DNA was marked as "M+"; methylation$^-$ DNA set has two samples, marked with "U-a" and "U-b"; NTC is the "No Template Control". Primer M/U is the methylation/unmethylation specific primers of every gene. (B) Dot graph showing the methylation pattern of genes revealed by MSP using control DNA. M –methylation PCR amplification only, U –unmethylation PCR amplification only, MU –both methylation and unmethylation amplifications (semi-methylation). Data are representative of three independent experiments.

2.3.3 Difference of methylation pattern between HepG2 and R-HepG2 cells in selected genes.

Genomic DNA of HepG2 and R-HepG2 cells were tested by MSP to show the promoter methylation pattern of the 8 selected genes. Among them, CDKN2A, hMLH1 and hMSH2 has no difference between the two cell lines. Rassf1A was methylated in HepG2 but showed no amplification of methylation or unmethylation in R-HepG2. MDR1, ESR1, Rassf2A and CDH1 showed a "hypo-methylation to hyper-methylation transition" in their promoters' methylation from the parental HepG2 to its daughter R-HepG2. MDR1 and CDH1 showed unmethylation in HepG2 and semi-methylation in R-HepG2; Rassf2A showed unmethylation in HepG2 and methylation in R-HepG2; ESR1 showed semi-methylation in HepG2 and methylation in R-HepG2. (Figure 2.10)

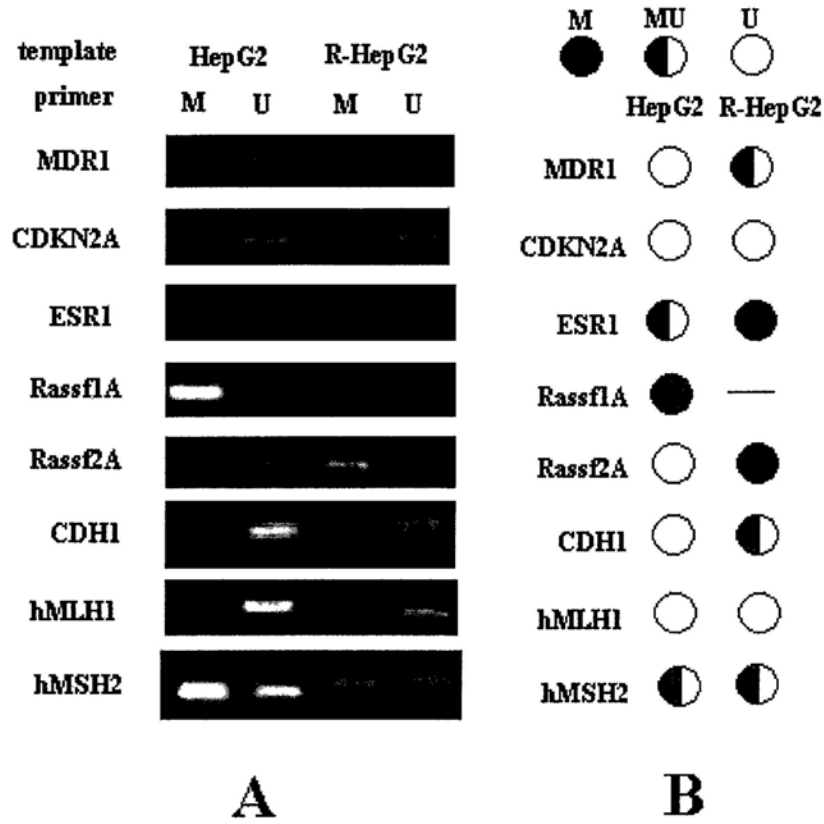
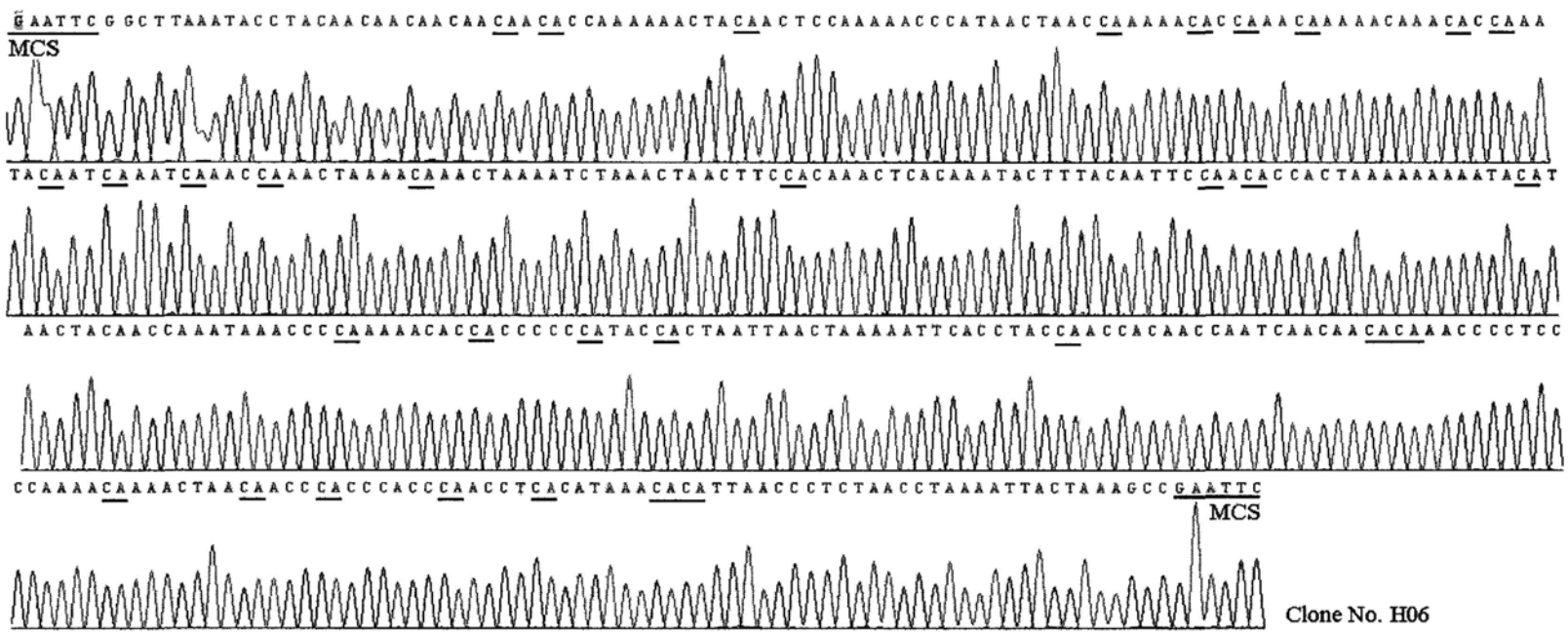
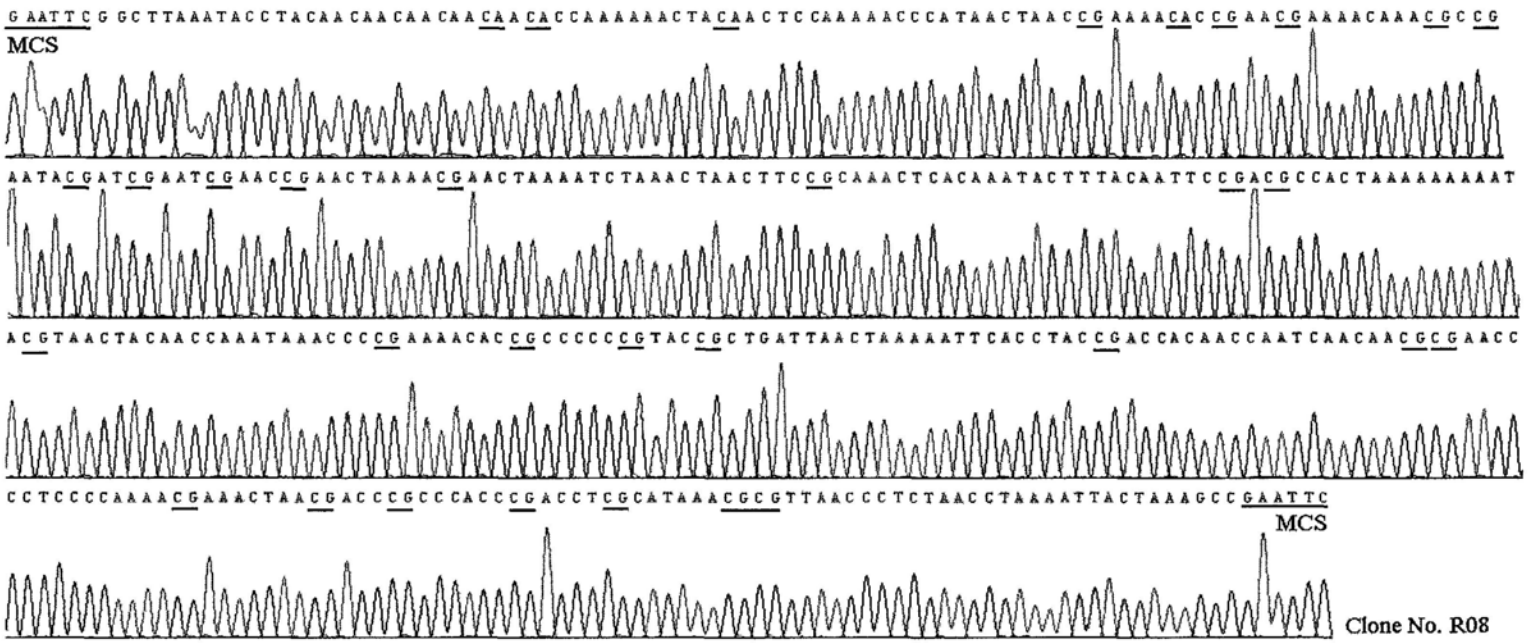


Figure 2.10 Agarose gel electrophoresis of methylation patterns of genes in HepG2 and R-HepG2 cells. (A) The methylation pattern of 8 selected genes in HepG2 cells and R-HepG2 cells were examined by MSP. Primer M/U is the methylation/unmethylation specific primers of every gene. (B) Dot graph showing the methylation pattern of genes revealed by MSP using HepG2 and R-HepG2 genomic DNA comparing to the controls. M –methylation PCR amplification only, U –unmethylation PCR amplification only, MU –both methylation and unmethylation amplifications (semi-methylation). Data are representative of three independent experiments.

2.3.4 Methylation status of CDH1gene in HepG2, R-HepG2 cells and 5aza-dC treated R-HepG2 cells by Bisulfite Sequencing

To further study the methylation pattern of CDH1 gene, bisulfite sequencing was carried out showing the methylation pattern of all 32 CpG dinucleotides in the CDH1 promoter region of the HepG2, R-HepG2 cells and 5aza-dC treated R-HepG2 cells. As shown in Figure 2.11 and Figure 2.12, the CDH1 promoter was hypomethylated in HepG2 cells but hypermethylated in R-HepG2 cells. Upon 10μM 5aza-dC treatment for 72 hours, the DNA methylation in CDH1 promoter was obviously decreased in R-HepG2 cells.


GAATTCGGCTTAAATACCTACAACAACAACAACAACACCAAAAAAACTACAACTCCAAAAACCCCATAACTAACCAAAAACACCAAACAAAAACAAACACCAAA
MCS
TACAATCAAATCAAACCAAACTAAAACAAACTAAAATCTAAACTAACTTCCACAAACTCACAAATACTTTACAATTCCAACACCACTAAAAAAAAATACAT
AACTACAACCAAATAAACCCCAAAAACACCACCCCCCATACCACTAATTAACTAAAAATTCACCTACCAACCACAACCAATCAACAACACAAACCCCTCC
CCAAAACAAAACTAACAACCCACCCACCCAACCTCACATAAACACATTAACCCTCTAACCTAAAATTACTAAAGCCGAATTC
MCS
Clone No. H06
(A)


GAATTCGGCTTAAATACCTACAACAACAACAACAACACCAAAAAACTACAACTCCAAAAACCCATAACTAACCGAAAACACCGAACGAAAACAAACGCCG
MCS
AATACGATCGAATCGAACCGAACTAAAACGAACTAAAATCTAAACTAACTTCCGCAAACTCACAAATACTTTACAATTCCGACGCCACTAAAAAAAAAT
ACGTAACTACAACCAAATAAACCCCGAAAACACCGCCCCCCGTACCGCTGATTAACTAAAAATTCACCTACCGACCACAACCAATCAACAACGCGAACC
CCTCCCCAAAACGAAACTAACGACCCGCCCACCCGACCTCGCATAAACGCGTTAACCCTCTAACCTAAAATTACTAAAGCCGAATTC
MCS
Clone No. R08

(B)

Figure 2.11 Examples of bisulfite sequencing results of selected clones. Sequencing results of CDH1 promoter region showed the methylation pattern of all 32 CpG dinucleotides in (A) clone H06 of HepG2 cells and (B) clone R08 of R-HepG2 cells. M13 reverse primer (5'-CAGGAAACAGCTATGAC-3') was used for sequencing so that the sequences shown were the antisense strand in 5'-3'order. The junction *EcoRI* sites at the ends of insertion fragments of multiple cloning sites were marked as MCS. All 32 CpG dinucleotides were marked with underline in the sequencing results.

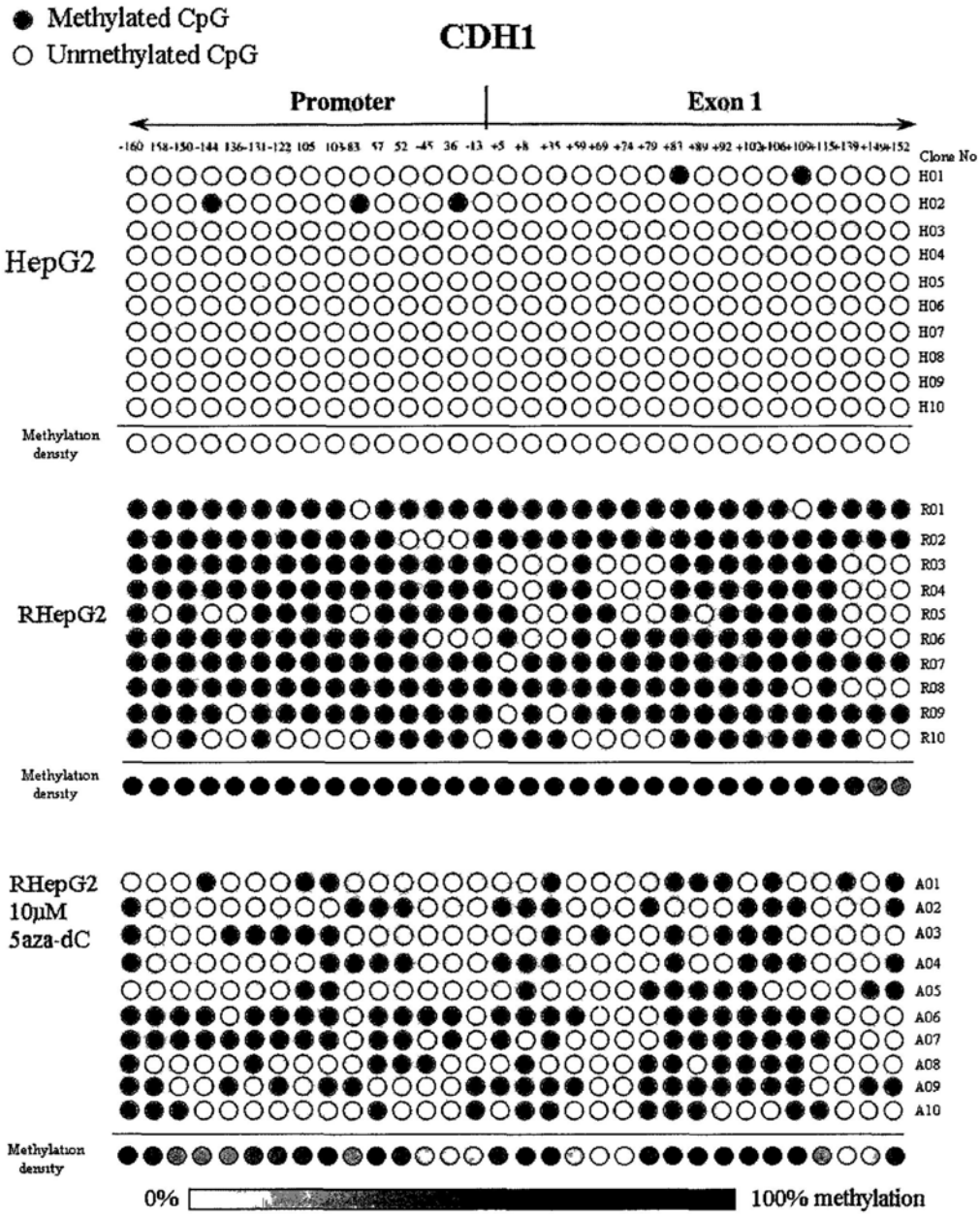


Figure 2.12 Bisulfite Sequencing results of Methylation patterns of CDH1 gene in HepG2, R-HepG2 cells, and 5aza-dC treated R-HepG2 cells. The locus of each CpG dinucleotides was shown with a number upstream (-) or downstream (+) of the transcription start nucleotide of CDH1-exon 1. Each circle represents a CpG dinucleotides unit in the CDH1 promoter region. The black circle represents a methylated CpG and the white one represents a unmethylated CpG. The grey circles in the methylation density part represent the methylation density of each locus. Data are representative of two independent experiments.

