# Functional Epigenetics Identifies Protein Phosphatase-1 Regulatory Subunit Genes as Candidate Tumor Suppressors Frequently Silenced by Promoter CpG Methylation in Multiple Tumors

LEUNG, Ching Hei

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The Bible says: *"And though the Lord give you the bread of adversity, and the water of affliction, yet shall not thy teachers be removed into a corner any more, but thine eyes shall see thy teachers: And though the Lord will give you the bread of trouble and the water of grief, you will no longer put your teacher on one side, but you will see your teacher: And thine ears shall hear a word behind thee, saying, This is the way, walk ye in it, when ye turn to the right hand, and when ye turn to the left. And at your back, when you are turning to the right hand or to the left, a voice will be sounding in your ears, saying, this is the way in which you are to go."*

*Isaiah 30:20-21*

For He is faithful, walking with me no matter what circumstance, guiding me both in spiritual and personal growth, showering me with his greatest mercy and endless love. Thank you, Lord. To God be the glory.

# 摘要

## 以功能腫瘤擬遺傳學方法鑒定蛋白磷酸酶 1 調控亞基爲候選抑癌基因，由啓動子 CpG 過度甲基化致在多種腫瘤中表達沉默

蛋白磷酸酶 1 調控亞基屬於小份子蛋白家族主要控制蛋白質磷酸酶 1 的底物特異性和亞細胞定位。蛋白質磷酸酶 1 調控亞基的表達下調與腫瘤的發生和發展有關，例如 ASPP 家族 (PPP1R13A 和 PPP1R13B)。在本研究中，我們對 PPP1R1B 和 PPP1R3C 的腫瘤遏抑功能進行了深入的研究。

通過半定量 RT-PCR， 我們發現 PPP1R1B 和 PPP1R3C 在多個腫瘤細胞中表達沉默。我們通過甲基化特異性 PCR (methylation specific PCR) 檢測 PPP1R1B 和 PPP1R3C 約 130bp 啓動子區域 CpG 島的甲基化修飾，結果證實這兩個基因啓動子區域過度甲基化是導致它們在腫瘤細胞中表達沉默的主要機制。亞硫酸氫鹽處理的基因組測序 (Bisulfite genomic sequencing) 進一步證實了這一結論。另一方面，利用去甲基化試劑（5-aza-2′dcoxycytidine）和 TSA 處理具有過度甲基化並基因表達沉默的腫瘤細胞，可以使沉默了的 PPP1R1B 和 PPP1R3C 基因重新表達並使其啓動子區域同時出現去甲基化。腫瘤細胞克隆生成實驗證明重新導入 PPP1R1B 和 PPP1R3C 的表達可以顯著抑制大約 40-50%和 50-60%的癌細胞生長。

在表達沉默的癌細胞中重新導入 PPP1R1B 表達，可致磷酸化的 p-ser473 Akt 和 p-ser552 β-catenin 的蛋白表達明顯下調。與此同時，AP-1 的轉錄活性也被明顯降低。在重新導入 PPP1R3C 表達的情況下，磷酸化的 pSer9-GSK-3beta 有上調的情況，並且在 HCT116 細胞裏的 p53 表達水平也同時升高。在 HCT116 細

胞裏 PPP1R3C 的表達可顯著增強 NF-κB 的轉錄活性，而其在 KYSE150 細胞中的表達卻可抑制 NF-κB 的轉錄活性。通過 GFP 融合蛋白追蹤目的基因的亞細胞定位，我們發現 PPP1R1B 蛋白分佈在細胞質中，而 PPP1R3C 蛋白則主要集中在細胞核膜周圍。

綜上所述，我們認爲 PPP1R1B 和 PPP1R3C 作爲重要的抑癌基因，其啓動子 CpG 島的過度甲基化而致使表達沉默與癌症的發生有潛在的顯著關聯。

# Abstract

Protein Phosphatase 1 regulatory subunits are a family of small molecules which define the substrate specificity and subcellular localization of protein phosphatase-1 upon their interactions. Downregulation of Protein Phosphatase 1 regulatory subunits were often associated with tumor initiation and progression, for example, ASPP family (PPP1R13A and PPP1R13B). In the present study, PPP1R1B and PPP1R3C were identified in which their tumor suppressor functions had been investigated.

Gene expression profiles obtained by means of semi-quantitative RT-PCR showed that both PPP1R1B and PPP1R3C were frequently silenced in multiple carcinomas. Bisulfite treated tumor DNA was subjected to Methylation-specific PCR (MSP) using primers flanking across the ~130bp CpG island of the promoter of the particular gene of interest. It was revealed that PPP1R1B and PPP1R3C gene silencing in the carcinoma cell lines were due to promoter CpG island hypermethylation. Such claim was further confirmed by bisulfite genomic sequencing (BGS). Treatment with 5' azacytidine and TSA restored PPP1R1B and PPP1R3C expression in carcinoma cells through demethylating the hypermethylated promoter. In terms of cancer growth inhibition, ectopic expression of PPP1R1B and PPP1R3C could significantly inhibit the proliferation of carcinoma cell lines by

40-50% and 50-60%, respectively, according to the result of anchorage-dependent colony formation assay.

Reduction in the level of p-ser473 Akt and p-ser552 β-catenin could be observed when PPP1R1B expression was restored in respective carcinoma cells. In addition, the transcription activity of AP-1 decreased in the presence of full-length PPP1R1B expression as determined by Dual-Luciferase reporter assay system. Ectopic expression of PPP1R3C increased the amount of inactive pSer9-GSK-3beta as shown in the western blot analysis and a concomitant increased in p53 level was observed in colorectal carcinoma HCT116 cells. Transcription activity of NF-κB in HCT116 cells was increased but decreased in KYSE150 cells (ESCC) in the presence of PPP1R3C expression. Subcellular localization study using the GFP-fusion protein revealed that PPP1R1B protein was distributed throughout the cytoplasm while PPP1R3C protein was mainly localized around the nuclear membrane.

Overall, we believed that PPP1R1B and PPP1R3C are the putative tumor suppressor genes in which their expression silencing through promoter CpG island hypermethylation may be strongly linked to the development of cancer.

# *List of Abbreviations*

| | |
|---|---|
| 5-aza-CdR | 5-aza-2'-deoxycytidine |
| aCGH | Array comparative genome hybridization |
| AP-1 | Activator protein-1 |
| ASPP | Ankyrin repeats-SH3 domain and proline rich region containing protein |
| BGS | Bisulphite genomic sequencing |
| Cdks | Cyclin dependent kinases family |
| cDNA | Complementary DNA |
| CpG | Cytosine-phosphate-Guanine |
| CREB | cAMP response element binding |
| DAPI | 4', 6-diamidine-2'-phenylindole dihyochloride |
| DARPP-32 | Dopamine and cAMP-regulated phosphoprotein $M_r$ 32kDa |
| DDR1 | Discoidin Domain Receptor 1 |
| DEPC | Diethyl pyrocarbonate |
| DMEM | Dulbecco's modified Eagle's medium |
| DISC | Death inducing signaling complex |
| DNA | Deoxyribonucleic acid |
| DNMT | DNA methyltransferases |
| dNTP | Deoxynucleoside triphosphate |
| dsRNA | Double-stranded RNA |
| ESCC | Esophageal carcinoma |
| EtBr | Ethidium bromide |

| | |
|---|---|
| FBS | Fetal bovine serum |
| FMR1 | Fragile X mental retardation 1 gene |
| FMRP | Fragile X mental retardation 1 protein |
| FRAXA | Fragile X syndrome |
| GADD34 | Growth arrest and DNA damage protein 34 kDa |
| GAPDH | Glyceraldehyde 3 phosphate dehydrogenase |
| GFP | Green fluorescence protein |
| GSK-3β | Glycogen synthase kinase-3beta |
| HAT | Histone acetyltransferases |
| HDAC | Histone deacetylase |
| HMT | Histone lsine methyltransferase |
| HPCE | High performance capillary electrophoresis |
| HPLC | High performance liquid chromatography |
| iASPP | Inhibitory ASPP |
| LC-ES/MS | Liquid chromatography-electrospray mass spectrometry |
| LOH | Loss of heterozygozity |
| LSD1 | Lysine-specific demethylase-1 |
| MDBP | Methylation DNA binding protein |
| MDM2 | Murine double minute 2 |
| mRNA | Messenger RNA |
| MS/MS | Tandem mass spectrometry |
| MSP | Methylation specific PCR |