2.4 DISCUSSION

In this part, MSRE-PCR, MSP and BS were used to reveal the methylation pattern of tumor related genes in HepG2 cells and its doxorubicin-induced multidrug resistant R-HepG2 cells. The same finding was that in all different methods targeted at the same promoters' CpG islands, their different sensitivity and targeting showed similar results with differences.

MSRE-PCR is a simple endonuclease digestion based method. It is suitable for initial screening of methylation because its digestion step is much easier to handle than the bisulfite modification of MSP or BS. But there are two disadvantages of this method: First, the targeting sequence of the endonucleases is 5'-CCGG-3' which excluded the majority of CpGs without "flanking 5'C and 3'G"s such as 5'-ACGG-3' and 5'-TCGA-3'. Although this limited recognition decreased the sensitivity of this method, the fact that methylation of CpG islands in promoter regions usually show patterns like hypermethylation or hypomethlation making it possible to represent the methylation pattern of the promoter which is located in because fully methylated CpG islands associated with many transcriptionally silent genes while the CpG islands associated with promoters with transcriptional activity (Herman and Baylin, 2003). Second, PCR is difficult to optimize because the primers usually target a high CG region in the promoter CpG island; the primers should be designed to avoid 5'-CCGG-3' and the PCR amplicons should have a size above 80bp to be detectable on agarose gel electrophoresis. For example, it was impossible

to design a primer N for MDR1 gene due to this cause so that when the difference in methylation is not "all to none", there was a lack of inner control to indicate the same DNA input. To solve these problems, careful DNA quantification was done by NanoDrop spectrophotometer; touchdown cycles were added into PCR programs to meet all possible annealing temperatures and 4/6 of these genes were also examined by MSP.

MSP was the "gold standard" method of methylation detection (Mill *et al.*, 2006) because of its unique combination of bisulfite modification and PCR which generates unique DNA sequence indicating the methylation patterns as well as the high sensitivity of any other assay excluding PCR. It has been widely applied especially in the medical and preclinical research mainly because of PCR's high sensitivity and ease of handling suits the bedside work. The MSP primer sets were designed to be specific only to methylated or unmethylated sequence after bisulfite modification. Each primer often covers 2-3 CpG dinucleotides units and there is usually one CpG unit designed at the 3' end of the primer which is the key to the specificity of MSP. However, the PCR is also the bottleneck of this method. Besides the designing difficulty of the primers, all unmethylated cytosines were converted to uracils in this once rich-CG region, thus the annealing temperature sometimes might be too low to make PCR successful. MSP is more qualitative than quantitative, it is able to show the change between methylation and unmethylation but it is difficult to show the accurate percentage of methylation or unmethylation in a DNA sample. Similar to

MSRE-PCR, MSP's targeting CpGs are limited to those 4-6 CpG dinucleotides in the promoter; it is better than MSRE-PCR but also could not show the distribution of every 5'methylcytidine in CpG islands.

Bisulfite Sequencing is often considered the gold standard in DNA methylation analysis. It has a time and labor consuming cloning step and a harsh bisulfite modification step which causes large-scale degradation of gemomic DNA. The bisulfite converted single stranded DNA is also prone to further denaturation unless stored at -80℃. However, bisulfite sequencing could provide methylation maps of single DNA molecules, the methylation status of all CpGs in a selected region of every DNA molecule could be revealed through sequencing. It could provide more quantitative data than other methylation assays.

In our study, the methylation difference of several tumor related genes in HepG2 and R-HepG2 cells were compared by MSRE-PCR, MSP and BS. **1. MSRE-PCR**: CDKN2B, MGMT, CDKN2A and hMLH1 promoters are unmethylated both in HepG2 and R-HepG2 cells; the methylation of MDR1 and CDH1 promoter seemed to have increased in R-HepG2 cells. **2. MSP**: CDKN2A, hMLH1 and hMSH2 has no difference between the two cell lines. Rassf1A was methylated in HepG2 but showed no amplification of methylation or unmethylation in R-HepG2 cells, this might be unmethylation because in unmethylation control B DNA Rassf1a showed no PCR amplification neither. In MDR1, ESR1, Rassf2A and CDH1, the methylation of

promoters are higher in R-HepG2 cells than in its parental HepG2 cells. This finding indicated that CDH1, MDR1, ESR1 and Rassf2A showed significant difference in their promoter methylation patterns between HepG2 and R-HepG2 cells. The methylation change of MDR1 between non-resistant cancer cells and multidrug resistant cancer cells had been reported in different cancer types such as breast cancer (Sharma and Vertino, 2004), prostate cancer (Enokida *et al.*, 2004), neuroblastoma (Qiu *et al.*, 2007) and leukemia (Nakayama *et al.*, 1998). Reports suggest that the full activation of MDR1 expression might also require a modification of chromatin structure on histone modification level (David *et al.*, 2004), histone modifications occurred within the 5' hypomethylated region of MDR1 (H3 acetylation and H3K4 methylation) directly correlated with MDR1 up-regulation (Baker *et al.*, 2005). The chromatin accessibility regulated by epigenetic modifications plays a large role in controlling the endogenous MDR1 expression state (Baker and El-Osta, 2004). Most of the researches on the epigenetic regulation of MDR1 were carried out in breast cancer or prostate cancer cell models, which were not the case of HCC cells. P-Glycoprotein which was encoded by MDR1 is one of several transport proteins active in the normal liver; it stained the canalicular membrane of hepatocytes and mediates the canalicular excretion of bulky lipophilic cations (Ros *et al.*, 2003). Our previous study (Tang *et al.*, 2007) and other researchers reported up-regulation of MDR1 in doxorubicin resistant HepG2 cells (Wang *et al.*, 2006; Jia *et al.*, 2009). In this study, I found that MDR1 in HepG2 was unmethylated but MDR1 in R-HepG2 was semi-methylated. This methylation pattern

of MDR1 seems contradictory with its expression profile in the two cell lines. This phenomenon is coordinate with some researches that the MDR1 repression in drug-sensitive cells is independent of its promoter methylation (Jin and Scotto, 1998; Baker *et al.*, 2005; El-Khoury *et al.*, 2007) which suggested that the regulations of MDR1 expression in some cells occurred at a transcriptional level. So the difference in MDR1 promoter methylation between HepG2 and R-HepG2 might not be responsible for the up-regulation of MDR1 in R-HepG2 cells since the depression of MDR1 on a DNA methylation level requires a highly methylated MDR1 promoter in both alleles which was not the case in our cell lines. ESR1 was semi-methylated in HepG2 and methylated in R-HepG2. However, ESR1 was not expressed in HepG2 cells (Barkhem *et al.*, 2002). This change in methylation pattern would be rather a marker for cancer progression than a factor for ESR1 expression. Tumor suppressor gene Rassf1A was the first identified member of the ras association domain family. It serves as a pro-apoptotic Ras effecter and cell cycle modulator at G1-S checkpoint (Donninger *et al.*, 2007). Our results show that the promoter of Rassf1A in HepG2 was methylated. But in R-HepG2 cells, neither methylation specific nor demethylation specific band was produced by MSP. This pattern was the same with the methylation$^-$ control DNA U-b. Thus I claim that the methylation of Rassf1A in R-HepG2 cells was probably lower than that in HepG2 cells. Further study should be carried out to elucidate this phenomenon. Rassf2A was a new member of the ras association domain family. Its aberrant hypermethylation had been noticed as an early event in carcinogenesis since this gene was identified (Hesson *et al.*, 2005). The

promoter methylation of Rassf2A was found 30% high in HCC tissue than adjacent normal liver tissue (Ren *et al.*, 2009). However, although there was obvious difference between HepG2's unmethylation and R-HepG2's methylation in Rassf2A promoter, I found that the mRNA of Rassf2A was transcribed in these two cell lines and showed little difference.

CDH1 encoded E-cadherin is a trans-membrane glycoprotein important for the maintenance of normal epithelial phenotypes (Tsuchiya *et al.*, 2006). It is expressed in the plasma membranes of normal epithelial cells and mediates hemophilic Ca2+ -dependent intercellular adhesion in adherents' junctions (Overduin *et al.*, 1995). Its cytoplasmic domain interacts with actin, α-catenin, p120 and β-catenin (Kemler, 1992). It acts as a tumor and invasion suppressor, the loss of E-cadherin function is an essential event in tumorigenesis and epithelial tumor invasion which increases metastatic potential. Its loss is also an indicator of poor prognosis in several types of cancer including the HCC (Pecina-Slaus, 2003; Li *et al.*, 2008). The E-cadherin expression is correlated with CDH1 promoter methylation and is silenced above 20% to 30% methylation in NCI-60 cancer cell lines which is a set of 59 human cancer cell lines (Reinhold *et al.*, 2007). It had been reported that CDH1 in HepG2 cells were normally expressed without any mutation (Saito *et al.*, 2001). In our study, the CDH1 promoter methylation in HepG2 is found to be lower than that in R-HepG2 cells by MSRE-PCR, MSP and BS. The relationship between these epigenetic changes in promoter methylation and the consequences of these changes in CDH1 expression and multidrug resistance in HepG2 and R-HepG2 cells would be studied

in the following chapters.

2.5 CONCLUSION

10 selected tumor related genes' (CDKN2A, CDKN2B, MGMT, MDR1, CDH1, ESR1, hMLH1, hMSH2, Rassf1A, Rassf2A) promoter methylation profiles were analyzed using different methylation assays (MSRE-PCR, MSP, BS) to examine any difference of their methylation pattern in doxorubicin sensitive HepG2 cells and its doxorubicin-induced multidrug resistant derivative R-HepG2 cells. Among these genes, CDKN2A, CDKN2B, hMLH1, hMSH2 and MGMT showed no difference in promoter methylation between the two cell lines. The methylation of CDH1, ESR1, Rassf2A and MDR1 in R-HepG2 cells were found to be higher than those in HepG2 cells. The hypermethylated CDH1 promoter could be demethylated by 5aza-dC treatment. The relationship between promoter methylation and corresponding protein expressions of these genes, and the significance of these changes in drug resistance would be examined in following chapters.

Chapter 3

The effect of epigenetic loss of CDH1 in R-HepG2 cells

3.1 INTRODUCTION

Genetic changes resulting in inactivation of tumor suppressor genes were believed to be essential in carcinogenesis and progression of human cancer. It is now widely accepted that epigenetic events are also frequent mechanism contributes to the silencing of tumor suppressor genes (Sawan *et al.*, 2008). In chapter two, changes of promoter methylation were found in several tumor related genes in R-HepG2 cells comparing with its parental HepG2 cells. Among them, CDH1 provoked our interest. The CDH1 gene is located on human chromosome 16q22.1. E-cadherin encoded by CDH1 is the main molecule of adherent junctions in epithelial cells. It has a large extracellular domain comprising five cadherin-motif subdomains, a single segment and a short conserved cytoplasmic domain, which interacts with several catenin proteins through a carboxy-terminal cytoplasmic domain of E-cadherin and the central armadillo domain of catenins (Berx and van Roy, 2001). Most of normal epithelial cells are adherence-dependent since they grow and divide only if attached to the extracellular matrix (ECM). Disruption of the interactions between normal epithelial cells and ECM or inappropriate anchorage can induce a programmed cell death called anoikis (Frisch and Francis, 1994). Resistance to anoikis promotes metastasis whereas loss of E-cadherin promotes metastasis in part by inducing anoikis resistance (Onder *et al.*, 2008). CDH1 is an important molecule in the epithelial-mesenchymal transition (EMT). Together with loss of cell adhesion and increased cell mobility, EMT is essential for cancer metastasis. E-cadherin is a very important molecule in cancer progression and EMT induction. Loss of function of

CDH1 is correlated with many types of cancers by increasing proliferation, invasion and metastasis (Jeanes *et al.*, 2008). To further elucidate the relationship between CDH1's epigenetic loss of function and chemotherapeutical resistance, I planned to answer these questions by comparing the differences between HepG2 cells and R-HepG2 cells in this chapter: (1) Does the change in CDH1 promoter methylation contribute to silencing of E-cadherin expression in R-HepG2 cells? (2) Is there any change in migration potential in R-HepG2 cells? (3) Can these changes in R-HepG2 cells be reverted in CDH1 transfected R-HepG2 cells? (4) Is there any change in expression of multidrug resistant genes in R-HepG2 cells? (5) Does CDH1 exhibit any effect on drug efflux repression and cell death induction in R-HepG2 cells with doxorubicin stress?

3.2 MATERIALS AND METHODS

3.2.1 Transformation of *E.coli* by plasmid DNA

Plasmid containing the full length recombinant CDH1 cDNA pCMV6-XL4-CDH1 (OriGene, #SC117413) and pcDNA3.1$^{(+)}$ (Invitrogen, #V79020) were transformed into *E.coli* JM109. The plasmid vector maps were shown in Figure 3.1, the transformation methodologies were as described in Chapter 2.

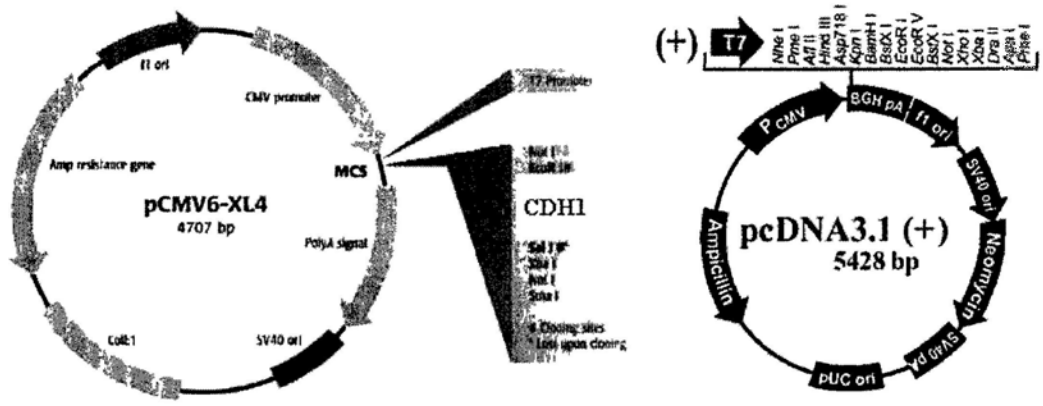


Figure 3.1 Plasmid vector maps of pCMV6-XL4-CDH1 and pcDNA3.1$^{(+)}$.

3.2.2 Cell lines and cell culture

Human HCC cell lines HepG2 and R-HepG2 were used in this part. R-HepG2-EV was the R-HepG2 transfected with pcDNA3.1$^{(+)}$ plasmid; R-HepG2-CDH1was the R-HepG2 transfected with pCMV6-XL4-CDH1. The cell culture methodologies were as described in Chapter 2.

3.2.3 Total RNA extraction

NucleoSpin® RNA II total RNA isolation kit (Macherey-Nagel, #740955.50) was used for RNA isolation of cell lines. All buffers and chemicals used were included in the kit. 5×10^6 cells were cultivated and harvested from 90mm petri-dish by trypsinization. 350μl RA1 and 3.5μl β-mercaptoethanol were added to the pellet. The mixture was vortexed vigorously to suspend the pellet and transferred to a NucleoSpin® Filter in a 2 ml collection tube, The suspension was centrifuged at 11000×g for 1 minute, then the filter was discard. 350μl 70%ethanol was added to the filtrate by pipetting up and down for 5 times. The liquid mixture was transferred to a NucleoSpin® RNA II column in a 2 ml collection tube and centrifuged at 11000×g for 30 seconds, the filtrate was discarded then. 350μl MDB was added to the column and centrifuged at 11000×g for 1 minute, the filtrate was collected and 95μl DNase Reaction Mixture (a mixture of 10ul rDNase and 90μl Reaction Buffer of rDNase) was added to the center of the filter membrane of the column. The tube was kept still at room temperature for 15 minutes and then 200μl RA2 was added to the column. The mixture was centrifuged at 11000×g for 30 seconds and the filtrate

was discarded. 600 ul RA3 was added and then centrifuged at 11000×g for 30 seconds, this step was repeated with 250μl of RA3 and 2 minutes' centrifugation. The column was transferred into a 1.5 ml tube provided with the kit. 60μl RNase-free water was added to the center of the membrane and centrifuged at 11000×g for 1 minute. This RNA eluate was immediately used for RT-PCR or stored at -80℃. Aliquot of the eluate underwent UV spectrophotometry at OD260/280 for quantification and quality control (Ratio 260/280≥1.8) of the RNA sample.

3.2.4 Extraction of protein lysate from cultured cells

5×10^6 cells were cultivated and harvested from 90mm petri-dish by trypsinization. 100µl Lysis Buffer [2% (M/V) Sodium Dodecyl Sulfate (SDS), 10% (M/V) Glycerol, 625mM Tris-HCl (pH 6.8), 0.1% (M/V) Aprotinin] was added to the vortexed cell pellet and mixed by inverting the tube for several times until there was no pellet visible. The mixture was kept still on ice for 30 minutes then in 100℃ water bath for 10 minutes and then centrifuged at 11000×g for 10 minutes. The supernatant was collected and the protein concentration was determined by Bicinchoninic Acid Assay. An equal volume of 2× sample buffer (125 mM Tris pH 6.8, 4% SDS, 10% glycerol, 0.006% bromophenol blue, 1.8% ß-mercaptoethanol) was added to all samples and boiled for 10 minutes. The protein samples were stored at -80℃.

Bicinchoninic Acid Assay (BCA) BCA Working Reagent (BWR) was a freshly prepared 50:1 mixture of BCA Reagent A [1%BCA, 2% $Na_2CO_3{\cdot}H_2O$, 0.16% $Na_2C_4H_4O_6(\cdot2H_2O)$, 0.4% NaOH, 0.95% $NaHCO_3$, in ddH_2O at pH 11.25] and BCA Reagent B (4%CuSO4·5H_2O, in ddH_2O). BSA standard solutions (0.1 – 20 g/L) were set up by diluting BSA stock solution (20g/L) with BWR. 3µl protein samples were mixed with 250µl BWR and incubated at 37℃ for 30 minutes. The absorbance at 540nm was measured by a microtiter plate reader (Bio-Rad) and the protein concentrations were calculated using the BSA standard curve.

3.2.5 Reverse transcript PCR (RT-PCR)

First Strand cDNA Synthesis. The cDNA synthesis was performed by TaqMan® Reverse Transcription Reagents (Applied Biosystems, N808-0234). The following reagents were mixed on ice for each reaction: RNase Inhibitor 1μl

10×RT buffer 5μl

2.5mM dNTP 10μl

25mM $MgCl_2$ 11μl

Random Hexamer 2.5μl

50U/μl Transcriptase 0.5μl

RNA template 2μg

RNase free H_2O was added to a final volumn of 50μl

The thermal cycling program for cDNA synthesis was: 25℃ 10 minutes, 37℃ 60 minutes, 95℃ 5 minutes. The cDNA synthesized was immediately used as template for second round PCR of selected genes or at -20℃ for storage.

10 multi drug resistant related genes were selected for the second round PCR, and primers were shown in Table. 3.1

Primer set	Sense primer, 5'->3'	Antisense primer, 5'->3'
MRP1	CAGCATGAGCTACTTGCCG	TTCCTCCTCGTCCATCACTGT
MRP2	CCACTTTGTTTTGAGCAAACTGT	CCAGCTCTATGGCTGCTAGAA
MRP3	ATTTGGAATCTAACATCGTGGCT	GCCGGTAGCGCACAGAATA
MRP4	GGACGCGAACATCTGCTCA	CTTCTGGCAGCACTGAATACAT
MRP5	AGAACTCGACCGTTGGAATGC	TCATCCAGGATTCTGAGCTGAG
MRP6	AGATGGTGCTTGGATTCGCC	GCCACACAGTAGGATGAATGAG
MRP7	CGACTCCGGGTCATCAAATAC	TCTACCACAATCTGGGCTGTA
MRP8	GTGAATCGTGGCATCGACATA	GGGCCATCTTGGAGAGTATAGG
BCRP	CCCGCGACAGTTTCCAATGA	GGCGTTGAGACCAGGTTTCA
MDR1	GGAACTCAGCTCTCTGGTGG	CTTCTCTGGCTTTGTCCAGG
GAPDH	CAGCCGAGCCACATCG	TGAGGCTGTTGTCATACTTCTC
CDH1	TGCCCAGAAAATGAAAAAGG	GGATGACACAGCGTGAGAGA

Table 3.1 Primers of multidrug related genes, CDH1 and GAPDH for RT-PCR

Taq PCRx DNA Polymerase Reagents (Invitrogen, #11508-017) were used for the second round PCR. The following reagents were mixed on ice for each reaction:

10×PCR Buffer Minus Mg^{++} 2μl

10mM dNTP 0.4μl

50mM $MgCl_2$ 0.6μl

10μM Primer mix 1μl

cDNA template 2μl

5U/μl Taq DNA Polymerase 0.1μl

Autoclaved distilled water was added to a final volume of 20μl

The thermal cycling program for second round PCR was: 94℃ 5 minutes; thermal cycles: 94℃ 50 seconds, 58℃ 50 seconds, 72℃ 50 seconds, 18-35 cycles (depend on target gene to avoid saturation); 72℃ 10 minutes. The PCR products were analyzed on 2% agarose gel electrophoresis.

3.2.6 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of protein lysate & Western blotting

The method for gel preparation, electrophoresis, PVDF protein transfer and Western blotting were carried out according to The Protein Protocols Handbook (Wang and Fan, 2002). 5% gel for MDR1 and 8% gel for other proteins were used. Briefly, 20μg of each protein lysate sample was loaded to the gel; then subjected to electrophoresis at 100 V for 150 minutes for protein separation by BIORAD Mini-PROTEAN® III Cell system. Proteins were then transferred to PVDF membrane at 1.2 mAmp/cm^2 for 45 minutes by Trans-Blot® SD Semi-Dry Transfer Cell system. The PVDF membrane was blocked by 10% skim milk for 30 minutes then incubated with desired primary antibody (1:1000) [GAPDH (Cell Signaling, #2118), CDH1 (Cell Signaling, #3195), MDR1 (Calbiochem, #517312)]overnight. The membrane was rinsed and incubated with horseradish peroxidase-conjugated secondary antibody for 30 minutes and then went through protein detection by ECL™ Western Blotting Detection Reagents (GE Healthcare, #RPN2106) and X-ray film exposure.

3.2.7 Plasmid DNA extraction (by large scale method)

Large scale of pCMV6-XL4-CDH1 and pcDNA3.1$^{(+)}$ were prepared for transient transfection. The cultured cells were lysed by alkali and the plasmids purified by precipitation with polyethylene glycol (PEG) (Sambrook *et al.*, 1989). Briefly, the overnight cultured cells were centrifuged and resuspended in solution I, lysed by solution II, bacterial genomic DNA and protein precipitated by solution III. This crude extract was precipitated by equal volume of isopropyl alcohol, washed by 70% ethanol then dissolved in 1×Tris-EDTA (TE, pH8.0). Equal volume of 5M LiCl was added to precipitate RNA; Ribonuclease A (RNase A, Invitrogen, #12091021) was added to digest RNA at a final concentration of 20μg/ml. Equal volume of 20%PEG-2.5M NaCl was added into the sample and was allowed to stand at 4℃ for 1 hour. The plasmid DNA was purified by isopropyl alcohol precipitation. This plasmid DNA was dissolved in 1×TE and stored at -20℃. DNA quantification was performed as described in chapter 2.

3.2.8 Transient transfection

3×10^5 cells/well were seeded in 6-well microplate (IWAKI, #3810-006) in antibiotics-free RPMI-1640 medium supplemented with 10% FBS and incubated in humidified incubator at 37℃ supplying 5% CO_2 for 16 hours to allow attachment. After incubation, medium was removed and the plates washed with FBS and PS free RPMI-1640 medium (plain medium). Finally 1.5 ml this plain medium was added to each well. DNA-Lipofectamine 2000 complex was then added to the wells and incubate the mixture in the humidified incubator at 37℃ supplying 5% CO_2 for 4 hours. The medium was removed and replaced with complete RPMI-1640. The cells were further incubated for 48 hours before harvest. The cells, RNA and protein were collected as described in this chapter and chapter 2.

DNA-Lipofectamine 2000 complex preparation: 4μg pCMV6-XL4-CDH1 or pcDNA3.1(+) plasmid was mixed with 250μl plain medium; 10μl Lipofectamine™ 2000 reagent (Invitrogen, #11668-019) was mixed with 250μl plain medium. These two mixtures were incubated separately in Eppendorf tube at room temperature for 5 minutes, then mixed and incubated at room temperature for another 20 minutes. The DNA-Lipofectamine 2000 complex was ready for use.

3.2.9 Quantification of doxorubicin intake by flow cytometry

Doxorubicin (Sigma-Aldrich, #D1515) was dissolved in sterile distilled water at 1mM and stored at -20℃ in aliquots.

After transient transfection and incubation for 48 hours as mentioned before, the medium was changed and 10μM of doxorubicin was added to the cells and incubated for 3 hours. The cells were washed twice with PBS and trypsinized into a PBS suspension. The samples were then analyzed by FACSCanto^{TM} Flow Cytometer (BD Biosciences). The signals were detected by PE-A channel and data analyzed by WinMDI v2.6 software.

3.2.10 Apoptosis analysis by flow cytometry

Annexin V-FITC Apoptosis detection kit (Bender MedSystems®, #BMS500F1/100) was used for the Apoptosis analysis. After transient transfection and incubation for 48 hours as mentioned before, the medium was changed and approximate amounts of doxorubicin (50μM, 100μM, 150μM) were added to the cells and incubated at 37℃ for 24 hours. The cells were harvested and resuspended in 1×binding buffer at a density of $2\sim5\times10^5$ cells/ml. 5μl Annexin V/FITC and 10μl propidium iodide (PI) were applied to the sample and incubated at room temperature for 15 minutes. 250μl 1×binding buffer was applied to the sample and this sample was analyzed by FACSCanto™ Flow Cytometer (BD Biosciences). The signals were detected by PE-A and FITC channels, and data analyzed by WinMDI v2.6 software.

3.2.11 Trans-well migration assay

After transient transfection and incubation for 48 hours as mentioned before, the cells were harvested and washed in RPMI-1640 plain medium to remove FBS. 1×10^5 cells were seeded into the upper chamber of the Cell Culture Insert (SPL, #36224) in 300μl plain medium. 800μl RPMI-1640 complete medium (with 10% FBS) was added into the lower chamber. This formatted 24-well plate was put back to CO_2 incubator and incubated for 20 hours. The insert was removed from the medium and washed twice in PBS. The cells on the insert were fixed with 2% paraformaldehyde for 20 minutes. The insert was washed with PBS and then stained by hematoxylin for 20 minutes. The non-migrated cells in the upper chamber were removed gently with cotton swabs. The migrated cells at the filter bottom of the insert were photographed. Number of migrated cells in 5 regions of the filter were counted and added up. This is the number of migrated cells per insert. Three inserts were used in each experiment.

3.3 RESULTS

3.3.1 Transcription and protein expression of CDH1 gene in HepG2, R-HepG2 and R-HepG2-CDH1 cells

In Chapter 2, I observed that the CDH1 promoter was hypermethylated in R-HepG2 cells and hypomethylated in HepG2 cells and this difference in promoter methylation might result in totally different expression profiles of the gene in the two types of cells. In this part, I observed the effect of plasmid pCMV6-XL4-CDH1 transient transfection on CDH1 transcription and E-Cadherin expression on R-HepG2 cells. As shown in Figure 3.2, CDH1 mRNA and E-cadherin expression could be detected in HepG2 cells and R-HepG2-CDH1 cells but there were neither mRNA transcription nor E-cadherin expression in R-HepG2 cells.

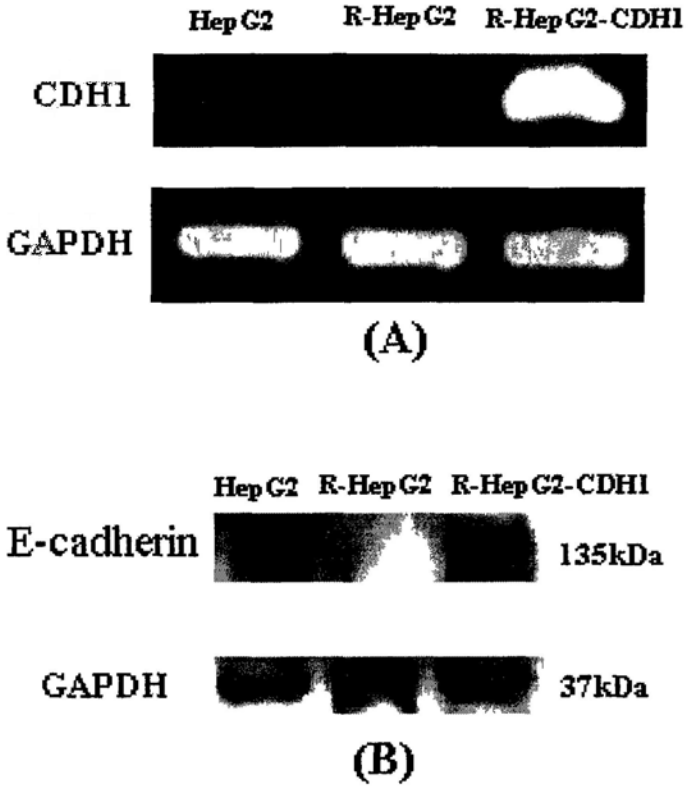


Figure 3.2 Agarose gel electrophoresis of RT-PCR and Western blotting of CDH1 in HepG2, R-HepG2 and R-HepG2-CDH1 cells with GAPDH as control. (A) The mRNA transcriptions of CDH1 in the three cell lines were analyzed by RT-PCR. (B)The protein levels of E-cadherin in the three cell lines were analyzed by Western blotting. Data are representative of three independent experiments.

In order to observe the loss of E-cadherin and its relations with drug resistance and cellular biological functions, I performed the following assays.

3.3.2 CDH1 cDNA transfection suppressed doxorubicin intake in doxorubicin resistant R-HepG2 cells

The doxorubicin intake difference between HepG2, R-HepG2, R-HepG2-CDH1 and R-HepG2-EV cells was analyzed by flow cytometry in PE-A channel. As shown in Figure 3.3, the amount of doxorubicin intake in HepG2 cells was the highest while the doxorubicin intake in R-HepG2 cells was the lowest. There was no significant difference between the doxorubicin intake in R-HepG2 and that in R-HepG2-EV cells. The doxorubicin intake was significantly increased in R-HepG2-CDH1 cells comparing to R-HepG2 cells. The result indicated that transfection of E-cadherin contributed to the doxorubicin intake. Our results revealed that regaining E-cadherin has an obvious effect on increased doxorubicin intake.