| | |
|---|---|
| MYPT1 | Myosin phosphatase targeting subunit (MYPT1) |
| NF-κB | Nuclear factor-κB |
| NPC | Nasopharyngeal carcinoma |
| PBMC | Peripheral blood mononuclear cell |
| PIG3 | P53-induced gene 3 |
| $PIP_3$ | Phosphatidylinositol (3, 4, 5)- triphosphate |
| PKA | Protein kinase A |
| PNUTS | Protein phosphatase-1 nuclear targeting subunit |
| PP1 | Protein phosphtase-1 |
| PPP1R1B | Protein phosphatase-1 regulatory subunit 1B |
| PPP1R3C | Protein phosphatase-1 regulatory subunit 3C |
| pRb | Retinoblastoma protein |
| PTEN | Phosphatase and tensin homolog deleted on chromosome 10 |
| PTG | Protein targeting to glycogen |
| IRF8 | Interferon regulatory factor 8 |
| RISC | RNA-induced silencing complex |
| RNAi | RNA interference |
| RT | Reverse transcription |
| SAHA | Suberoylanilide hydroxamic acid |
| SDS-PAGE | Sodium dodecyl sulphate polyacrylamide gel electrophoresis |
| SERCA | Sacroplasmic or endoplasmic reticulum $Ca^{2+}$-ATPases |
| siRNA | Small interference RNA |

| | |
|---|---|
| SLE | Systemic lupus erythematosus |
| t-DARPP | Truncated isoform of DARPP-32 |
| TGF-β | Transforming growth factor-β signaling |
| TNF-α | Tumor necrosis factor alpha |
| TRAIL | Tumor-necrosis factor-related apoptosis-inducing ligand |
| TSA | Trichostatin A |
| TSG | Tumor suppressor gene |
| UGS | Upper gastrointestinal adenocarcinomas |

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# Chapter 1

# General Introduction

## 1.1 Maintaining normal physiological status: orchestration between cell cycle and apoptosis

Cell cycle and apoptosis are the two major cellular processes which control the fate of the cells. To sustain normal growth and development, millions of cell divisions are required in which the daughter generations of the cells are produced upon each round of cell cycle. For every round of the cell cycle, errors in the DNA replication process may arise that are corrected by DNA repairing enzyme. However, aging of the cells will increase the frequency of the errors in DNA replication. In addition, UV damage and insult of DNA by chemical carcinogen can lead to extensive DNA damages which may be uncorrectable. If the cells suffer irreversible DNA damages, apoptosis will be executed in order to eliminate any harmful cells from the body.

*Cell cycle*

Cell cycle consists of four distinct phases: $G_1$ phase, S phase (synthesis), $G_2$ phase ($G_1$, S and $G_2$ are collectively known as interphase) and M phase (mitosis). Before the start of a new round of cell cycle, cells are in the quiescence state called $G_0$ phase, or the resting phase. In $G_0$ phase, cell division remain temporarily or reversibly stopped for a period of time until receiving intracellular stimuli such as growth factor hormone receptor binding, after which the cells will then enter a new round of cell cycle starting at the $G_1$ phase.

*$G_1$ phase* (Gap 1)

It is the first phase of the cell cycle. It is also called the growth phase. In this phase, cells acquire nutrients and are at a high rate of biosynthesis in order to prepare all the necessary materials such as enzymes for the DNA synthesis in the subsequent phase of cell cycle. Increase in the size of the cells is one of the major features in this phase. There is no definite period of time for the $G_1$ phase. It is known that duration of $G_1$ phase can vary from one kind of cells to another (Smith et *al.*, 1973).

*S phase* (synthesis)

When all the materials required for DNA synthesis are ready, cells will be transited from $G_1$ phase to S phase. During S phase, the amount of DNA in the cell is effectively doubled because all of the chromosomes have been replicated. At the end of S phase, each chromosome has two sister chromatids though the ploidy of the cell remains the same. Another interesting feature of this phase is that most of the histone production occurs in this phase (Wu et *al.*, 1981; Nelson et *al.*, 2002; Cameron et *al.*, 1963).

*$G_2$ phase* (Gap 2)

After the completion of S phase, the cell will enter the $G_2$ phase. $G_2$ phase is the last interphase before cell undergoes mitosis. Similar to $G_1$ phase, the rate of biosynthesis is high in $G_2$ phase. One of the major products of the biosynthesis in this phase is microtubules which are required during the process of mitosis. At this phase, the cell will continue to grow. $G_2$ phase is the last checkpoint to ensure that all the

materials required for mitosis are ready. Protein synthesis will be inhibited during $G_2$ phase to prevent the cell from prematurely undergoing mitosis.

*M phase (mitotic)*

Immediately after the $G_2$ phase, cells will enter into M phase. Mitosis and cytokinesis are the two tightly coupled processes in M phase. In this phase, cell growth stops and the parent cells are ready to divide into 2 identical daughter cells. The sequential events of mitosis in M phase are prophase, prometaphase, metaphase, anaphase and telophase. In between the phases of mitosis, pairs of chromosomes (replicated in S phase) will condense and attach to microtubules that pull the sister chromatids to the opposite sides of the cell. Cytokinesis is the process right before the telophase at which the parent cells are divided into two daughter cells with identical genetic composition (also identical to the parent cell) and equal shares of cellular components.

*Apoptosis*

In Greek, Apoptosis translates to "dropping off" of petals or leaves from plants or trees. In contrast to necrosis, apoptosis is a form of traumatic cell death as a result from acute cellular injury, Apoptosis is the process of programmed cell death in which less inflammatory response will be produced. Activation of caspases family, DNA fragmentation (production of double-stranded, low molecular weight DNA fragments and single strand breaks), cell surface blebbing are the hallmark features of apoptosis. At late stage apoptosis, cellular and nuclear shrinkage, chromatin condensation and prominent nuclear fragmentation with formation of apoptotic

bodies can be observed (Kroemer et *al.*, 2005; Janicke et *al.*, 1998; Kroemer et *al.*, 2005).

There are two major pathways for triggering apoptosis: the extrinsic pathway, i.e. the death receptor ligand binding induced apoptosis and the intrinsic pathway, which is also called mitochondria-mediated apoptosis. Extrinsic pathway or death receptor-ligand binding pathway, as the name implies, involves the binding of a death receptor ligand (e.g. Fas ligand) with the death receptor (e.g. Fas-APO-1 receptor) and forms the death inducing signaling complex (DISC). DISC will then stimulate the proteolytic maturation of pro-caspase 8 to active caspase 8. For the intrinsic pathway, it involves the release of cytochrome C from mitochondria to cytosol. Release of cytochrome C will trigger the Apaf-1-mediated activation of pro-caspase 9 and result in the formation of apoptosome. Both active caspase 8 and apoptosome can trigger the proteolytic cleavage of pro-caspase 3 to form active caspase 3 (Kroemer et *al.*, 2005). Active caspase 3 is essential for late stage apoptotic response such as DNA fragmentation and cell surface blebbing (Janicke et *al.*, 1998).

Cell cycle and apoptosis orchestrate perfectly throughout the normal lifespan. The proper executions of apoptosis and cell cycle are required to maintain the normal physiological functions of the body. Disregulation of either process is devastating and will result in diseases. A typical example is cancer, in which continuous progression of cell cycle is accompanied by the suppression of apoptosis, leading to uncontrolled cell proliferation.

## 1.2 Carcinogenesis: Battle between Oncogene and Tumor suppressor gene

Cancer is a class of diseases in which a group of cells display uncontrolled growth, invasiveness (intrude and destroy adjacent tissues), and sometimes metastasis (spread to other locations in the body via lymph or blood). These abnormal features are resulted from the aberrant changes in the genetic materials of the cell. The process for a normal cell transforming into a cancer cell is called carcinogenesis. Carcinogenesis is a multi-steps process in which a series of mutations has occurred and has led to abnormal gene expression and hence the functioning of the normal cells. Oncogenes and tumor suppressor genes (TSGs) are the two classes of genes important to carcinogenesis. Dysregulation of oncogenes expression will initiate the neoplastic transformation while the existence of the tumor suppressor genes serve as a counteracting force to act against the influence from dysregulated oncogenes and are able to suppress tumor formation. Thus, inactivation of tumor suppressor genes also contributes to tumor development and progression.