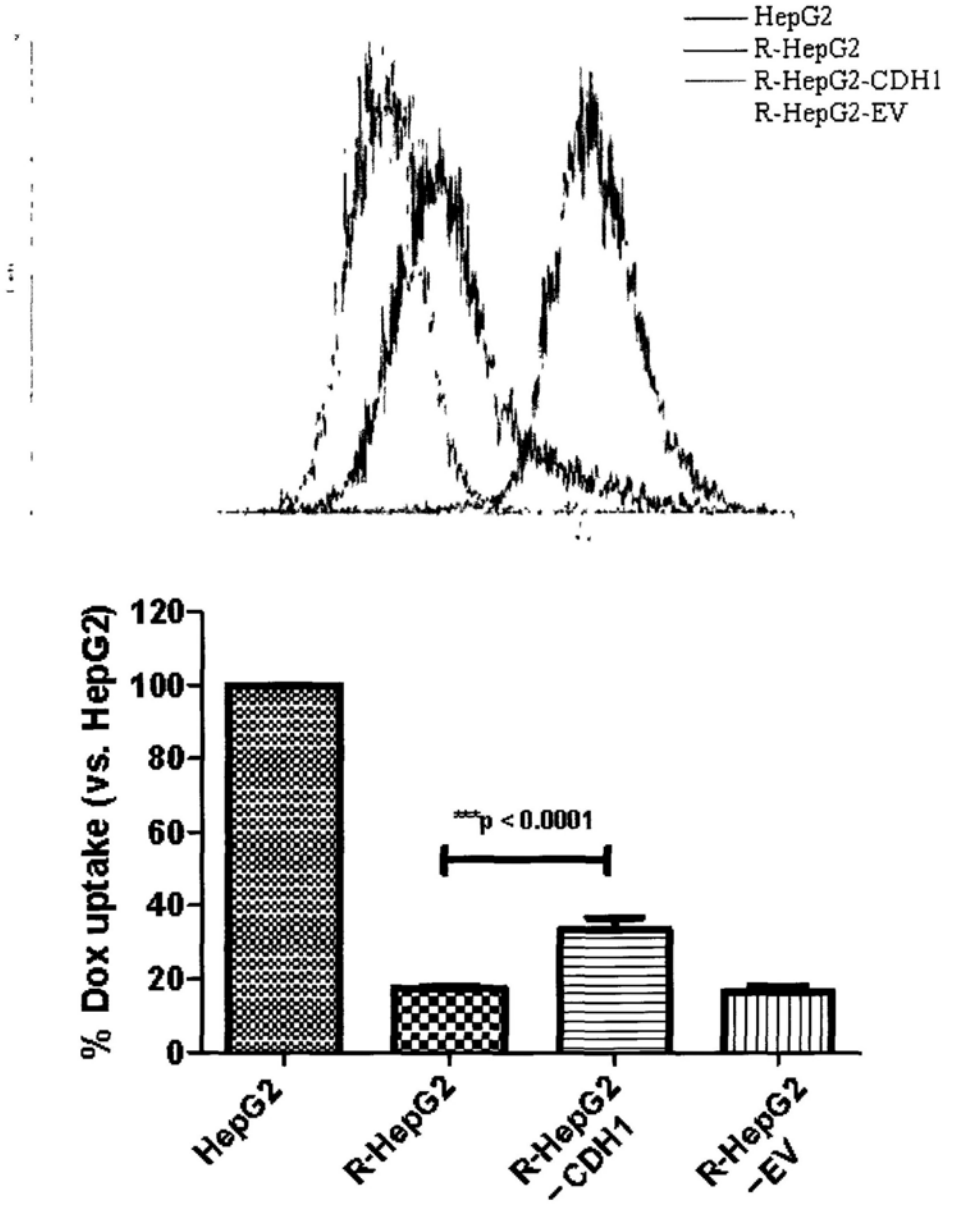


Figure 3.3 Effect of CDH1 on doxorubicin intake in HepG2, R-HepG2 and R-HepG2-CDH1 cells. The fluorescence of doxorubicin in PE-A channel was analyzed in HepG2, R-HepG2, R-HepG2-CDH1 and R-HepG2-EV cells. Red line: doxorubicin intake of HepG2 cells. Black line: doxorubicin intake of R-HepG2 cells. Green line: doxorubicin intake of R-HepG2-CDH1 cells. Blue line: doxorubicin intake of R-HepG2-EV cells (A). Bars, SD. Data are representative of three independent experiments (B).

3.3.3 CDH1 cDNA transfection suppressed migration potential of R-HepG2 cells

The migration potential and invasiveness are important markers of cancer progress and EMT. As CDH1 encodes one of the most important cell-cell adhesion molecules –E-cadherin which is a suppressor of cell migration and invasion, the trans-well migration assay was carried out to analyze the migration potentials of HepG2, R-HepG2 cells and R-HepG2 cells with transfected CDH1. This assay was also carried out on R-HepG2-CDH1 cells to observe any change of the migration potential in the doxorubicin resistant R-HepG2 cells. R-HepG2-EV cells which were the R-HepG2 cells transient transfected with pcDNA3.1$^{(+)}$ plasmid were the empty vector (EV) control sharing the same plasmid CMV promoter with pCMV6-XL4-CDH1. As shown in Figure 3.4, the number of migrated R-HepG2 cells was about 4 times more than that of HepG2 cells, the number of migrated R-HepG2-EV cells was similar to that of non-transfected R-HepG2 cells, and the number of CDH1 transfected R-HepG2-CDH1 cells was slightly higher than that of HepG2 cells.

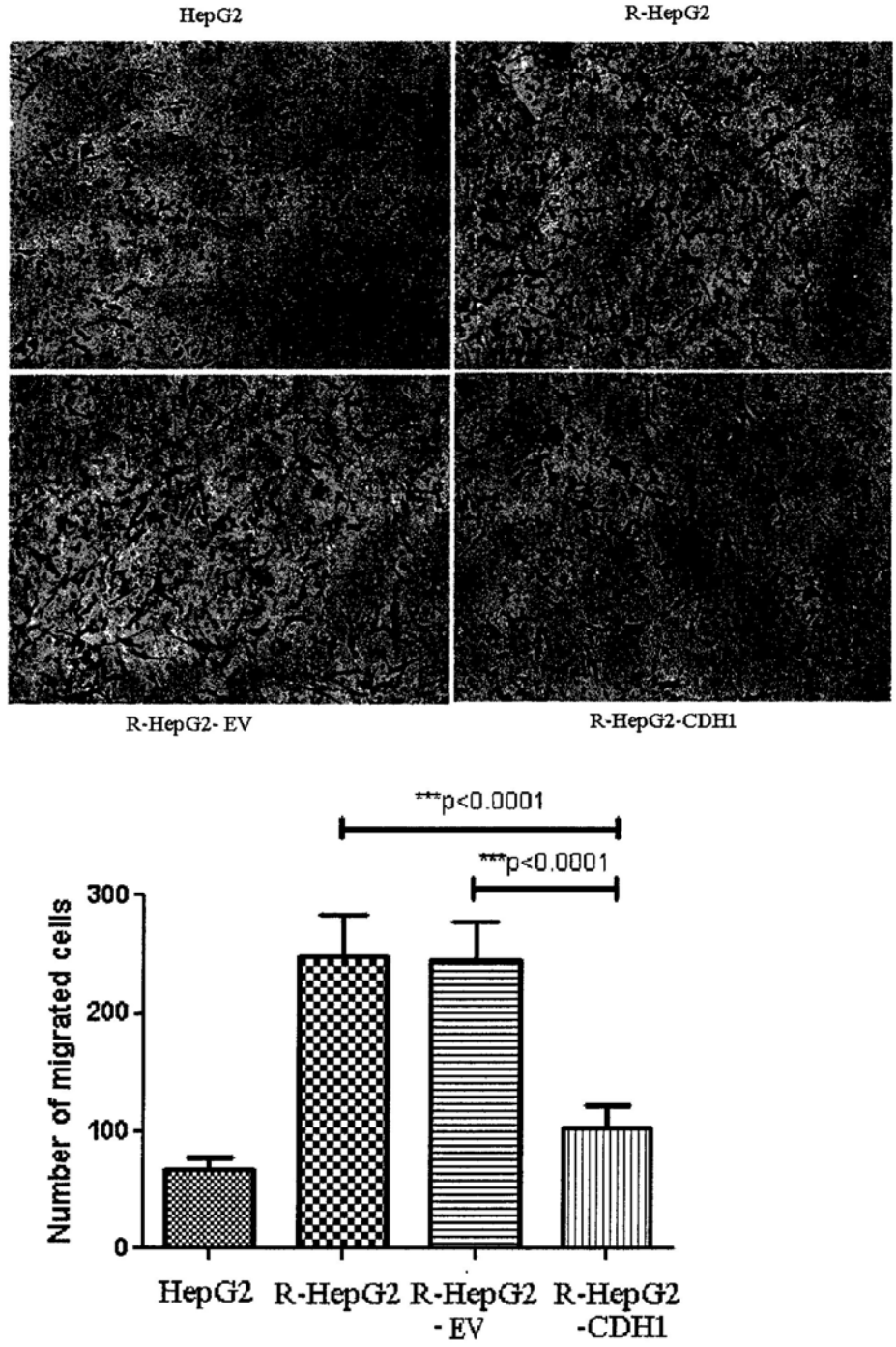


Figure 3.4 The trans-well migration assay of HepG2, R-HepG2, R-HepG2-EV, and R-HepG2-CDH1 cells. (A) Low-magnification photographs of representative wells are shown. The migrated cells were stained with hematoxylin into dark blue color. (B) Number of migrated cells in 5 regions of the trans-well filter were counted and added up. Three wells were analyzed for each treatment. Bars, SD. Data are representative of three independent experiments.

3.3.4 CDH1 cDNA transfection promoted apoptosis in doxorubicin resistant R-HepG2 cells

The percentages of apoptotic cells in R-HepG2, R-HepG2-EV and R-HepG2-CDH1 cell lines with the presence of approximate amount of doxorubicin (50μM, 100μM, 150μM) were analyzed after Annexin V-PI staining by flow cytometry. As shown in Figure 3.5, the percentages of apoptotic cells in R-HepG2-CDH1 cells were higher than that of their paired R-HepG2 and R-HepG2-EV cells at all three doxorubicin concentrations.

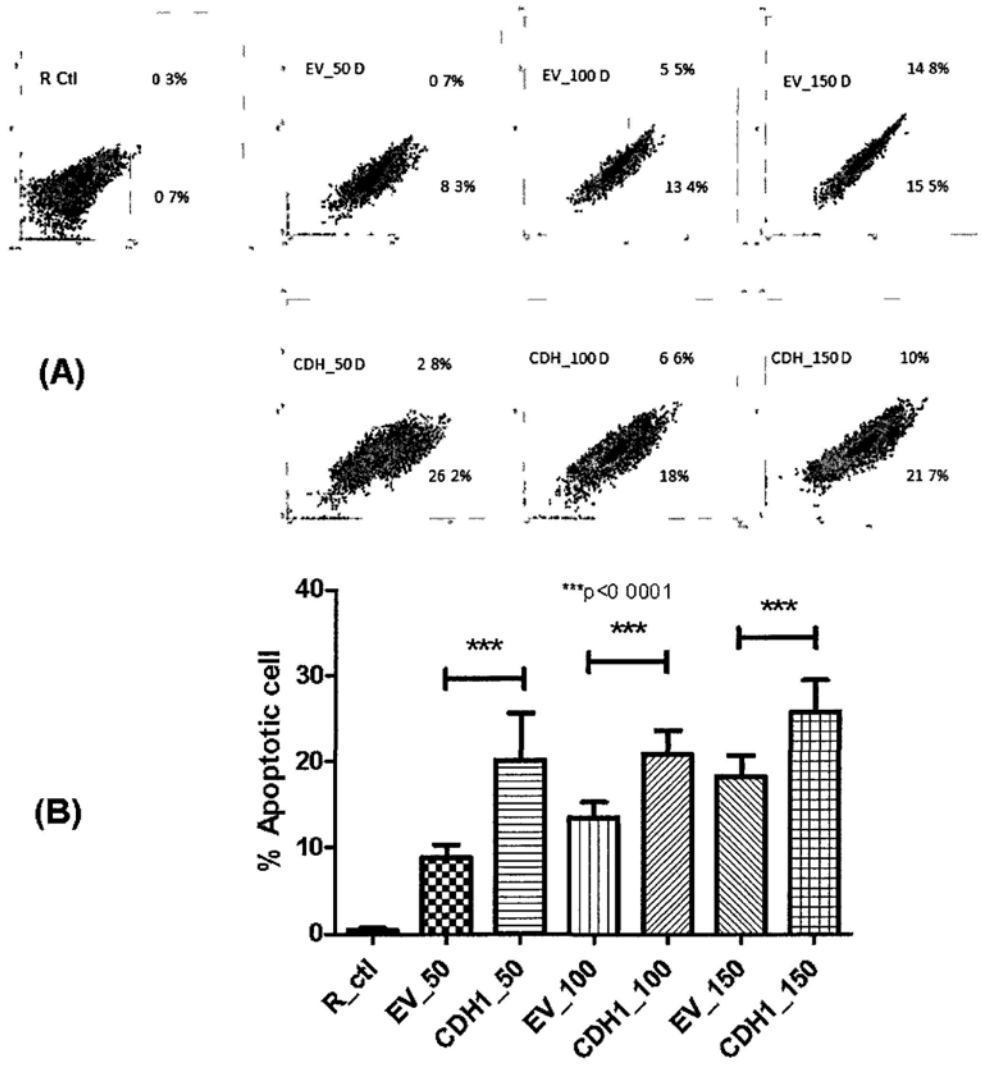


Figure 3.5 Effect of CDH1 on apoptosis in R-HepG2 and R-HepG2-CDH1 cells. The R-HepG2 cells with/without CDH1 transfect were exposed to different amounts of doxorubicin 48 hours after transfection The percentage of apoptotic cells was measured by flow cytometry further 24 hours after doxorubicin exposure (A) Flow cytometry analysis (B) Percentage of apoptotic cells in cells with different treatments Each result is representative of at least three independent experiments Abbreviations R_ctl (non-transfected R-HepG2 cells in absence of doxorubicin), EV_50/100/150 D (R-HepG2-EV cells with 50/100/150μM doxorubicin), CHD1_50/100/150 D (R-HepG2-CHD1 cells with 50/100/150μM doxorubicin) Data are representative of three independent experiments

3.3.5 Transcription of 10 MDR related genes in R-HepG2 cells

10 MDR related genes were selected to analyze their transcription change in R-HepG2 cells comparing to the transcription of these genes in the non resistant HepG2 cells. As shown in Figure 3.6, among the 10 selected genes, there was no obvious change in MRP2, MRP3, MRP4, MRP7, MRP8 genes. The transcription of MDR1, MRP1, MRP5 were higher in R-HepG2 cells while the transcription of MRP6 and BCRP were lower in R-HepG2 cells comparing to those in HepG2 cells.

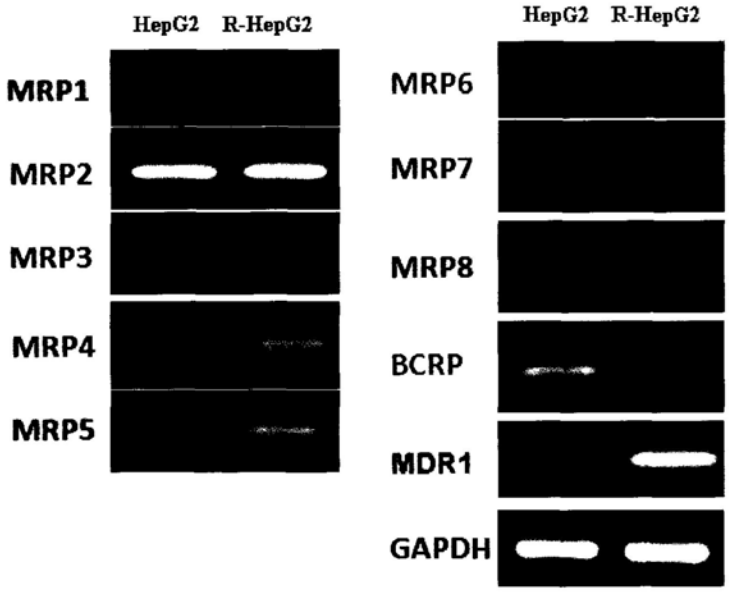


Figure 3.6 Agarose gel electrophoresis of RT-PCR results in 10 drug resistance related genes with GAPDH as control in HepG2 and R-HepG2 cells. The names of up-regulated genes in R-HepG2 cells were in red, down-regulated genes in blue and the genes with no significant change were in black. Data are representative of three independent experiments.

3.3.6 P-glycoprotein expression in HepG2, R-HepG2, and R-HepG2-CDH1 cells

To investigate the relationship between CDH1 and MDR1 by examining whether the quantity of MDR1 would be interfered by CDH1's reoccurrence in the MDR cells, Western blotting was carried out to show the expression of P-glycoprotein in HepG2, R-HepG2 and R-HepG2-CDH1 cells. As shown in Figure 3.7, there was little P-glycoprotein expression in HepG2 cells. P-glycoprotein was expressed in both R-HepG2 and R-HepG2-CDH1 cells but the P-glycoprotein expression level was lower in R-HepG2-CDH1 cells. Our results suggested that the increase of E-cadherin expression could suppress the expression of P-glycoprotein .

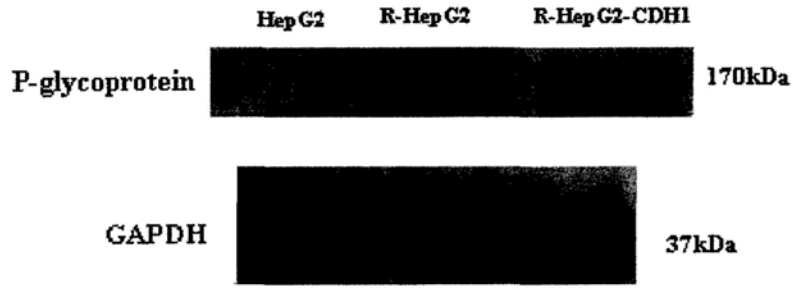


Figure 3.7 Western blotting of MDR1 in HepG2, R-HepG2 and R-HepG2-CDH1 cells with GAPDH as control. The protein levels of P-glycoprotein in the three cell lines were analyzed by Western blotting. Data are representative of three independent experiments.

3.4 DISCUSSION

Among the 10 selected multidrug resistant related genes, the transcription of MDR1, MRP1, MRP5 were higher in R-HepG2 cells while the transcription of MRP6 and BCRP were lower in R-HepG2 cells comparing to HepG2 cells. MDR1 was the most obvious up-regulated gene among these up-regulated genes. This result indicated the essential role of this gene in cancer progression and chemo-resistance. The amount of doxorubicin intake in R-HepG2-CDH1 cells was significantly higher than that in R-HepG2 cells. This suggested that CDH1 might possess an important role in keeping the intracellular drug concentration as the mechanism for decreased multidrug resistance in R-HepG2-CDH1 cells and the decreased P-glycoprotein expression in R-HepG2-CDH1 cells might partially explained this phenomenon. All these revealed that besides its functions in cell-cell adhesion, CDH1 has a potential role in anti-multidrug resistance development.

Development of MDR during chemotherapy is a major cause of treatment failure and a big barrier to successful management in systematic cancer treatment (Gottesman *et al.*, 2002). Researches on the systemic chemotherapy of HCC have shown quite low response rate, namely not higher than 24% (Boucher *et al.*, 2002; Park *et al.*, 2006).Epigenetics had proved itself as a mechanism driving polygenetic clinical drug resistance through methylation of genes such as pro-apoptotic genes Apaf1, Caspase8; DNA repair related genes hMLH1, FancF and MGMT (Glasspool *et al.*, 2006). In this part, I found that the hypermethylation in CDH1 promoter

epigenetically silenced CDH1 gene in R-HepG2 cells. CDH1 was transcribed and E-cadherin expressed in HepG2 cells but no transcription or expression detected in R-HepG2 cells.

The significant difference in CDH1 expression between HepG2 and R-HepG2 cells also resulted in different migration activities. The R-HepG2 cells were approximately 4 times more active in migration comparing to HepG2 cells. But in R-HepG2-CDH1 cells, they were approximately 2.4 times less migration active than the R-HepG2 cells. In the presence of CDH1, the migration activity of the multidrug resistant cells could be significantly suppressed. The suppressed cell migration phenotype also contributed to the reversion of EMT. This supported the role of CDH1 as a metastasis suppressor. I also investigated the percentage of apoptotic cells in R-HepG2-EV (empty vector control) and R-HepG2-CDH1 cells in presence of different amounts of doxorubicin. The half maximal inhibitory concentration (IC_{50}) of doxorubicin on HepG2 and R-HepG2 had been reported by our group that the IC_{50} for HepG2 was 5μM while the IC50 for R-HepG2 was 210μM (Chan *et al.*, 2000). There was approximately 1 fold more apoptotic cells in R-HepG2-CDH1 than R-HepG2-EV cells at all three doxorubicin concentrations. As mentioned in the introduction, CDH1 contributed to the induction of anoikis –a type of apoptosis. This suggested that loss of CDH1 helped the progressed multidrug resistant cells to evade apoptosis.

The methylation of CDH1 as a marker of cancer progression has been long found but researches on the mechanisms behind it mainly focused on catenins-actins and Wnt pathway (Pecina-Slaus, 2003). The involvement of CDH1 in cancer invasion and metastasis is well known but its involvement in anti-multidrug resistance through suppression of MDR1 was not reported. A few researches supported our findings: Research on a multidrug resistant breast cancer cell line demostrated that E-cadherin was not detected by immunolabeling at the cell junctions of the drug resistant cells (Faute *et al.*, 2002). Another research reported that the transcriptional suppressor of CDH1 --Zeb-1 might be responsible for maintaining drug resistance on transcription level in pancreatic cancer cells (Arumugam *et al.*, 2009). Our findings elaborated that loss of CDH1 through promoter hypermethylation was an important mechanism involved in cancer cell death, metastasis and multidrug resistance. I revealed the relationship between CDH1, MDR1 were related to multidrug resistance: suppression of CDH1 through promoter methylation in multidrug resistant cells contributed to multidrug resistance by increasing cell migration, increasing MDR1 expression, increasing drug efflux and exhibiting much higher IC_{50}; re-expression of CDH1 in multidrug resistant cells would suppress cell migration, suppress MDR1 expression, prevent drug efflux and increase apoptosis.

3.5 CONCLUSION

Among the 10 multidrug resistant genes, up-regulation of MDR1 was one of the major causes of multidrug resistance in our R-HepG2 cells. Transient transfection of CDH1 cDNA plasmid pCMV6-XL4-CDH1 could increase the CDH1 transcription and E-cadherin expression in R-HepG2 cells. The transfection of CDH1 into R-HepG2 cells could also increase the doxorubicin intake, increase the apoptotic population of cells exposed to doxorubicin, suppress the cell migration, and decrease the P-glycoprotein expression.

Chapter 4

The alteration of transcriptional expression in multiple genes in R-HepG2 and R-HepG2-CDH1 cells

4.1 INTRODUCTION

In our previous study I observed the transcriptional differences of MDR related genes in HepG2 and R-HepG2. To study the differences of expression between these cells and CDH1 cDNA transfected R-HepG2-CDH1 cells, I selected 14 genes for further investigation. As reported by us and our colleagues using microarray to study the transcriptional differences in our HepG2 and R-HepG2 cell model, among all 9982 analyzed genes and expressed sequence tags (ESTs), a total of 70 genes/ESTs were up-regulated and 59 genes/ESTs were down-regulated in R-HepG2 cells comparing to its parental HepG2 cells (Wang *et al.*, 2009) (Table 4.1). Among these genes of significant transcription changes, 9 interesting targets were selected: ID1, NINJ1, JUP, FYN, CTGF, ABCB4, ASNA1, CDKN2A, and THBS1, which might be involved in multidrug resistance and might be related to CDH1 for further study. To further study the relationship between the absence of CDH1 and multidrug resistance, 5 CDH1 related genes (SNAI1, SNAI2, TWIST1, SIP1, Rassf2A) were also selected to study their expression in HepG2, R-HepG2 and R-HepG2-CDH1 cells. The known functions of these 14 selected genes are: Inhibitor of DNA Binding 1 (ID1), a gene contributing to cell growth, metastasis and angiogenesis (Gao *et al.*, 2008); Nerve Injury-induced Protein 1 (NINJ1), a gene encoding the ninjurin1 protein which is a cell surface protein which mediating cell communication and enhancing the entry, migration and activity of leukocytes (Lee *et al.*, 2010); Junction Plakoglobin (JUP), a component of the cadherin-catenin complex, is involved in signaling pathways and tumorigenesis through this complex's interaction with epidermal growth factor

receptor (EGFR) (El-Bahrawy and Pignatelli, 1998); FYN Tyrosine Kinase Proto-oncogene (FYN), one of the Src family kinases, has multiple functions including multidrug resistance and cell migration (Cary *et al.*, 1996); Connective Tissue Growth Factor (CTGF), a gene which is expressed after induction by growth factors or oncogenes such as transforming growth factor-β (TGF-β) (Yang *et al.*, 2009); ATP-Binding Cassette Subfamily B Member 4 (ABCB4), a cell membrane protein relates to multidrug resistance (Roninson *et al.*, 1986); Human Arsenite-Stimulated ATPase (ASNA1), the catalytic component of a multisubunit oxyanion pump that is responsible for resistance to arsenicals and antimonials (Bhattacharjee *et al.*, 2001); CDKN2A, as described in Chapter 2, is a regulator of CDK4 kinase and p53 which is responsible for cell cycle G1 phase arrest (Liggett and Sidransky, 1998); Thrombospondin I (THBS1), a gene related to anti-angiogenesis and adhesion (Staniszewska *et al.*, 2007); Human homolog of Drosophila Snail 1 (SNAI1), a gene of a zinc finger transcription factor which is known to control the EMT by repressing E-cadherin (Cano *et al.*, 2000); Human homolog of Drosophila Snail 2 (SNAI2 or SLUG), a gene similar to SNAI1 which also triggers EMT (Savagner *et al.*, 1997); Human homolog of Drosophila Twist 1 (TWIST1), a gene encodes a transcriptional regulator whose expression is related to invasiveness and loss of E-cadherin expression (Yang *et al.*, 2004); SMN-Interacting Protein 1 (SIP1), a gene encoding a two-handed E box binding zinc finger protein which could down-regulate E-cadherin and induces invasion (Comijin *et al.*, 2001) and Rassf2A, a tumor suppressor gene of the Ras association domain family proteins

which was found lost through promoter hypermethylation in various cancers (Vos *et al.*, 2003).

The aim of this part of study was to look for CDH1 responsive genes which may contribute to development of MDR in R-HepG2 cells and responsive to the re-introduction of CDH1 in CDH1 transfected R-HepG2 cells to help better understanding the complicated mechanisms of MDR.

Gene name	R-HepG2 to HepG2	Gene name	R-HepG2 to HepG2	Gene name	R-HepG2 to HepG2	Gene name	R-HepG2 to HepG2	Gene name	R-HepG2 to HepG2
HSPA6	4 6	MSN	2 17	CTAG2	6 26	SRI	3 46	ANXA3	2 61
HSPA8	3 36	ABCB4	8 26	MAGEA9	5 8	ACTR3	4 75	RAIP1	2 18
HSPA1A	3 31	ABCB1	7 81	MAGEA11	5 44	TAGLIN	9 68	G3BP	2 77
HSPCB	2 06	ABCB8	2 11	MAGEA6	5 03	TAGLIN2	2 74	VEGF	-3 47
ACTG2	7 17	ABCF1	2 48	MAGEA1	4 93	CCT5	2 19	CDKN2	-2 31
ACTB	2 65	GAGE2	14 38	MAGEA2	4 73	CGA	10 64	JUP	-3 05
ACTG1	3 68	GAGEB	11 64	MAGEA12	3 74	Sp140	7 67	CSRP1	4 35
SGK	4 57	HMG1	2 03	NCL	2 05	CYR61	3 7	CEBPB	-2 88
PIG8	2 02	ID1	3 09	ASNA1	3 33	PSMD11	2 02	ZNF173	-2 35
USP14	2 49	NINJ1	-2	SERPINB1	-3 4	TPM4	6 62	CDK2	2 02
ZYX	5 7	WDR1	5 15	DXS1357E	2 28	CAPN2	2 03	CTGF	6 76
EFNA1	-3 01	RGS2	-2 19	VEGFB	2 79	ADRM1	2 53	FYN	3 44
RDC1	-2 14	LGALS3	-2 53						

Table 4.1 Part of differently expressed genes in R-HepG2 detected by DNA micro-array analysis. (Wang *et al* , 2009)

4.2 MATERIALS AND METHODS

4.2.1 Cell lines and cell culture

Human HCC cell lines HepG2 and R-HepG2 were used in this part of study. R-HepG2-CMV was the R-HepG2 transfected with pcDNA3.1$^{(+)}$ plasmid; R-HepG2-CDH1was the R-HepG2 transfected with pCMV6-XL4-CDH1. The cell culture methodologies were described in Chapter 2.

4.2.2 Total RNA and protein extraction

Total RNA from cell lines was extracted by NucleoSpin® RNA II total RNA isolation kit. Protein lysates from cell lines were extracted and stored at -80℃. The methodologies were described in Chapter 3.

4.2.3 RT-PCR

RT-PCR was carried out on the selected genes by TaqMan® Reverse Transcription Reagents and Taq PCRx DNA Polymerase Reagents were as described in Chapter 3. The primers were shown in Table 4.2 below.

Primer set	Sense primer, 5'->3'	Antisense primer, 5'->3'
ID1	ACGAGCAGCAGGTAAACGTG	GAAGGTCCCTGATGTAGTCGAT
NINJ1	TCAAGTACGACCTTAACAACCCG	TGAAGATGTTGACTACCACGATG
JUP	ACGACTCGGGTATCCACTCG	GGTTTTCTTGAGCGTGTACTGG
FYN	GCAGAACAGCAAGGGGTTCAT	GGAGCAGGGAAACCATCATCA
CTGF	CAGCATGGACGTTCGTCTG	CCAACCACGGTTTGGTCCTT
ABCB4	GCAGAAGGCACACATCTATGG	ACCAGAATAACATCTCTGAAGCG
ASNA1	CAGCCTGAAGTGGATCTTCGT	CCTTGGTAGGCACCTTTGAGAA
CDKN2A	ATGGAGCCTTCGGCTGACT	GTAACTATTCGGTGCGTTGGG
THBS1	TGCCTGATGACAAGTTCCAAG	CCAGAGTGGTCTTTCCGCTC
SNAI1	GCCTTCAACTGCAAATACTGC	CTTCTTGACATCTGAGTGGGTC
SNAI2	AAGCATTTCAACGCCTCCAAA	AGGATCTCTGGTTGTGGTATGAC
TWIST1	GTCCGCAGTCTTACGAGGAG	GCTTGAGGGTCTGAATCTTGCT
SIP1	TCGGCTATTGCCGGTAGAG	GCGGGTTGGCATCCTGAAA
Rassf2A	AAGGGGTGGAGAGTGATATGAAGAG	AGGGACGTTTGGTGGCTGTAGT
CDH1	TGCCCAGAAAATGAAAAAGG	GGATGACACAGCGTGAGAGA
GAPDH	CAGCCGAGCCACATCG	TGAGGCTGTTGTCATACTTCTC

Table 4.2 Primers of selected genes for RT-PCR

4.2.4 SDS-PAGE & Western blotting

Western blotting was carried out on FYN (Cell Signaling, #4023) in HepG2, R-HepG2 and R-HepG2-CDH1 cells. The methodologies were as described in Chapter 3.

4.2.5 Plasmids extraction and transient transfection

Transfection of pCMV6-XL4-CDH1 into R-HepG2 cells and the pCMV6-XL4-CDH1 plasmid extraction were carried out by methods as described in Chapter 3.

4.3 RESULTS

4.3.1 Transcription of 14 genes in HepG2 R-HepG2 and R-HepG2-CDH1 cells

The genes investigated include 5 CDH1 and multidrug resistance related genes: SNAI1, SNAI2, TWIST1, SIP1, Rassf2A; and 9 genes of significance from our microarray results (Wang *et al.*, 2009), namely ID1, NINJ1, JUP, FYN, CTGF, ABCB4, ASNA1, CDKN2A, THBS1. The transcription level of these genes in the three cell lines were shown in Figure 4.1 and Figure 4.2, the transcription levels of SNAI2, ASNA1, TWIST1 and FYN were higher in R-HepG2 than those in HepG2 cells; the transcription of other selected genes had no obvious difference between HepG2 and R-HepG2 cells. The transcription levels of ASNA1, TWIST1 and FYN were obviously lower in R-HepG2-CDH1 than those in R-HepG2 cells; the transcription of other selected genes had no obvious difference between R-HepG2 and R-HepG2-CDH1 cells.