### 1.2.1 *Oncogenes*

Activation of oncogenes contributes to the survival and proliferation of the cells. Overexpression of oncogene will turn a normal cell into a cancer cell. Prior to mutation, oncogene genes existed in the form called proto-oncogene. Proto-oncogenes encode proteins and are essential for regulating cell growth and differentiation. Upon mutations or overexpression, Proto-oncogene will convert into an oncogene and allow the cells to survive and proliferate in an uncontrolled manner (classical feature of cancer) (Todd et *al.*, 1999). The first identified Proto-oncogene is Src. It is an oncogene in a chicken retrovirus originally discovered in 1970. Src is a family of proto-oncogenic tyrosine kinases which plays a role in the regulation of

embryonic development and cell growth. The protein encoded by this gene is a tyrosine-protein kinase whose activity can be inhibited by kinase phosphorylation. Mutations in this gene led to the malignant progression of colon cancer (Stehelin et *al.*, 1977). After the discovery of oncogene src, many other oncogenes were subsequently identified, for examples, RAS, WNT, MYC, ERK, and TRK.

### 1.2.2 *Tumor suppressor genes*

As mentioned earlier, TSGs are able to counteract the signal from oncogenes. It serves as a gatekeeper to prevent a normal cell from turning into a cancer cell. Downregulation or even loss of function of TSGs will promote carcinogenesis and tumor progression. The proteins encoded by TSGs are often the negative regulators of cell cycle accomplished by repressing genes which are essential for cell cycle progression. They also induce cell cycle arrest for repairing DNA damage or promote the induction of apoptosis if damages are unable to be repaired. Some of them are involved in cell adhesion or inhibition of cell migration which are strongly associated with metastasis of the cancer cells (Yoshida et *al.*, 2000; Hirohashi et *al.*, 2003). Retinoblastoma protein (pRb) is the first discovered tumor suppressors that is associated with the development of human retinoblastoma. The other famous tumor suppressor is p53, which is encoded by TP53 gene. Mutation of p53 was found in many kinds of cancer such as leukemias, lymphomas, sarcomas, and neurogenic tumors. Individuals with genetic disease called Li-Fraumeni syndrome are inherited with one copy of defected p53 gene and thus are highly susceptible to development of various kinds of cancer (Li et *al.*, 1969). APC, CD95, ST5, ST7, and ST14 are the other examples of tumor suppressors which all contribute to carcinogenesis if these genes are mutated.

For years, identification of the oncogene and tumor suppressor gene has been the top agenda in cancer research. Since the completion of the human genome project, identification of oncogenes and TSGs are easier and many functional analyses have been done. The importance of the identification and investigating the functions of oncogenes and tumor suppressor genes is to acquire a better understanding of the molecular pathogenesis of carcinogenesis, which will provide insight in the development of novel diagnostic methods, therapeutics approaches for cancer and also better prognosis can also be predicted for the cancer patients.

### 1.2.3 *Mechanism of TSGs inactivation*

In 1971, Alfred Knudson proposed the famous "two-hit" hypothesis about the inactivation of TSGs during carcinogenesis. It has been known that functions of a particular TSG can still be exhibited even if one of the alleles is inactivated by genetic mutation because tumor suppressor functions can be provided by the remaining allele. According to Knudson's two hit hypothesis, functions of the TSGs will be loss only when both alleles are inactivated upon genetic mutation. In other words, the "first-hit" (germline mutation or somatic mutation) predisposes cells to the development of malignancy. The "second-hit" to the remaining allele by somatic mutation will completely abolish the function of TSG and thus initiate tumor formation (Knudson, A. G., 2001). Transition, Transversion, single point mutation and aberrant chromosomal copy numbers (deletion or amplification) are some of the genetic mutations which lead to inactivation of the TSGs. Searching for gene with the loss of heterozygozity in a specific kind of human cancer allows us to identify the putative TSGs. For many years, it has been thought that inactivation of the allele by genetic mutation was the only way causing loss of heterozygozity. It has been

suggested that some of the TSGs are inactivated despite no genetic mutation has been found. This prompted the scientists to rethink that inactivation of TSG may follow a very different mechanism other than simply the genetic mutation. The answer for such question has later become clearer upon the flourish of studies about the transcriptional silencing of the gene through epigenetic mechanism.

### 1.3 Epigenetics and its influence in cellular functions

Epigenetics refers to all meiotically and mitotically heritable change in gene expression that occurred without a change in DNA sequence. DNA methylation, Histone modification and RNA interference are the epigenetic modifications which regulate the gene expression within the cells. These epigenetic modifications are stable over rounds of cell division (Egger et *al.*, 2004). Gene expression controlled by epigenetic mechanism has significant impact in various cellular processes including the mammalian embryonic development. It has been suggested that different cells and tissues acquire different programmes of gene expression which are substantially regulated by epigenetic modifications. These bring a unique epigenetic signature which reflects genotype, developmental history and environmental influences and lead to diverse phenotype of the cell and organism (Morgan et *al.*, 2005). It is now known that many human diseases such as cancer are caused by aberrant epigenetic modifications.

*Histone modification*

Modficaition of histone can be either through methylation or acetylation. Histone modification is important for gene transcription because it affects the chromatin structure which only opened and loosely-packed chromatin allows various

transcription factors to access to the promoter of the target genes and then activates gene transcription. Activation or repression of transcription depends on the type and the site of the histone modification. Lysine and arginine are the amino acid residues susceptible to the modification in the histone molecules. Methylation of argnine residues can be mono-methylated, symmetric di-methyalted or asymmetric dimethylated catalyzed by protein argnine methyltransferases (PRMTs), while methylation of lysine can be mono-methylated, dimethylated or trimethylated catalyzed by histone lysine methyltransferases (HMTs) (Yoo et *al.*, 2006). For example, transcription of the gene is permitted when lysine-4 (H3-K4) of the histone is methylated. It was reported that demethylation of H3-K4 by lysine-specific demethyalase-1 (LSD1) would lead to dowregulation of its target genes (Shi et *al.*, 2004). In contrast, methylation of H3-K9 marks transcriptionally inactive chromatin as it leads to close packing of chromatin so that genes are difficult to be accessed (Rice et *al.*, 2001; Cheung et *al.*, 2005). It was reported that LSD1 specifically co-localized with the androgen receptor to remove H3-K9 methylation and allowed the activation of androgen receptor target genes (Metzger et *al.*, 2005).

Histone lysine acetylation, in particular, acetylation of lysine residues on histone H3 and H4 is associated with active transcription as it leads to active and open chromatin structure while deacetylation of histone on lysine residues will result in chromatin compaction and inactivation of the genes. The acetylation status of the histone is orchestrated by the activity of histone acetyltransferases (HAT) and histone deacetylase (HDAC). Loss of histone deacetylation is suggested to be the primary event and as the first step in gene silencing, whereas the accumulation H3-K9 methylation plays a secondary role (Mutskov et *al.*, 2004). It is also suggested that histone deacetylation not only results in gene silencing but also negatively

affected DNA repairing (Masumoto et *al.*, 2005), which is critical in accelerating molecular events leading to the development of cancer. In fact, it is reported that losses of both the acetylation of lysine-16 and trimethylation of lysine-20 on histone H4 have been found in some cancer cells (Fraga et *al.*, 2005).

*RNA interference (RNAi)*

RNAi is a posttranscriptional gene silencing mechanism that defenses the host from invasion by foreign DNA such as virus and transposons (Wolffe et *al.*, 1999). A Decade ago, it was found that injection of the double-stranded RNA (dsRNA) into animals was effective to silence a specific gene which such silencing was stable even for next few daughter generations (Fire et *al.*, 1998). RNAi is an RNA-dependent gene silencing process that is controlled by the RNA-induced silencing complex (RISC) and is initiated by short double-stranded RNA molecules (microRNA and small interference RNA) in a cell's cytoplasm, where they interact with the catalytic RISC component argonaute (Fire et *al.*, 1998). Generally, both the foreign dsRNA and endogenous dsRNA (pre-microRNAs expressed from RNA-coding genes in the genome) will be cleaved into short fragments by the enzyme *Dicer* in the cytoplasm.

The short double-stranded fragments produced by the *Dicer* are called small interfering RNAs (siRNAs). These siRNAs can be separated into single strands and integrated into an active RISC complex. After integration into the RISC, siRNAs base-pair to their target mRNA and induce cleavage of the mRNA, thereby preventing it from being used as a translation template (Ahlquist, P., 2002).

*DNA methylation*

DNA methylation has been the most extensively studied. In mammal, a family of enzymes called DNA methyltransferases (DNMTs) including DNMT1, DNMT3a and DNMT3b) and methylation DNA binding protein (MDBP) are two essential components in the DNA methylation system. During the process of gene silencing process, DNMTs are responsible for establishing and maintaining DNA methylation patterns. They will transfer the methyl group to C-5 position of CpG dinucleotides. Then, MDBP will be recruited which recognizes and binds to the methylated CpG. It is also known that methylated DNA can also attract the binding of transcriptional MeCP1 and MeCP2 form transcription repressor complex. It prevented the binding of DNA polymerase and transcription factor on DNA of the promoter region in a sequence independent manner (Jones, P.A., 1999; Robertson, K. D., 2005). Besides, the complex of methylated DNA sequence and MeCP2 can recruit and initiate histone deacetylation.

Studies revealed that DNA methylation may have an important role in evolution. In eukaryotes, transcription repression by DNA hypermethylation is useful for the defense against harmful DNA elements such as transposons, viral genomes, etc. In mammals, cytosine methylation is a nuclear-host defense system to eliminate the threat posed by endogenous parasitic mobile genetic elements (Yoder et *al.*, 1997), for example, inactivation sequences of the transposons, including retroviruses and Alu elements in the differentiated cells (Schmid, C. W., 1996). Robertson, K. D. et *al* (Robertson et *al*, 1997) has found that that Epstein-Barr virus DNA is methylated in normal lymphocytes of healthy people. It has been suggested that de novo methylation is used as a cellular defense mechanism to silence integrated foreign DNA or genes such as the promoter of adenovirus (Singal et *al.*, 1999).

Apart from host defense against foreign DNA invasion, DNA methylation was also found to be vital for the genomic imprinting and embryonic development. It is suggested that phenotypes of the cell and organism (influenced by the genotype, developmental history and environmental interaction) are determined by the epigenetic signature of the individual cell type (Morgan et *al.*, 2005). Cycle of demethylation can be found during early development of parental germ cells and in oocyte after fertilization. Coupling with remethylation in germ cells maturation and in oocyte after fertilization is especially important for erasing any acquired epigenetic modifications, those influenced by the individual genetic and environmental factors. It also provides totipotency, correct initiation of embryonic gene expression and early lineage development in the embryo (Morgan et *al.*, 2005; Reik et *al.*, 2001)

### 1.4 Techniques used in studying DNA methylation and Histone modification

The very first approach for detecting DNA methylation is the combined use of high-performance liquid chromatography (HPLC) or high-performance capillary electrophoresis (HPCE) and the restriction enzyme digestion. Briefly, this method involves the digestion of the particular DNA sequences by restriction enzyme that can distinguish between methylated and unmethylated recognition sites in genes of interest. The digestion products will then be resolved in HPLC and HPCE in which the total amount of 5-methylcytosine can be quantified (Fraga et *al.*, 2002). However, drawbacks like incomplete restriction enzyme digestion and the requiring of relatively large amount of DNA samples in the purification step by Southern blotting have limited the use of this technique and so as the prevalence of epigenetic researches (Esteller, M., 2007).

Utilization of bisulphite treatment of DNA has brought revolutionary changes to epigenetic researches. Investigation of DNA methylation using bisulphite treatment of DNA often couples with genomic sequencing or amplification of sequence-of-interested by PCR methods, also called methylation specific PCR (MSP). The advantage of this method is the study of DNA methylation is still feasible even if there is only limited amount of sample available (since DNA samples can be amplified by PCR). Distinguishing methylated DNA sequences from the unmethylated one is much easier and reliable because unmethylated cytosines can be reproducibly changed to uracil but leaves methylated cytosines unchanged after bisulphite treatment of DNA (Esteller, M., 2007).

Chromatin immunoprecipitation is another technique that leads to recent advances in epigenetic research particularly in epigenomic profiling of cancer cells. It basically involves the precipitation of DNA by an antibody recognizing 5-methylcytosine. The precipitated DNA can be used as a probe for the hybridization to genomic microarray platforms which makes the analysis of DNA methylome simple and also allows rapid identification of multiple CpG cites. This approach was called ChIP-on-chip (Weber et *al.*, 2005; Keshet et *al.*, 2006). All the techniques mentioned have proved to be successful in identifying methylated genes. By relying on these techniques, it gives us a better understanding of epigenetics and unveils the causes of some disease processes such as tumorigenesis. However, it should be noted that approaches like ChIP-on-chip and MSP do not reveal to us a predominant methylation pattern of the sample DNA. Therefore, bisulphite genomic sequencing is often employed as a last resort to further confirm the result and also provides a complete picture of the heterogeneous methylation patterns that exists in cancer cells (Esteller, M., 2007).

At present, chromatographic separation and ChIP are the methods used for the study of histone modification. Different kinds of histone (H3, H4, H2A, H2B and H1) can be resolved in HPLC. The relative amounts of histone modification and the identity of particular histone can be determined by coupling with HPCE or liquid chromatography-electrospray mass spectrometry (LC-ES/MS) (Fraga, et *al.*, 2005). ChIP is an approach used when the information of a particular DNA sequence that couples histone modification would like to be known. The immunoprecipitated DNA could be subjected to PCR amplification for further analysis. Similar to those approaches used in studying DNA methylation, methods such as western blots, immunostaining or tandem mass spectrometry (MS/MS) should be performed if detailed information such as specific histone modification at each amino acid residue is needed to be characterized (Fraga et *al.*, 2005; Seligson et *al.*, 2005).

## 1.5 Transcriptional silencing through epigenetic mechanism and its disease association

DNA methylation, especially methylation of CpG Island in the promoter region is so powerful to silence the gene expression. Gene silencing through DNA methylation is especially important in the normally differentiated cell because it represses the transcription of the self-renewal inducing gene. Another example is the X-inactivation in female. The X-inactivation is essential for the dosage compensation. One of the X-chromosomes is inactivated by epigenetic mechanism so that the female with two X chromosomes will not produce twice as many X chromosome gene products as the male, which only possess a single copy of the X chromosome. So, it can be imagined that diseases will be resulted if there is disregulation in the

methylation or abnormal reprogramming of the epigenetic printings, (Reik et *al.*, 2001; Morgan et *al.*, 2005; Robertson et *al.*, 2005).

Fragile X syndrome (FRAXA), a common cause of inherited mental retardation. Affected individuals are unable to produce the fragile X mental retardation 1 protein (FMRP) which is believed to regulate synaptic plasticity by controlling translation at synapses (Jin et *al.*, 2004). The inability of producing FMRP is due to the *de novo* methylation of the expanded CGG repeat (increases from normally 6-52 copies to 52-200 copies in the permutation state) located in 5'-untranslated region of *fragile X mental retardation 1 gene* (FMR1) which lead to transcriptional repression (Crawford et *al.*, 2001; Oberle et *al.*, 1991). Another example is the autoimuune disease, Systemic lupus erythematosus (SLE). It has been suggested that genome of *SLE* T cells are globally hypomethylated which is likely the cause for the production of autoantibody against the host nuclear components (Lu et *al.*, 2002; Oelke et *al.*, 2004). In particular, several genes relevant to the SLE phenotype are methylated in normal T cells but are found to be demethylated in SLE patients.

In recent years, researches about the relationship between the epigenetic silencing of TSG in tumor and causes of carciongenesis have been extensively conducted. The first report about the relationship between cancer and epigenetics could be dated back to 1983 (Feinberg et *al.*, 2004). It was once suggested that the genomes of cancer cells was hypomethylated when comparing with the normal counterpart. It was believed that the loss of DNA methylation led to gene activation and frequently included the oncogene. For example, Feinberg A. P. and Vogelstein B. (Feinberg A. P. and Vogelstein B., 1983) discovered that the oncogene HRAS and "CT" genes were hypomethylated in a number of primary human tumors which these genes would be methylated (silenced) in normal tissue or would only express in testis.

Later, it was believed that global hypomethylation in cancer genomes was an incorrect interpretation as the experimental design was only biased to certain region (Feinberg et *al.*, 2004). Redistribution of the methylation on cytosine nucleotides is now agreed to be the critical event that actually happened for the early development of primary tumor. The rationale is that CpG of the normally methylated oncogenes are demethylated while region like CpG island of the promoter of TSG are hypermethylated which ultimately causes the transcriptional repression of TSG.