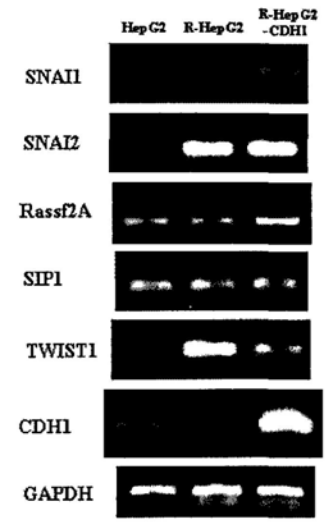


Figure 4.1 Agarose gel electrophoresis of RT-PCR results in 5 CDH1 related genes with GAPDH as control in HepG2, R-HepG2 and R-HepG2-CDH1 cells. The names of genes were shown on the left of the graphs. The RT-PCR results were compared with CDH1's RT-PCR results in the selected cell lines. Data are representative of three independent experiments.

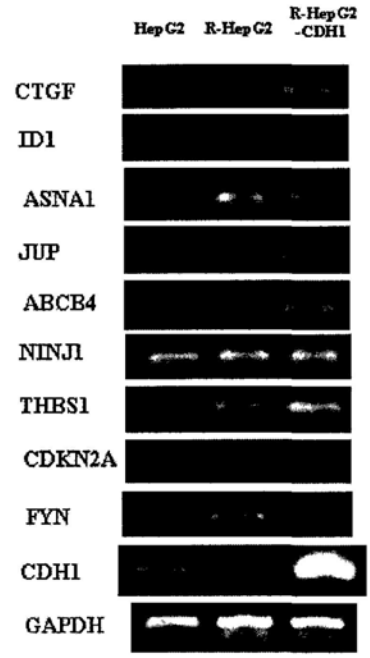


Figure 4.2 Agarose gel electrophoresis of RT-PCR results in 9 genes of significance from the micro-array data with GAPDH as control in HepG2, R-HepG2 and R-HepG2-CDH1 cells. The names of genes were shown on the left of the graphs. The RT-PCR results were compared with CDH1's RT-PCR results in the selected cell lines. Data are representative of three independent experiments.

4.3.2 Protein expression of FYN in HepG2, R-HepG2 and R-HepG2-CDH1 cells

To further study the relationship between CDH1 and FYN, Western blotting was carried out on FYN in HepG2, R-HepG2 and R-HepG2-CDH1 cells, with the hope of showing that expression of FYN was related to CDH1 expression. As shown in Figure 4.3, the expression of FYN in HepG2 cells was low but abundant FYN was expressed in R-HepG2 cells. With CDH1 cDNA transfection, the FYN expression was void in R-HepG2-CDH1. The protein expression result was in accordance with the transcription results shown in Figure 4.2.

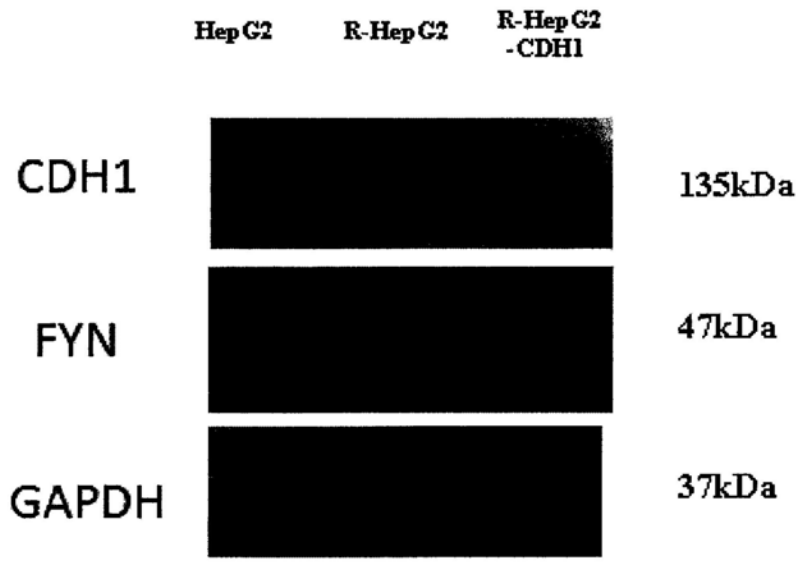


Figure 4.3 Western blotting results of CDH1, FYN, and GAPDH in HepG2, R-HepG2, R-HepG2-CDH1 cells. The names of the genes were shown on the left and the molecular weight on the right. Data are representative of three independent experiments.

4.4 DISCUSSION

Loss of E-cadherin was a common event in advanced tumors, unlike many other tumor suppressor genes which were lost through gene mutation/deletion. There were mainly two mechanisms for CDH1 namely promoter hypermethylation and E-cadherin suppression by its upstream transcriptional suppressors such as SNAI1, SNAI2 and TWIST1. Frequent hypermethylation of CDH1was found in many types of cancer including HCC (Kanai *et al.*, 1997). Aberrant over-expression of E-cadherin transcriptional repressors such as SNAI1 or SNAI2 could suppress CDH1 transcription through their binding to E-box motifs in the CDH1 promoter (Nieto, 2002). In our study, I found that E-cadherin was lost through CDH1 promoter hypermethylation in our doxorubicin-induced multidrug resistant R-HepG2 cells while the CDH1 gene of their parental doxorubicin sensitive HepG2 cells were hypomethylated and the E-cadherin expressed. The transfection of CDH1 gene into the R-HepG2 cells partially inhibited the MDR by suppression of MDR1 expression, suppression of doxorubicin efflux and sensitization of doxorubicin response.

In this chapter, among the 14 analyzed genes being examined, the transcription of SNAI2, TWIST1 and FYN were found to be much higher in R-HepG2 cells than those in HepG2 cells. While CDH1 was transfected into R-HepG2 cells, the transcription of SNAI2 was not suppressed but the transcriptions of TWIST1 and FYN were suppressed. An inverse correlation between Slug, the protein encoded by SNAI2, and CDH1 was related to EMT and reported to promote metastasis

(Savagner *et al.*, 1997). In our model, SNAI2 transcription was significantly higher than that in R-HepG2 cells than that in HepG2 cells. The transcription of SNAI2 was maintained in R-HepG2-CDH1 cells suggesting that this EMT inducer might have taken part in the genesis of MDR in the R-HepG2 cells.

It had also been well illustrated that expression of Twist resulted in loss of E-cadherin thus mediated cell-cell adhesion and contributed to metastasis by promoting EMT. The appearance of Twist served as a transcriptional suppressor of CDH1 (Yang *et al.*, 2004). However, in our cell model, I found that the transcription of TWIST1 gene could be suppressed by re-appearance of E-cadherin in CDH1 transfected R-HepG2 cells suggesting a negative regulatory role of CDH1 on TWIST1. Further study should be carried out to answer the relationship between the expression of E-cadherin upstream regulators such as SNAI2, TWIST1 and the promoter methylation of CDH1. In this chapter, I found that the transcription of TWIST1 was reversely correlated to CDH1 transcription which suggested the point of other researchers that TWIST1 was an upstream suppressor of CDH1. Furthermore, I found that in CDH1 cDNA transfection in R-HepG2 cells, the transcription of TWIST1 was decreased which suggested that the transcription level of CDH1 might also suppress the transcription of TWIST1. However for SNAI2, although its transcription level was very high in R-HepG2 cells while negative in HepG2 cells, the cDNA transfection of CDH1 seemed to have no effect on its transcription in R-HepG2 cells suggesting that it might serve as an upstream

suppressor of CDH1 but unlike TWIST1, its transcription could not be suppressed by CDH1 cDNA transfection.

Fyn, encoded by gene FYN, is a member of the Src family proteins –a family of proto-oncogenic tyrosine kinases. Src family kinases (SFK) interact with lots of membrane, cytosolic and nuclear proteins through tyrosine phosphorylation of those proteins, transmit signals in multiple physiological homeostatic pathways. Increased activity of SFKs is a frequent event in many cancers which promotes cell survival, mitogenesis and metastasis (Guarino, 2010). The biological functions of Fyn are diverse. It has important roles in mitogenic signaling and regulation of cell cycle entry, growth and proliferation, intergrin-mediated interactions as well as cell-cell adhesion. FYN is over-expressed in many cancers including glioblastoma, head & neck squamous cell carcinoma, prostate cancer and melanoma (Saito *et al.*, 2010). Fyn over-expression activates the anti-apoptotic activity of Akt thus promoting cell growth and suppressing apoptosis. Fyn has been reported to play a role in EMT through the phosphorylation of its substrate FAK in E-cadherin knockdown HEK293 cells, resulting in the stabilization of β1-integrin-mediated focal adhesions and increases of cell spreading and migration (Lehembre *et al.*, 2008). Transient transfection of FYN into Fyn negative RPW1 cells (pancreatic carcinoma) could suppress the expression of α-catenin but the expression of E-cadherin was unaffected (Piedra *et al.*, 2003). In our study, I found that FYN was neither transcribed nor translated in HepG2 cells but expressed in R-HepG2 cells. Upon CDH1 transient

transfection, FYN expression was suppressed. In studies of this chapter, I found that both transcription and protein expression of FYN was void in HepG2 but high in R-HepG2 cells, and with CDH1 cDNA transfection, the transcription and protein expression of FYN was significantly suppressed. Together with other reports, it would be possible that loss of E-cadherin function as an upstream transcriptional suppressor of FYN is one cause of the suppressed drug sensitivity in multidrug resistant cancer cells. The onset of MDR might be an outcome of regulation change in multiple genes of multiple signal pathways, including both proto-oncogenes and tumor suppressor genes.

ASNA1, an ATPase targeting tail-anchored protein, was reported to be responsible for the drug resistance of cisplatin and arsenite in melanoma cells and ovarian cancer cells as well as promotion of growth of these cells. Down-regulation of ASNA1 displays a significant increase in apoptosis and increase in sensitivity to these drugs (Hemmingsson *et al.*, 2009; Hemmingsson *et al.*, 2009). In this part of our study, I found that the transcription of ASNA1 was similar to the transcriptional profile of TWIST1 and FYN, with low expression in HepG2 and R-HepG2-CDH1 cells while high expression in R-HepG2 cells. It seems that in our cell model, the onset of drug resistance might be an outcome of regulatory change in multiple genes of multiple signal pathways, including both proto-oncogenes and tumor suppressor genes.

In our study, I found that genes MDR1, SNAI2, TWIST1, FYN, ASNA1 which were related to multidrug resistance and EMT were silenced in the drug sensitive and E-cadherin positive HepG2 cells but were activated with the epigenetically loss of CDH1 in R-HepG2 cells. In MDR1, TWIST1, FYN and ASNA1, up-regulation in transcription could be reverted by CDH1 cDNA transfection into R-HepG2 cells. This suggested that there might be multiple genes responsible for the origin of doxorubicin resistance in HepG2 cells in our resistant cell model. As a core component of the EMT progress, epigenetic loss of CDH1 might have acted as an essential step in both EMT and drug resistance besides its suppression effect on the P-glycoprotein. There are increasing amount of reports relate drug resistance of tyrosine kinase inhibitors to EMT. For example, epithelial but not mesenchymal gene had been associated with sensitivity to erlotinib, a tyrosine kinase inhibitor targeting on the epidermal growth factor receptor (EGFR), mediated growth inhibition in NSCLC (Yauch *et al.*, 2005). Further clinical trials confirmed this that NSCLC patients with negative E-cadherin expression had worsened overall situation than the NSCLC patients with high E-cadherin expression after erlotinib treatment (Thomson *et al.*, 2005). Multidrug resistance of cancers has become a major threat to successful cancer treatment; more evidences show that genes and their involved pathways related to the EMT may also have potential roles in drug resistance, cancer invasion and metastasis. The study on EMT has emerged as one of the hot spots in biomedical research. A better understanding of the mechanisms behind the process may give a better explanation of the cancer progression, metastasis, drug resistance and

treatment evaluations. Hence EMT and its markers have great potential in drug sensitivity prediction, treatment selection and provision of new targets for drug developing. Besides the classical combined chemotherapy, substances targeting the EMT-related mechanisms to re-sensitize the MDR cells or specifically demethylated those epigenetically silenced epithelial markers such as CDH1 should be studied as a new mechanism on chemo-resistant cancer therapy.

4.5 CONCLUSION

To further explore the mechanisms related to multidrug resistance in our doxorubicin-induced multidrug resistant R-HepG2 cell model, I found up-regulation of SNAI2, TWIST1, ASNA1 and FYN, and down-regulation of CDH1 in R-HepG2 cells comparing to those in its parental drug sensitive HepG2 cells. Transfection of CDH1 cDNA could suppress the expression of the transcription of TWIST1, ASNA1 and FYN in R-HepG2-CDH1 cells. The epigenetic loss of CDH1 might be an essential event of cells transforming in cancer progression related to poor malignancy and multidrug resistance.

Chapter 5

Searching for drug candidates targeting the promoter methylation modulation of cancer related genes in breast cancer cell lines

5.1 INTRODUCTION

Most of the anti-cancer agents target at the fast dividing cells by inhibiting the metabolic pathways crucial to cell division which are not specific to the cancer cells thus the fast dividing normal cells would also suffer from the cytotoxicities of anti-cancer agents. In recent years, more and more reports focused on the role of epigenetic mechanisms in cancer formation and progression. Our previous works in this research also showed that promoter methylation of genes showed opposed patterns between drug sensitive parental HepG2 cells and multidrug resistant sub-linage R-HepG2 cells which imply that the promoter methylation mechanism might contribute to the formation of multidrug resistant sub-population of cancer cells during chemotherapy. Based on these new understandings of epigenetics, new therapeutic strategy was considered targeting on the molecules of the epigenetic mechanisms. Anti-cancer agents targeting the epigenetic mechanisms were discovered and applied in cancer therapy such as the famous DNMT inhibitors 5-aza-cytidine (Vidaza) and 5-aza-2'-deoxycytidine (Dacogen). These nucleoside analogues could bind to the DNMT enzyme and rapidly deplete its levels in the cell, leading to complete reversal of CpG hypermethylation (Hurtubies and Mompaler, 2004). Researchers showed that CDH1 methylation was a common event in cancers (Yoshiura *et al.*, 1995). Restoring E-cadherin by Dacogen in CDH1 hypermethylated breast cancer cells could reduce metastasis (Nam *et al.*, 2004). These DNMT inhibitors and other drugs targeting the histone acetylation mechanisms of epigenetics proved their value in cancer therapy. Since our group had been working

on discovering new anti-cancer drugs from traditional Chinese medicine (TCM), in this chapter, I tried a simple screening method using the MSP technology targeting the promoter methylation of tumor related genes and tested 3 anti-cancer agents of our interest to see whether they had any effect on modulation of promoter methylation.

Several anti-cancer agents were tested in this part. In the previous study of our group, the significant anti-tumor effects of Pheophorbide A (Pa, Figure 5.1), the active anti-cancer compound which was purified from a traditional Chinese anti-cancer herb *Scutellaria barbata*, have been demonstrated on human HCC cell lines including HepG2 and R-HepG2 cells. Pa inhibited the growth of HepG2 and R-HepG2 through G_2/M phase cell cycle arrest and apoptosis induction, and the expression of a number of genes were found to be regulated during the Pa-treatment (Tang *et al.*, 2007). It was demonstrated that Pa has photo-cytotoxic anti-proliferative effect in both HCC and breast cancer cell lines in previous studies of our group. With 610nm red light exposure on the Pa-treated cells, cytosolic reactive oxygen species (ROS) would be generated to kill various cancer cells (Tang *et al.*, 2009). Another compound Polyphyllin D (PD, Figure 5.2) was found to be an active anti-cancer component of a traditional anti-cancer Chinese herb *Paris polyphylla*. It was chemically synthesized by our collaborators and designated as HK-18. This compound had saponin backbone and showed high cytotoxicity to cancer cell lines through triggering the mitochondrial apoptotic pathway by mitochondrial

fragmentation (Lee *et al.*, 2005; Ong *et al.*, 2008). A compound with same backbone but different side chains of HK-18, which was designated as HK-27 (Figure 5.3), was also synthesized by our collaborators and this compound showed significant cytotoxicity on cancer cell lines as well (Lee *et al.*, 2009). I tested these 3 agents for any effect on the promoter methylation of selected genes.