Promoter hypermethylation is thought to be the culprit for silencing of tumor suppressor gene and hence leads to the carcinogenesis. As mentioned before, Knudson's "two hit" theory (Knudson et *al.*, 2001) stated that tumor suppressor function of the TSG will lose when both copies of alleles are inactivated. At present, epigenetic silencing of TSGs is increasingly recognized as an important mechanism leading to the loss of tumor suppressor functions of TSGs. It has been suggested that epigenetic modification can also contribute to the double hit gene inactivation (Sigalotti et. *al.*, 2007). In other words, one of the copy of the gene or both silenced through epigenetic mechanism should also play an important role in carcinogenesis (Fig. 1). In particular, promoter CpG island hypermethylation is the epigenetic modification and has been found to be the most frequent events associated with inactivation of TSGs (Jones et *al.*, 1999). The retinoblastoma gene RB was the first TSG identified to be silenced by hypermethylation. In 1989, Gerger. V. et *al.* (Gerger et *al.*, 1989) reported that RB promoter is methylated in a significant subset of sporadic and even hereditary retinoblastoma and later they suggested that there may be a link between such phenomenon and RB expression silencing (Sakai et *al.*, 1991). For years, vast amount of TSGs have been identified to be silenced by promoter CpG hypermethylation in tumor.

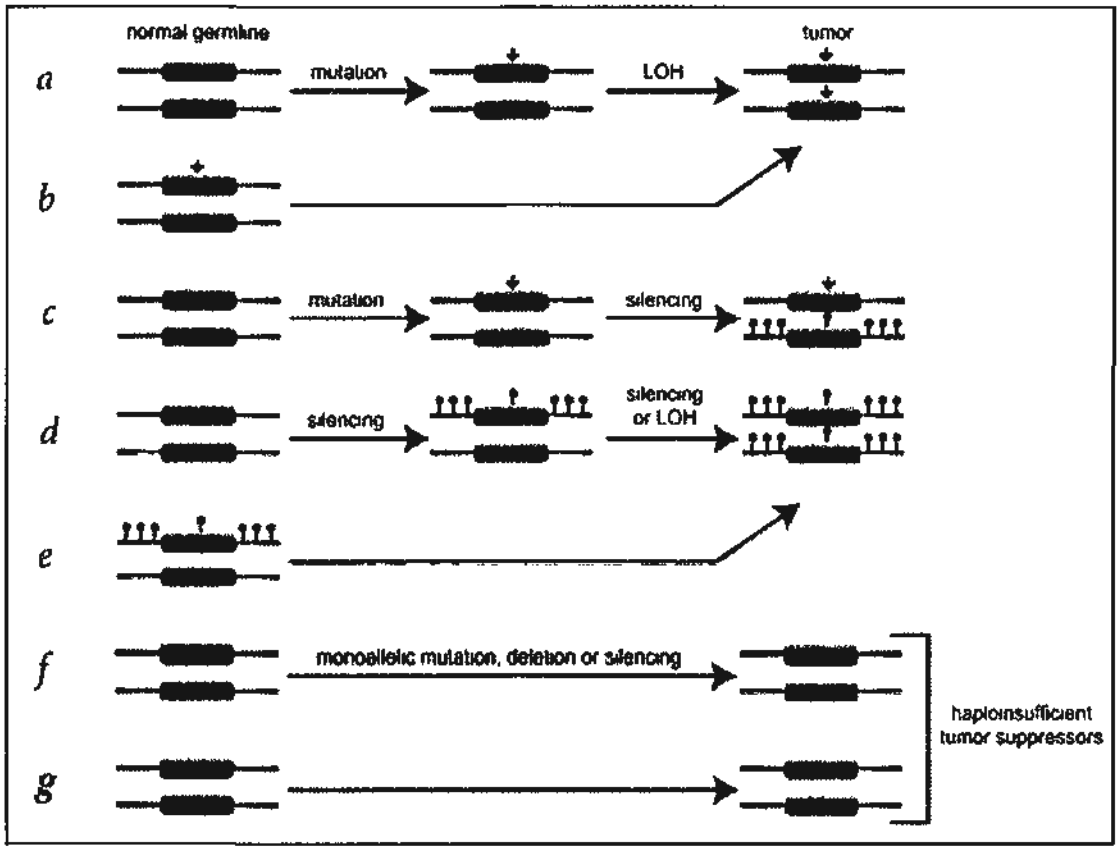



**Figure 1.1 Loss of TSG functions in cancers.** ***a,b***. The classical Knudson's two-hit model indicating inactivation of TSG and hence tumor development is solely caused by genetic mutation on both alleles. Individuals inherited with a germline mutation are more susceptible to tumor development. ***c***. LOH can be a combination of a genetic mutation on one allele and followed by gene silencing through promoter methylation on another allele. ***d.*** Biallelic silencing of TSG. ***e.*** Individuals with germ-line epimutation also highly susceptible to tumor development. ***f*** & ***g***. Haploinsufficient tumor-suppressor genes. Loss of a single gene copy by mutation, deletion or silencing, is adequate to drive cancer formation. Such phenomenon can be due to the inheritance of a partially or completely non-functional allele. (Adopted and modified from ref. (Balmain et *al.*, 2003). Functional alleles are represented by red coloured Bar while inactivated alleles are represented by blue coloured bars.

Recently, researchers have focused not only on the identification of epigenetic silenced TSG but also tried to investigate the association between the epigenetic silencing of TSG and the functional consequences to the cells which are pivotal for the development of cell malignancy. TP53 and PTEN are the examples. Loss of function of TP53 and PTEN (Phosphatase and tensin homolog deleted on chromosome 10) are often observed in tumor, many studies about these two genes have been conducted in order to unveil its functional role in carcinogenesis.

PTEN possess dual specificity phosphatase activities which allows it to dephosphorylate both tyrosine and serine/threonine residues by its protein tyrosine phosphatase domain, though its lipid phosphatase activity is much well-known. Phosphatidylinositol (3, 4, 5)-triphosphate ($PIP_3$) is proven to be the main PTEN substrate. As $PIP_3$ is a secondary messenger for intracellular signal transduction that can trigger diverse downstream pathways and activate the cell proliferation or suppressing apoptosis, the normal functioning for the phosphatase activity of PTEN on cleaving $PIP_3$ will have a significant influence on regulating cell cycle arrest and apoptosis (Di Cristofano et *al.*, 2000). It was suggested that ectopic PTEN expression was able to inactivate *Akt* kinase activity and also induced a specific apoptosis pathway initiated by cell detachment from extracellular matrix called anoikis in glioma and breast cancer cells (Lu et *al.*, 1999; Tamura, M., 1999). It has been reported that mutations of PTEN are often associated with aggressive metastatic tumor phenotype. Freeman et *al.* (Freeman et *al.*, 2003) has also suggested that PTEN can regulate the stability and hence the p53 protein level and the p53 transcriptional activity by modulating p53 DNA binding activity, thereby influencing the cellular response to stress or death signal. It has also been reported that PTEN has a role in the regulation of cell differentiation, cell adhesion and migration. For

example, Tamura et *al.* (Tamura et *al.*, 1999) has found that reintroduction of PTEN into the glioblastoma cell line U-87MA led to direct phosphorylation of *FAK* by PTEN, thus inhibiting integrin-mediated cell spreading and migration. For many years, deletion or mutation of gene in tumor is the only known mechanisms that results in the loss of PTEN expression or function and so as the carcinogenesis. However, deletion or somatic mutation of the gene could not be observed in some cases even if the silencing of TSG has been found in the tumor samples. Zhou et *al.* (Zhou et *al.*, 2000) detected absent or low PTEN expression in 65% of melanoma metastases despite the presence of wild-type PTEN. In a study from Mirmohammadsadegh et *al.* (Mirmohammadsadegh et *al.*, 2006), they found significant positional PTEN promoter methylation in 62% of circulating DNA isolated from sera of patients with metastatic melanoma. The percentage of methylation of a selected CpG island in blood showed a correlation with methylation levels in the corresponding melanoma tissue. Such high percentage of methylation was correlated with the low transcriptional level of PTEN in melanoma tissue. It is believed that epigenetic silencing by promoter CpG island hypermethylation is the third mechanisms for the loss of PTEN function. In fact, epigenetic silencing of the PTEN gene has also been shown in advanced human prostate cancer (Whang et *al.*, 1998) and hematologic malignancies (Dahia et *al.*, 1999) as well as endometrial carcinoma (Salvesen et *al.*, 2001).