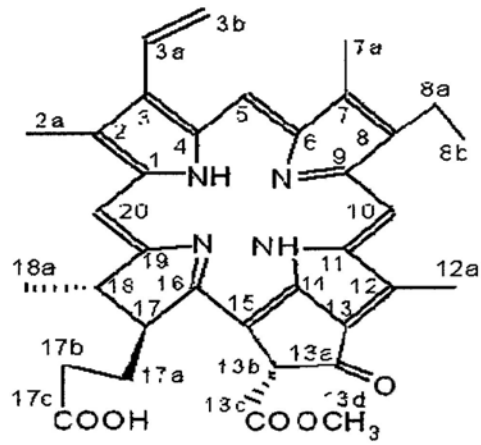


Figure 5.1 The chemical structure of Pheophorbide A

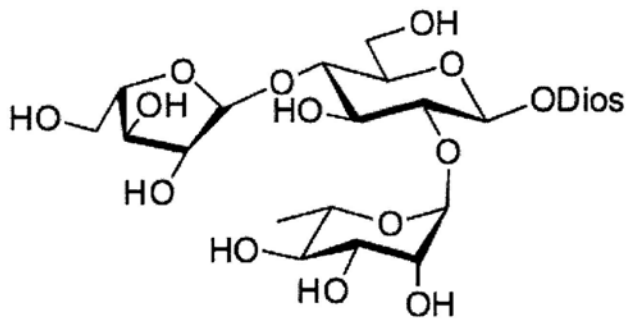


Figure 5.2 The chemical structure of HK-18 (Lee *et al.*, 2009)

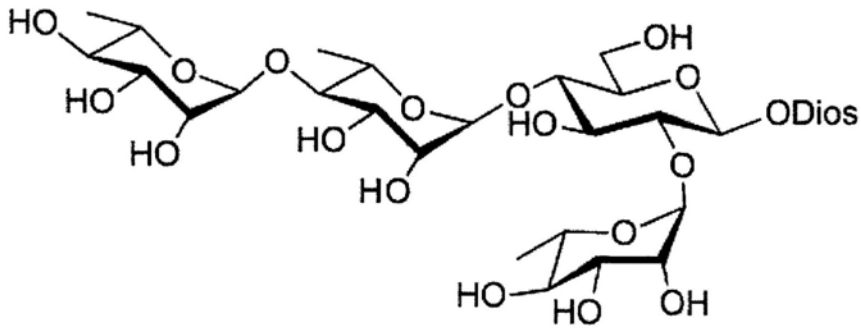


Figure 5.3 The chemical structure of HK-27 (Lee *et al.*, 2009)

Based on the study of chapter 2, five genes were selected in this part of pilot study including: CDH1, ESR1, Rassf1A, Rassf2A, and hMLH1. These genes were tumor suppressor genes reported to be silenced through promoter hypermethylated in breast cancer (Berx and van Roy, 2001; Widschwendter *et al.*, 2004; Peters *et al.*, 2007; Hesson *et al.*, 2005; Viswanathan *et al.*, 2006). Cells were first treated with different doses of the selected anti-cancer agents and then analyzed for their promoters' methylation by MSP. For Pa, cells were treated with and without photo-sensitization in two groups for analysis. Pa with photo-sensitization is considered to be a photodynamic therapy (PDT) of Pa on various tumor cells (abbreviated as Pa-PDT) (Tang *et al.*, 2006; Tang *et al.*, 2007; Tang *et al.*, 2009).

5.2 Materials and Methods

5.2.1 Cell lines & drug treatments

Human breast tumor cell lines MCF-7 and MDA-MB-231 were used in this part of study. The cell culture methodologies were described in Chapter 2. IC_{50}s were determined by 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (MTT assay) by methods as described in previous work of our group and were shown in Table 5.1.

$3x10^5$ cells of each cell line were seeded into 90 mm petri dish and incubated with RPMI-1640 full medium at 37℃ in humidified incubator supplying 5% CO_2 overnight. Three doses (C_1, C_2, C_3 in Table 5.1) of each drug were applied to the medium respectively at a final concentration of $0.5xIC_{50}$, $0.75xIC_{50}$, IC_{50} of each drug and a solvent control group was also included. The treatment of Pa was divided into two groups including a non-photo-sensitization group (Pa group) and a photo sensitization group (Pa-PDT group) according to different IC_{50}s. The Pa photosensitized cell groups were subjected to Pa-PDT treatment as described in Figure 5.4. The cells were incubated at 37℃ for 3 hours after drug loading. The cells were then photo-illuminated for 20 minutes using a 600W quartz-halogen lamp with infrared radiation attenuated by a 10 cm layer of water and a color filter cut-on 610nm. The light intensity was $70mW/cm^2$, 20 minutes of irradiation = $84J/cm^2$ (Tang *et al.*, 2006). The cells were then incubated for 48 hours before harvest. The media and drugs of other groups were changed every 24 hours and the cells were

incubated for 72 hours before harvest. All petri dishes containing cells and drugs were sheltered from light by aluminum foil during incubation.

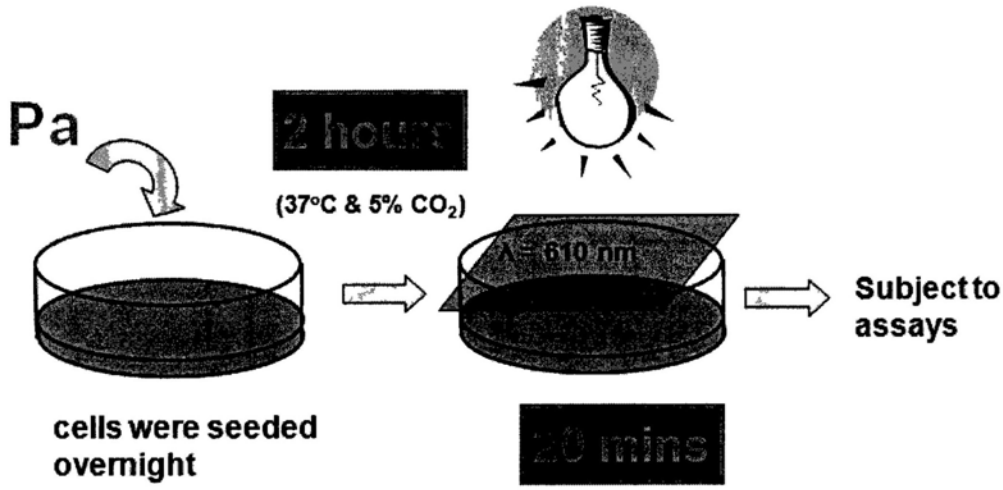


Figure 5.4 The protocol of Pa-PDT treatment

Cell line	MCF-7			
Drug / Concentration(μM)	Pa	Pa-PDT	HK-18	HK-27
C_1 (=0.5×IC_{50})	2.5	-	1.5	0.525
C_2 (=0.75×IC_{50})	3.75	-	2.25	0.788
C_3 (=IC_{50})	5.0	0.51	3.01	1.05
Cell line	MDA-MB-231			
Drug / Concentration(μM)	Pa	Pa-PDT	HK-18	HK-27
C_1 (=0.5×IC_{50})	3.125	-	0.6	0.405
C_2 (=0.75×IC_{50})	4.688	-	0.9	0.608
C_3 (=IC_{50})	6.25	0.5	1.19	0.81

Table 5.1 Final concentration of anticancer drugs used in the drug treatment of MCF-7 and MDA-MB-231 cells. C_1, C_2 and C_3 represented the different final concentrations of drug treatments for 48 hours which equals to 0.5×IC_{50}, 0.75×IC_{50}, and 1×IC_{50} of different drugs respectively.

5.2.2 Preparation of genomic DNA and MSP of selected genes

Genomic DNA of cells was harvested after the drug treatment. All cells were sheltered from light by aluminum foil during DNA preparation. The methodology of DNA extraction and MSP of CDH1, ESR1, Rassf1A, Rassf2A, and hMLH1 were described in Chapter 2.

5.3 RESULTS

5.3.1 Promoter methylation of 5 genes in MCF-7 and MDA-MB-231 cells with Pa treatment

MSP was adapted to examine the promoter region DNA methylation of 5 selected tumor suppressor genes (CDH1, ESR1, Rassf1A, Rassf2A, and hMLH1) in MCF-7 and MDA-MB-231 cell lines subjected to 3 different concentrations of Pa treatment for 72 hours. The results were shown in Figure 5.5, in both cell lines, promoters of Rassf1A and Rassf2A were hyper-methylated. hMLH1 was hypo-methylated. The promoter methylation statuses of ESR1 and CDH1 were different in these two cell lines, with ESR1 and CDH1 semi-methylated in MDA-MB-231 and hypo-methylated in MCF-7. After the Pa treatment, there was no change in promoter methylation of ESR1, Rassf1A, Rassf2A and hMLH1 in the two cell lines. However, the methylation in CDH1 gene decreased in Pa treated MDA-MB-231 cells at C2 and C3 concentrations. In MCF-7 cells with drug treatment, the CDH1 promoter maintained at hypo-methylated status.

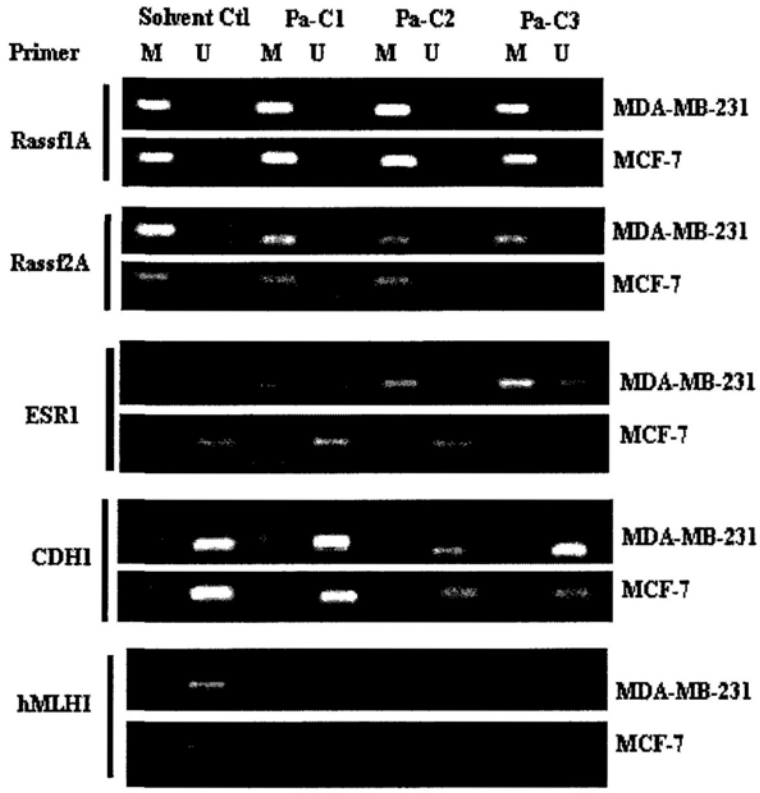


Figure 5.5 Effect of Pa on promoter methylation of Rassf1A, Rassf2A, ESR1, CDH1, and hMLH1 genes in MDA-MB-231 and MCF-7 cells. MSP analyses of Rassf1A, Rassf2A, ESR1, CDH1, and hMLH1 were performed upon Pa treatment at 3 concentrations as indicated in Table 5.1 for 72 hours.

5.3.2 Promoter methylation of 5 genes in MCF-7 and MDA-MB-231 cells with Pa-PDT treatment

MSP was adapted to examine the promoter region DNA methylation of 5 selected tumor suppressor genes (CDH1, ESR1, Rassf1A, Rassf2A, and hMLH1) in MCF-7 and MDA-MB-231 cell lines subjected to Pa-PDT treatment and further incubation for 48 hours. The results were shown in Figure 5.6, after the Pa-PDT treatment, there was no change in promoter methylation of all 5 genes in the two cell lines.

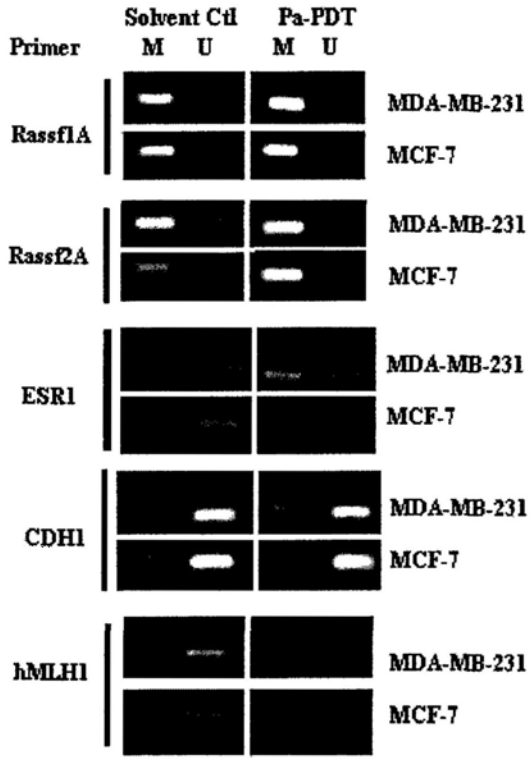


Figure 5.6 Effect of Pa-PDT on promoter methylation of Rassf1A, Rassf2A, ESR1, CDH1, and hMLH1 genes in MDA-MB-231 and MCF-7 cells. MSP analyses of Rassf1A, Rassf2A, ESR1, CDH1, and hMLH1 were performed upon Pa-PDT treatment at the concentrations indicated in Table 5.1 for 48 hours.

5.3.3 Promoter methylation of 5 genes in MCF-7 and MDA-MB-231 cells with HK-18 treatment

MSP was adapted to examine the promoter region DNA methylation of 5 selected tumor suppressor genes (CDH1, ESR1, Rassf1A, Rassf2A, and hMLH1) in MCF-7 and MDA-MB-231 cell lines subjected to 3 different concentrations of HK-18 treatment for 72 hours. The results were shown in Figure 5.7, after the HK-18 treatment, there was no change in promoter methylation of ESR1, Rassf1A and Rassf2A in the two cell lines. However, the methylation in CDH1 gene decreased in HK-18 treated MDA-MB-231 cells. In MCF-7 cells with drug treatment, the CDH1 promoter maintained at hypo-methylated status. The unmethylation specific amplificons of hMLH1 gene in MCF-7 at C1 and C2 were decreased comparing to the solvent control group, but the methylation specific amplificons were negative in all 3 concentrations of HK-18.

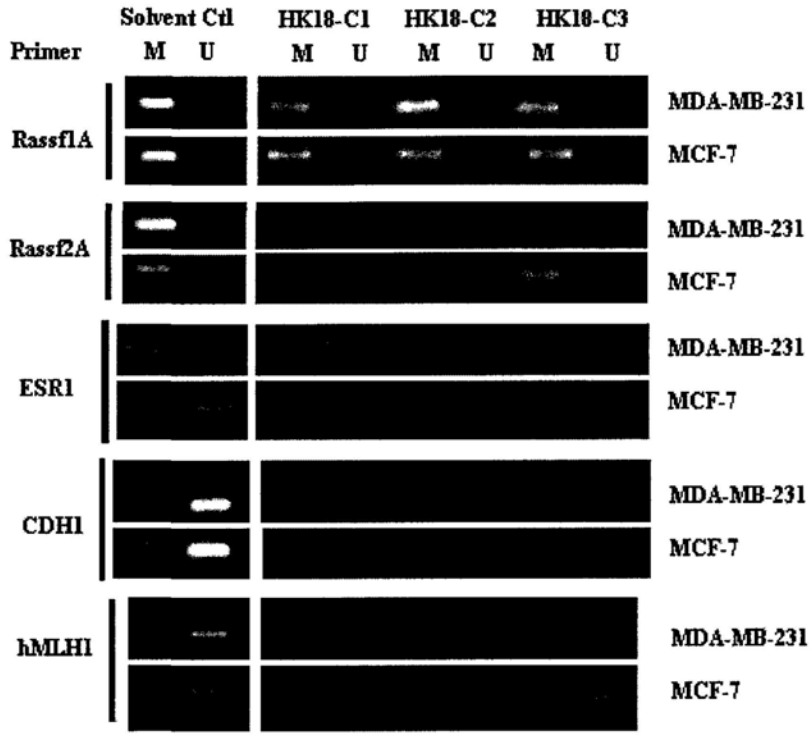


Figure 5.7 Effect of HK-18 on promoter methylation of Rassf1A, Rassf2A, ESR1, CDH1, and hMLH1 genes in MDA-MB-231 and MCF-7 cells. MSP analyses of Rassf1A, Rassf2A, ESR1, CDH1, and hMLH1 were performed upon HK-18 treatment at 3 concentrations indicated in Table 5.1 for 72 hours.

5.3.4 Promoter methylation of 5 genes in MCF-7 and MDA-MB-231 cells with HK-27 treatment

MSP was adapted to examine the promoter region DNA methylation of 5 selected tumor suppressor genes (CDH1, ESR1, Rassf1A, Rassf2A, and hMLH1) in MCF-7 and MDA-MB-231 cell lines subjected to 3 different concentrations of HK-27 treatment for 72 hours. The results were shown in Figure 5.8, after the HK-27 treatment, there was no change in promoter methylation of ESR1, Rassf1A, Rassf2A and hMLH1 in the two cell lines. However, the methylation in CDH1 gene decreased in HK-27 treated MDA-MB-231 cells. In MCF-7 cells with drug treatment, the CDH1 promoter maintained at hypo-methylated status.

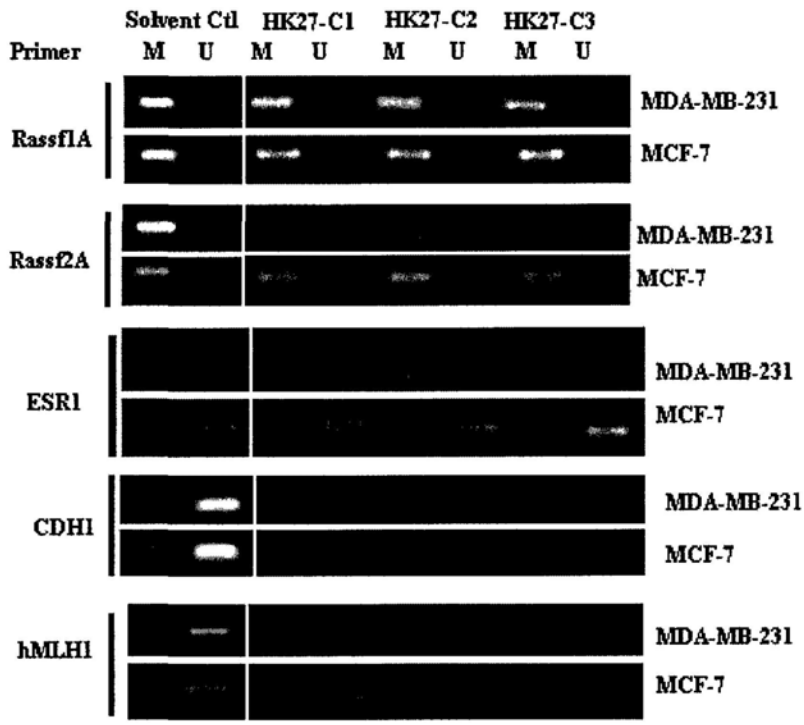


Figure 5.8 Effect of HK-27 on promoter methylation of Rassf1A, Rassf2A, ESR1, CDH1, and hMLH1 genes in MDA-MB-231 and MCF-7 cells. MSP analyses of Rassf1A, Rassf2A, ESR1, CDH1, and hMLH1 were performed upon HK-27 treatment at 3 concentrations indicated in Table 5.1 for 72 hours.

5.4 DISCUSSION

It was commonly accepted that heritable information is transmitted to offspring through sequences of DNA. The change of DNA sequence occurred randomly during transmission was not influenced by environmental factors except for mutagens (Franklin and Mansuy, 2009). However, the researches on epigenome revealed that inheritance of DNA sequence was not the only carrier of heritable information. The information of epigenome, unlike DNA sequences, could be modulated by environmental factors such as nutrition, aging and chemicals, and this information could be passed down through generations together with the information of DNA sequences. From our results in this part of study, the promoter methylation of CDH1 was found to be decreased in response to multiple drug treatments in MDA-MB-231 cells and this result suggests that promoter demethylation of CDH1 might be an active event in the cells with CDH1 promoter hypermethylation in response to drug treatments. I also observed that in MDA-MB-231 cells treated with Pa, there was demethylation of CDH1 in lower Pa concentration (1/2 & 3/4 of IC_{50}) treatments but no such effect in the cells treated with the concentration of Pa's IC_{50}. This result suggests that the change of CDH1 promoter methylation might have some kinds of preference or selection of the surrounding environment. I also found that the ESR1 promoter DNA methylation was higher in the estrogen receptor alpha silenced MDA-MB-231 cell line than the estrogen receptor alpha expressed MCF-7 cell line which suggested that the promoter hypermethylation of ESR1 in MDA-MB-231 might be one of the major causes of the loss of estrogen receptor alpha expression.

However, further study is needed to elucidate the impact of this change in methylation on CDH1 expression, the cause of these changes and the mechanisms behind this phenomenon in response to drug treatments.

As Pa is a photosensitizer, Pa-PDT treatment was extensively studied in previous works of our group (Tang *et al.*, 2006; Tang *et al.*, 2009). In this part of study, I tested any potential of Pa-PDT on methylation based on the Pa-PDT settings on cell lines of our previous works. I found no change in promoter methylation in all 5 genes analyzed. This might be due to the nature of photosensitizers and their cytotoxic mechanisms which contributed little on the promoter methylation mechanisms. After light excitation, the toxicity due to large amount of photon-mediated generation of ROS could induce cell death in a short time on lots of intracellular targets. The time of light exposure was optimized to 20 minutes by our previous studies but this time might be too short on an epigenetic basis because the change in DNA methylation –if there were any, needed at least one – two times of cell division to make the epigenetic change stable and be able to pass down through generations. These cells undergo PDT induced cell death pathways might not have a chance to gain methylation modification before they die. Moreover, the pathway of cell death in individual cell under PDT might be diverse, so the change in promoter methylation as one part of the epigenetic mechanism might do little in PDT.

The epigenetic modifiers such as Vidaza and Dacogen were challenged by their

side effects. For instance, Dacogen was known targeting at the DNMTs which also meant that the demethylation effect of it was global and non-gene-specific. In other words, hypo-methylation could activate epigenetically silenced tumor suppressor genes as well as oncogenes (Smith *et al.*, 2009). Furthermore, DNA methylation plays an essential role in maintaining T-cell function. Loss of DNMT during mitosis might induce T-cells to change their pathologic significance thus cause a lupus-like autoimmune disease (Richardson, 2003). CpG methylation was known to be more reactive to environmental factors than the sequential change of DNA molecule (Shen *et al.*, 2002). It was reported that arsenic exposure could induce malignant transformation in prostatic epithelial cells (Benbrahim-Tallaa *et al.*, 2005) and mice liver (Chen *et al.*, 2004) through global and individual gene hypo-methylation. Consequently, to look for a more "gene and methylation" specific agent that would specifically alter the methylation of a specific gene or a specific group of genes would be a better strategy of treatment. To setup a screening method for testing drug candidates of the epigenetic mechanisms, whole genome methylation analysis techniques should be included such as the methylation sensitive microarray. This would help us finding CpG island clusters which might be modulated by drugs thus precisely predict the target genes of the drugs through epigenetic mechanisms. *In vivo* study on tumor-bearing mouse model would not be excluded as well because that would better mimic the environmental change that the cancer cells experienced during anti-cancer therapy. The change of methylation in promoters could be stabilized after at least one cell cycle in single cell, thus low-level drug with longer

exposure time in both *in vitro* and *in vivo* study would be considered to induce any possible change in promoter methylation which would provide longer time for methylation change to take place.

5.5 CONCLUSION

Our preliminary study on drug candidates (Pa, Pa-PDT, HK-18, HK-27) on breast cancer cell lines MCF-7 and MDA-MB-231 targeting the promoter methylation mechanism showed that of all 5 cancer related genes (CDH1, ESR1, Rassf1A, Rassf2A, and hMLH1) analyzed, only the promoter methylation of CDH1 was found to be decreased in response to multiple drug treatments (Pa, HK-18, HK-27) in MDA-MB-231 cells. Further studies should be carried out to better elucidate the mechanism of metaphor.

Chapter 6
General Discussion

The origin and progression of cancer was a process involving multiple genes and multiple steps. The activation of oncogenes and inactivation of tumor suppressor genes were recognized as essential basis of tumor development. Many researchers reported that tumor suppressor genes silenced by promoter hypermethylation had been found in different types of cancer (Jiang *et al.*, 2009; Dammann *et al.*, 2005; Hall *et al.*, 2008; Hasegawa *et al.*, 2002; Herman and Baylin, 2003). Recent researches proved that epigenetic mechanisms were essential in cancer development but the research on the involvement of epigenetic mechanisms in the development of MDR during cancer chemotherapy was limited. The involvement of promoter DNA methylation in MDR development was investigated in the present pilot study.

Researches on DNA methylation change in MDR related genes in cancer showed that the methylation profiles of these genes were altered in cancer which indicated that change in DNA methylation in related genes might be one of the causal factors of MDR (Ding *et al.*, 2004). In the present study, I analyzed the promoter CpG methylation of 8 genes between doxorubicin sensitive HepG2 and multidrug resistant R-HepG2 cells by MSP and found 4 of them showed promoter hypermethylation in the R-HepG2 cell line. This result suggests that under the stress of chemotherapeutics epigenetic modifications might take place in multiple genes in cancer cells.

CDH1 and its protein product E-cadherin are important factors in cancer

metastasis and EMT (Martin e al., 2010). In this study, it was observed that the transcription of CDH1 and expression of E-cadherin were down-regulated in R-HepG2 cells. When CDH1 cDNA was introduced into R-HepG2 cells by transient transfection, the expression of E-cadherin increased. With the increased expression of E-cadherin, the R-HepG2-CDH1 cells showed less resistant properties by increased doxorubicin uptake, increased apoptotic cell population exposed to doxorubicin, suppressed cell migration and decreased P-glycoprotein expression.

It was also observed in this study that the transcription of MDR related genes MDR1, MRP1 and MRP5 had increased in R-HepG2 cells comparing to those in parental HepG2 cells. The transcription of SNAI2, TWIST1, ASNA1 and FYN were higher in R-HepG2 cells than those in HepG2 cells. CDH1 cDNA transfection suppressed the transcription of TWIST1, ASNA1 and FYN. The suppression of these genes which were related to metastasis (Lehembre *et al.*, 2008), drug sensitivity (Grosso *et al.*, 2009), CDH1 suppression (Yang *et al.*, 2004) and resistance to apoptosis (Hemmingsson *et al.*, 2009) suggested that the expression of CDH1 might be a dominating suppressor of those EMT and cancer multidrug resistance mechanisms involved genes. Our results showed that loss of E-cadherin might be related to the mechanisms provoking the MDR phenotype. Hence, up-regulation of CDH1 in drug resistant cancer cells might be a valid therapeutic strategy for sensitizing MDR cancer cells to chemotherapeutics.

MSP analysis of promoter methylation is a sensitive, selective and fast method in DNA methylation study. It was widely applied in researches in which CpG island methylation was involved. But the examined CpG sites were limited by this method and there were methylation differences between sub-populations of cells. Despite of time consuming, bisulfite sequencing is still the gold standard in DNA methylation studies.

Some interesting phenomena were observed that further studies are needed to provide a better explaination. I observed that the promoter of Rassf2A was hypomethylated in HepG2 cells and hypermethylated in R-HepG2 cells but this difference in methylation did not influence the transcription of Rassf2A because the RT-PCR result showed that Rassf2A was transcribed in both cell lines without significant difference. Since the loss of pro-apoptotic mediator Rassf2A through promoter methylation had been reported in gastric, breast, lung, oral and colorectal cancers (Maruyama *et al.*, 2008; Cooper *et al.*, 2008; Imai *et al.*, 2007; Park *et al.*, 2006) and known as a gene whose methylation was "always tumor-specific" (Hesson *et al.*, 2005), no report noticed the transcription of Rassf2A in Rassf2A promoter hypermethylated cells. To explain this, bisulfite sequencing should be carried out to show the whole promoter region DNA methylation of the cells, RT-PCR result should be confirmed by Western blotting. The hypermethylation of Rassf1A was one of the most common events in human cancers (van der Weyden, 2007), the loss of its promoter hypermethylation was not reported in multidrug resistant cancer cells.

Since our findings related promoter methylation of CDH1 with the MDR phenotype, the potential of CDH1 promoter methylation in the classification of tumors could not be neglected. Such classification might be useful in prediction of tumor prognosis and potential response to therapy. The classification could also be applied in deciding the selection of drugs on different individuals of different cancer types. Work on clinical cancer samples with patient backgrounds of chemo-treatment failure would provide sound basis for this part of research. The potential of CDH1 promoter methylation in early diagnosis of cancer might hopefully be answered by our future research on clinical materials.

Collectively, the promoter methylation of CDH1 is an important molecular event in the development of drug resistance in cell culture. This event regulates the expression of other genes in order to decrease the drug sensitivity in chemotherapy. Application of agents which would modulate CDH1 promoter methylation to increase the expression of E-cadherin or agents which would inhibit the expression of genes such as MDR1, FYN, TWIST1 and ASNA1 would provide a novel therapeutic strategy in cancer chemotherapy.

Chapter 7
Conclusion

In present study, I have performed various experiments and conclusions are drawn as follows:

1. MSP technology was applied to investigate the promoter DNA methylation status of 8 tumor related genes in human HCC cell line HepG2 and its doxorubicin-induced multidrug resistant derivative R-HepG2 cell line. I observed that the promoter methylation was higher in ESR1, Rassf2A, CDH1, and MDR1 in R-HepG2 cells than HepG2 cells. The promoter methylation showed no obvious difference in CDKN2A, hMLH1, and hMSH2. The methylation status of CDH1 in the two cell lines analyzed by MSRE-PCR was the same with our results by MSP.

2. Bisulfite sequencing was performed to show the methylation status of total 32 CpG in the CDH1 promoter region which also confirmed the MSP result. The results were the first report that the CDH1 promoter region of the R-HepG2 cell line was hypermethylated.

3. CDH1 transcription and E-cadherin expression were maintained in CDH1 promoter hypomethylated HepG2 cells but both were lost in CDH1 promoter hypermethylated R-HepG2 cells. The loss of CDH1 in R-HepG2 cells was caused by promoter hypermethylation.

4. RT-PCR of 10 multidrug resistant related genes revealed that

transcription of MDR1 was obviously increased in R-HepG2 cells, transcription of MRP1 and MRP5 were slightly increased in R-HepG2 cells, transcription of MRP6 and BCRP were slightly decreased in R-HepG2 cells, comparing to those in parental HepG2 cells.

5. Up-regulation of P-glycoprotein expression was one of the major causes of multidrug resistance, including doxorubicin in our R-HepG2 cells. Transient transfection of CDH1 cDNA plasmid pCMV6-XL4-CDH1 could increase the CDH1 transcription and E-cadherin expression in R-HepG2 cells. The CDH1 transfected R-HepG2-CDH1 cells showed increased amount of doxorubicin uptake, increased apoptotic population of cells exposed to doxorubicin, suppressed cell migration, and decreased P-glycoprotein expression comparing to the resistant R-HepG2 cells.

6. Increase of SNAI2, TWIST1, ASNA1, FYN transcription, and decrease of CDH1 transcription were found in R-HepG2 cells comparing to those in its parental HepG2 cells. Transfection of CDH1 cDNA could suppress the transcription of TWIST1, ASNA1 and FYN in R-HepG2-CDH1 cells.

7. Our preliminary study on potential drug candidates Pa, Pa-PDT, HK-18, and HK-27 on breast cancer cell lines MCF-7 and MDA-MB-231 targeting the promoter methylation mechanism showed that of all 5 cancer related genes

CDH1, ESR1, Rassf1A, Rassf2A, and hMLH1 analyzed, only the promoter methylation of CDH1 was found decreased in response to treatments Pa, HK-18, and HK-27 in MDA-MB-231 cells respectively.

8. Collectively, our results suggested that the epigenetic loss of CDH1 might be an essential event in cancer progression and multidrug resistance. Our future perspectives will focus on restoration of E-cadherin as a novel strategy to reverse multidrug resistance in cancer especially against the loss of CDH1 by the epigenetic mechanism.

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