By virtue of the invention and advancement of techniques for studying epigenetics, especially for promoter CpG island hypermethylation, it is now known that many tumor suppressor genes are silenced by promoter CpG island hypermethylation in the early stage of tumor or silenced in later stage which is

| Gene name | Chromosome location | Description | References |
|---|---|---|---|
| EDNRB | 9p21 | Promoter hypermethylation of EDNRB might contribute to development of NPC | Lo et *al.*, 2002 |
| Casp1 | 11q23 | DNA methylation or histone deacetylation is associated with gastric carcinoma | Jee et *al.*, 2005 |
| ASPP1 | 14q32.33 | abnormal promoter methylation. Hypermethylation in CpG island is very likely linked with tumor cells with wild type p53 | Trigiante et *al.*, 2006 |
| RASSF1 [Ras association (RalGDS/AF-6) domain family 1] | 3p21.3 | RAS effector protein, Ras-signalling, cell cycle arrest, apoptosis, DNA repair, inhibits the cyclin D1 accumulation. It was found to be inactivated by promoter hypermethylation | Lo et *al.*, 2001 |
| CCAR1 cell division cycle and apoptosis regulator 1 | 10q21.3 | A novel, perinuclear phosphoprotein, is a regulator of apoptosis signaling by epidermal growth factor receptors. It is a suppressor of breast cancer growth, and its expression is diminished in tumors, in part, by methylation-dependent silencing | Zhang et *al.*, 2007 |
| *PCDH10* | 4q28.3 | Protocadherin, cell adhesion and cell-cell interaction | Ying et *al.*, 2006 |

Table 1.1 Examples for the Genes related with apoptosis which are hypermethylated in their promoter region in carcinomas.

believed to be the cause for the aggressive phenotype of tumor. Now that epigenetic silencing of tumor suppressor gene is known to be one of the causes for loss of function of a TSG and improper execution of apoptosis relating with carcinogenesis. Researchers have turned their focus on investigating the epigenetic silencing of those genes which their functions are necessary for apoptosis (Table 1.1).

With increasing number of epigenetic silenced TSGs has been discovered and a better understanding of the associated functional consequences in cancer cells have been unveiled, the outcome of specific kind of cancer patients become more predictable and hence the cancer patients will have a better prognosis because tailor-made treatment can be designed for them. Such research topic is still intensively studied and continuous effort should be worthwhile.

### 1.6 Introduction to Protein-Phosphatase 1 regulatory subunits

Reversible phosphorylation of serine, threonine and tyrosine residues play a vital part in controlling the activity or function of about one-third of all eukaryotic protein (Ceulemans et *al.*, 2004). Activation or inactivation of a protein depends on its phosphorylation status, which is influenced by the activities of the protein kinases and phosphatases. It is known that the number of protein phosphatases encoded in human genome is much less than that of protein kinases (approximately 150 for protein phosphatases compare with around 500 for protein kinases). In particular, only around 40 members of protein phosphatase family are serine/threonine phosphatase including the protein phosphtase-1 (PP1). The numerical inferiority for serine/threonine protein phosphatase lead to the question of how such a small number of protein phosphatases regulates a wide variety of proteins by dephosphorylation and balances the influence exerted by protein kinases. In fact, the

versatility of protein phosphatase largely depends on the interaction between its catalytic subunit and different regulatory subunits. Different regulatory subunits will affect the activity, conformation, substrate specificity and ultimate subcellular localization of the protein phosphatase.

### 1.6.1 *Structure of PP1*

As mentioned, PP1 holoenzyme consists of a catalytic subunit and a regulatory. The catalytic subunit of PP1 is encoded by the gene PP1c (in humans, it also includes PP2A, PP4, PP6, PP2B/calcineurin, PP5 and PP7) (Cohen, P. T. W., 1997; Barton et *al.*, 1994). The interaction between the catalytic subunit and the regulatory subunit relies on the short, conserved PP1c-binding motif, the RVxF motif. In human genome, there are more than 50 encoded regulatory subunits (PPP1R) which influence the targeting or substrate specificity for PP1. Some of them may simply serve as an allosteric factor of PP1 which affects the conformation and also the activity of PP1 while the other may target PP1 to different proteins or subcellular localization depending on the phosphorylation status of the regulatory subunits (Cohen, P. T. W., 2002; Ceulemans et *al.*, 2004; Bollen M., 2001).

### 1.6.2 *PPP1R and functional versatility of PP1*

The cellular functions of PP1 include the control of cell proliferation, cell death, glycogen metabolism, cooperation of the muscle contraction and relaxation, protein synthesis, regulation for the Receptors, Ion Channels and Ion Pumps, etc. The regulatory subunits responsible for these particular cellular responses have been identified and the detailed mechanisms are well-studied.

*Glycogen metabolism*

Cooperation of the glycogen synthase and glycogen phosphorylase contributes to the body's normal glycogen metabolism. The switch-on/off of their activities depends on their phosphorylation status. It is known that phosphorylation of the glycogen synthase will lead to its inactivation while phosphorylation of phosphorylase by phosphorylase kinase (active in phosphorylated form) will turn on the activity of phosphorylase. The role of PP1 in glycogen metabolism is mainly the regulation on the phosphorylation level of glycogen synthase, glycogen phosphorylase and also the phosphorylase kinase (Ceulemans et *al.* 2004). Upon association with specific class of regulatory subunit named G subunits, PP1 is able to anchor to the glycogen particles and also the glycogen bounded glycogen synthase, glycogen phosphorylase and the phosphrylase kinase, in which PP1 would dephosphorylate them. Involvement of PP1 in glycogen metabolism can easily be found in liver and skeletal muscle where there are abundant sources of glycogen.

*Regulation of the functions of the Receptors, Ion Channels and Ion Pumps*

The intracellular uptake and release of $Ca^{2+}$ is important for many aspects including the cardiac muscle contraction. The intracellular uptake and release of $Ca^{2+}$ is mediated by sacroplasmic or endoplasmic reticulum $Ca^{2+}$-ATPases (SERCAs). It has been suggested that membrane protein phospholamban can inhibit the activity of SERCA pumps but Ser-16 and/or Thr-17 phosphorylation of phospholamban will relieve such inhibition. For PP1, it regulates the SERCA pump activity by dephosphorylating the phosphorylated phospholamban (Colyer, J., 1998).

Transforming growth factor-β signaling involves two types of receptors, TGFβR-I and TGFβR-II. Recruitment of PP1 and subsequently dephosphorylation of

TGFβR-I will antagonize TGFβR-II. This will reset the TGFβ signaling complex to the basal state so that unwarranted signaling in the absence of ligand will be prevented (Bennett et *al.*, 2002). Signaling of C1C-2 channel is also regulated by PP1. It has been suggested that cyclin dependent kinase inhibitor, olomoucin can increase C1C-2 currents. Such effect can be significantly reduced when PP1/PP2A holoenzyme interacts with calyculin (Furukawa et *al.*, 2002).

*Cell cycle arrest and apoptosis*

*pRb* is known for its importance in inducing cell cycle arrest. *pRb* protein is inactivated upon its phosphorylation by cyclin dependent kinases family (Cdks). PP1 participates in inhibiting cell cycle progression in a Rb dependent fashion. It is suggested that PP1 controls the G1/S transition by maintaining the level of dephosphorylated (active form of Rb) in order to prevent the excessive cell growth. Calyculin A and inhibitor-2 are the PP1 regulatory subunits which can inhibit the activity of PP1. Under the action of Calyculin A or inhibitor-2, the amount of phosphorylated Rb will increase and hence prevent the induction of cell cycle arrest and apoptosis (Dou et *al.*, 1995; Wang et *al.*, 2001). Similarly, PP1 can induce apoptosis by interacting with the Bcl-2/$X_L$/w protein family (Ceulemans et *al.* 2004). Although Bcl-2/$X_L$/w proteins are often served as positive regulator of cell survival, they can induce apoptosis by activating the caspase family when there is the presence of dephosphorylated Bad (Ayllon et *al.*, 2000; Ayllon et *al.*, 2001; Ayllon, V. et *al.*, 2002). It was found that Bcl-2/$X_L$/w proteins contain PP1-binding RVxF motif and are able to form ternary complex with PP1 and *Bad*. It is likely that *Bad* is dephorphorylated by PP1 in this ternary complex. Such notion was supported by the

evidence that inhibition of PP1 prevented the dephosphorylation of Bad and induced the apoptosis in interleukin deprivated hematopoietic cells (Ceulemans et *al.*, 2004).

### 1.6.3 *Protein phosphatase-1 regulatory subunit (PPP1R) and carcinogensis*

In several genome wide differentiation expression studies, downregulation of the members of PPP1R could be frequently observed. Takakura et *al* (Takakura et *al.*, 2001) conducted a genetic and expression analysis on several PPP1R genes in 55 human cancer cell lines including lung, colorectal, gastric and ovarian cancer cell lines in order to examine their association with carcinogenesis. In that study, PPP1R5, PPP1R7 and PPP1R8 genes were detected with possible missense mutations while differential expression PPP1R1A, PPP1R5 (also known as PPP1R3C), and PPP1R6 were observed in a few cancer cell lines. It is now known that many PPP1Rs' members were putative tumor suppressor genes and have a significant role in carcinogenesis and impact on the prognosis of cancer patient upon receiving the treatment for cancer.

#### *PPP1R10*

PPP1R10 is also known as protein phosphatase-1 nuclear targeting subunit (PNUTS). It was first isolated from mammalian cell lysates in a stable complex with PP1α and PP1γ (Allen et *al.*, 1998). In some of the *in vitro* study, PPP1R10 was proved to be an allosteric inhibitor of PP1 (Kim et *al.*, 2003). PPP1R10 was also reported to involve in restructuring nuclei in telophase following the assembly of nuclear membranes and augements *in vitro* chromosome decondensation in a PP1-dependenet manner (Landsverk et *al.*, 2005). *In vitro*, it is believed that PPP1R10 binds to PP1 and inhibits PP1 from dephosphorylating p53 at ser-15 or ser-37 in

stress condition such as UVC treatment (Li et *al.* 2006). Ser-15 and/or ser-37 phosphorylated p53 are essential for the stability and apoptosis inducing capacity of p53. Thus, PPP1R10 is likely to serve as a regulator to augment the protein level and function of p53 in response to stress (Haneda et *al.*, 2004). Lee et *al.* (Lee et *al.*, 2007) has suggested that PPP1R10 is a hypoxia inducible gene in which it modifies the phosphorylation status of p53 and MDM2 in response to hypoxia condition. They over-expressed PPP1R10 in HEK293 cells and found that phosphorylation at ser-15 and nuclear localization of p53 were increased. Such effect and transactivating activity of p53 on response gene including p21 were further enhanced when HEK293 cells were treated with hypoxia-mimetic agent, desferrioxamine (DFO). On the other hand, they also suggested that the presence of PPP1R10 would disrupt PP1 from interacting with MDM2 and hence increased the phosphorylation of MDM2 at serine-395. Phosphorylation of MDM2 at serine-395 is known to be important for promoting MDM2 degradation. As MDM2 is responsible for the degradation of p53 through ubiquitination, PPP1R10 appears to have a protective effect on p53 level and p53 transactivation activity especially under hypoxic stress condition. In the same study, it was showed that overexpression of PPP1R10 increased the hypoxia-induced cell death. Thus, PPP1R10 might possess a tumor suppressive function in which mutation or silencing of it would lead to cancer cell transformation or contribute to the aggressive phenotype of malignant cells such as the hypoxia-resistant tumor cells, which have lower sensitivity to cancer treatment.

*ASPP family: ASPP1 (PPP1R13B), ASPP2 or called TP53BP2 (PPP1R13A) and iASPP (PPP1R13L)*

The name ASPP stands for ankyrin repeats-SH3 domain and proline rich region containing protein or also called apoptosis stimulating proteins of p53 family. The family consists of three members: ASPP1, ASPP2 and inhibitory ASPP (iASPP). They are specific regulators of p53 protein and also the p53 family member, p63 and p73. ASPP1 and ASPP2 are tumor suppressor genes. The ankyrin repeats, SH3 domain and the proline rich region of all these members allows them to bind with the targeting partner like p53, Bcl-2, RELA/p65 and PP1, etc (Trigiante, et *al.*, 2006). When ASPPs interact with p53 or with p53 family member p63 and p73, the apoptotic function of p53 protein will be selectively enhanced without affecting the cell cycle arrest function (Trigiante et *al.*, 2006; Bergamaschi et *al.*, 2004).

Several studies about the function of ASPP1 and ASPP2 on p53 have showed that both ASPP1 and ASPP2 can stimulate the binding and transactivation of p53, p63 and p73 on promoter of pro-apoptotic gene such as Bax, p53-induced gene 3 (PIG3) and PUMA (Samuels-Lev *et al.* 2001) which contributes to the cellular sensitivity to apoptosis. Besides, expressing ASPP1 and ASPP2 can enhance the sensitivity of cancer cell to chemotherapeutic drug or radiation treatment. Mori et *al.* (Mori et *al.*, 2000) showed that the mRNA level of ASPP2 was positively correlated with cellular sensitivity to the cytotoxic effects of UV and X-ray radiation. Similarly, reduction of endogenous ASPP protein by using the antisense oligonucleotides to target ASPP1 and ASPP2 can reduce the efficacy of chemotherapeutic drugs such as cisplatin and etoposide (Trigiante, et *al.*, 2006). This further supports the notion that ASPP1 and ASPP2 expression has a crucial influence to cellular sensitivity to cancer treatment.

In contrast to ASPP1 and ASPP2, iASPP is an oncogene. Structurally, iASPP is similar to ASPP1 and ASPP2 except the N terminus of iASPP has no homology with ASPP1 and ASPP2 (Trigiante, et *al.*, 2006). Thus, iASPP can bind to p53 family protein as ASPP1 and ASPP2. However, its binding to p53 will inhibit the apoptotic function of p53 family proteins and increase the cellular resistance to apoptosis. Overexpression of iASPP could be observed in a variety of cancer, including breast carcinomas and certain leukemia (Bergamaschi et *al.*, 2003; Zhang et *al.*, 2005). It has been suggested that iASPP overexpression promotes leukemogenesis and disease progression of acute leukemia (Zhang et *al.*, 2005; Sullivan, A., 2007). It can also be understood that tumor cells with increased iASPP expression have higher resistant to chemotherapeutic drugs or radiation treatment (Trigiante, et *al.*, 2006; Sullivan, A., 2007).

*Epigenetic silencing of ASPP1 and ASPP2*

Now that epigenetic mechanism like CpG island hypermethylation in promoter region can lead to gene silencing, it is able to explain how downregulation or absence of a gene expression will be resulted even though there is no genetic mutation. By far, mutations of ASPP1 and ASPP2 genes have not yet been found (Agirre et *al.*, 2006; Liu et *al.*, 2005; Slee et *al.*, 2003). Reports about the loss of transactivating functions of wild type p53 were pointed to the epigenetic silencing of ASPP genes. Liu et *al.* (Liu et *al.*, 2005) investigated the methylation status of ASPP genes in breast carcinoma cell lines MCF-7, A549 and hepatocarcinoma cell lines HepG2 which these cell lines posses the wild type p53. They found that the mRNA levels of ASPP1 and ASPP2 were significantly downregulated. Results of the methylation-specific PCR showed that the CpG island of the 5'-untranslated region of both genes

were hypermethylated which was likely to be the reason for the downregulation of ASPP genes and hence the loss of pro-apoptotic function of p53. Agirre et *al.* (Agirre et *al.*, 2006) has the similar claim that methylation of the promoter of ASPP1 would lead to the inactivation of ASPP1 in acute lymphoblastic leukemia. Among the samples from 180 acute lymphoblastic leukemia patients, hypermethylation of ASPP1 promoter was found in 25% of cases with decreased ASPP1 mRNA expression. In addition, relapse and mortality rate were significantly higher in patients with methylated ASPP1. Thus, aberrant methylation of ASPP1 is one of the factors associated with poor prognosis of acute lymphoblastic leukemia patients.

### 1.7 Brief introduction of the present study

Although there is increasing number of reports about the aberrant expression for member of the protein PPP1R family, only limited studies have studied the functional role of those putative tumor suppressor genes. Previously, several candidates among the PPP1R family have been identified with downregulation in tumor samples comparing with that of the normal control by means of array comparative genomic hybridization. These candidates are PPP1R1B, PPP1R3B, PPP1R3C, PPP1R12A and PPP1R15A. In the present study, differentiation expression study of those candidates in selected esophageal carcinoma (ESCC) and nasopharygeal carcinoma cell lines was conducted by means of reverse transcriptase PCR (RT-PCR) to narrow down the possible targets. In the present study, we would like to focus on tumor suppressor genes which are silenced through epigenetic mechanisms. Methylation specific PCR and bisulfite genomic sequencing were performed to verify whether promoter CpG hypermethylation is the cause of the expression loss of the gene.

Full length cDNA of the putative tumor suppressor genes were cloned from the cDNA of normal tissue sample which will be used for the investigation of the functional role of the genes. To confirm the tumor suppressor role of the candidate genes, anchorage-dependent colony formation assay has been performed so as to reveal their growth inhibition ability on cancer cell lines. Total cell protein lysates from the candidate genes-transfected cancer cell lines were collected and western blots analysis was then carried out to find out the intracellular signaling pathway involved by the candidate genes. In addition, Renilla-firefly dual luciferase reporter assay system was used to study the effect of these candidate genes on specific target in transcriptional level. Subcellular localization of the protein of the putative tumor suppressor genes has also been determined by using the green fluorescence protein (GFP) tagged protein. Based on all the information collected, schematic models for the tumor suppressor role of the putative tumor suppressor genes will be proposed.

# Chapter 2

# Screening and Determination of epigenetic silenced protein phosphatase-1 regulatory genes with tumor suppressor functions

## 2.1 Introduction

### 2.1.1 *Brief introduction to PPP1R genes investigated in this study*

Although investigations on the expression level of protein phosphtase-1 regulatory subunits associating with carcinogenesis and their tumor suppressor functions have been very limited, most of their primary physiological roles in mammals have been well-studied. In fact, the discoveries of those regulatory subunits usually come from the investigation on particular physiological process. The physiological roles of the targeted protein phosphtase-1 regulatory subunits family members will be briefly described in the following section.

*Protein phosphatase 1, regulatory (inhibitor) subunit 1B, PPP1R1B*

*(NCBI reference sequence: NM_032192.2)*

PPP1R1B is encoded in human chromosome 17 locating at 17q12. The whole gene contains 8 exons with a total length of about 1841 bp. The full length PPP1R1B protein is DARPP-32 (dopamine and cAMP-regulated phosphoprotein $M_r$ 32kDa). PPP1R1B is abundant in brain and kidney while the subcellular localization of 1R1B is reported to be mainly in the cytosol (Hemmings et *al.*, 1986; Hemmings et *al.*, 1984). Thr-34 phosphorylated DARPP-32 is a potent PP1 inhibitor which serves to

promote locomotor activity in neurological level. In response to processes such as neurotransmitter stimulation of the receptor, changes in membrane potential or receptor modulation, inhibition of PP1 by DARPP-32 can relay the neuronal signal to activate different signaling pathways in postsynaptic region (Cohen, P. T. W., 2002).

In brain, DARPP-32 as well as PPP1α and PPP1γ1 are particularly enriched in the striatum (Ouimet et *al.*, 1984; Walaas et *al.*, 1984). As the name implied, DARPP-32 plays an important role in the dopamine-regulated signaling cascades, particularly in the areas of the brain receiving high dopaminergic input. It has been suggested that knockout of DARPP-32 in mice would diminish or lead to lack of responses to dopamine, psychostimulant and antipsychotic drugs, altered dopamine-induced activities of several ion channels, etc (Fienberg et *al.*, 1998). The importance of DARPP-32 in the striatum makes DARPP-32 often chosen as the target for the therapeutic use, abuse of the drugs and as afflicted area in motoric disorder such as Huntington's disesase. For instances, acute administration of amphetamines or cocaine can stimulates locomoter activity via increased release and inhibited reuptake of neurotransmitter like dopamine and serotonin which accompanied by an increased phosphorylation of DARPP-32 on Thr-34 (Greengard et *al.*, 1999). It was found that DARPP-32 knock out mice were less sensitive to ethanol reinforcement and self-administer less ethanol than wild type mice (Risinger et *al.*, 2001).

*Protein phosphatase 1, regulatory (inhibitor) subunit 3B, PPP1R3B*

*(NCBI reference sequence: NM_024607.2)*

PPP1R3B (or PPP1R4) is encoded in human chromosome 8 which locates at 8p23.1. The whole gene contains 2 exons with total length about 5564bp. It belongs

to a group of regulatory subunits (G subunits) which all are glycogen targeting and involves in glycogen metabolism. PPP1R3B is also denoted as $G_L$ because it mainly distributes in liver and the glycogen particles. $G_L$ can associate and form complex with PP1. $G_L$-PP1 complex is able to dephosphorylate glycogen synthase and turn glycogen synthase into an active form for the glycogen synthesis (Alemany et *al.*, 1986; Hubbard et *al.*, 1993; Doherty et *al.*, 1995). During fasting or unfeeded state, activity of $G_L$-PP1 complex is inhibited by phosphorylase *a* in allosteric manner. When glucose and insulin level increases, it will inactivate and decrease the level of phosphorylase *a*, respectively, which then alleviates the allosteric inhibition if $G_L$-PP1 complex (Armstrong et *al.*, 1998; Bollen et *al.*, 1998). It was suggested that low level or loss of $G_L$ with association of impaired glycogen synthase activity would be observed in diabetic or adrenalectomized and starved rats (Doherty et *al.*, 1998; Bollen et *al.*, 1983a; Bollen et *al.*, 1983b). Level of $G_L$ can be restored by refeeding the rat or supplied with insulin treatment which highlights the importance of $G_L$ and PP1 in the glycogen metabolism especially the activation of glycogen synthase.

*Protein phosphatase 1, regulatory (inhibitor) subunit 3C, PPP1R3C*

*(NCBI reference sequence: NM_005398.3)*

PPP1R3C (PPP1R5) is encoded in human chromosome 10 which is located at 10q23-q24. The whole gene contains 2 exons with a total length of about 2571bp. PPP1R3C is also called Protein Targeting to Glycogen (PTG). It belongs to the same class as PPP1R3B (G subunits). PPP1R3C is also denoted as $G_C$. It is widely distributed but especially abundant in liver, muscle and glycogen particles. PPP1R3C contains a binding site for glycogen and glycogen synthase (Den Hertog, J., 2003).

Similar to PPP1R3B, it can bind to PP1 to inhibit the phosphorylase phosphatase activity of PP1 and increase the glycogen accumulation (Doherty et *al.*, 1996).

PTG is linked to an autosomal recessive disorder Lafora disease, which is characterized by progressive neurological deterioration, myoclonus and epilepsy. Polyglucosan inclusion bodies consisting of very few branches are called the Lafora bodies. Lafora bodies accumulate in tissues such as neurons of the individuals with Lafora disease. In a normal individual, laforin binds to PTG and facilitates the interaction with malin for the ubiquitination of PTG, which is subsequently degraded in the proteosome (Dubey et *al.*, 2008; Worby et *al.*, 2008). Such process is thought to be essential for decreasing the glycogen accumulation. It is suggested that the etiology of lafora disease may be due to loss of malin or mutated laforin which result in poor interaction with PTG. It will then cause the reduction in PTG degradation and lead to increase of glycogen accumulation with inefficient branching, which is a possible reason for the formation lafora bodies (Dubey et *al.*, 2008).

*Protein phosphatase 1, regulatory (inhibitor) subunit 12A, PPP1R12A*

*(NCBI reference sequence: NM_002480.2)*

PPP1R12A is encoded in human chromosome 12 which is located at 12q15-q21. The whole gene contains 25 exons with a total length of about 4616bp. It is also known as myosin phosphatase targeting subunit (MYPT1) or simply called $M_{110}$. It is mainly distributed in smooth muscle and non-muscle cells myofibrils-myosin. Cooperation of muscle contraction and relaxation relies on the dynamic changes of actomyosin fibers which is tightly regulated by its reversible phosphorylation involving the activity of PP1 (Fernandez et *al.*, 1990). In response to a rise in intracellular $Ca^{2+}$ levels, Ser-19 of myosin P-light chains will be phosphorylated by

$Ca^{2+}$/calmodulin-regulated myosin light chain kinase to mediate smooth muscle contraction. For the smooth muscle relaxation, dephosphorylation of myosin P-light chains at ser-19 of is required which is catalyzed by the myosin phosphates. In muscle tissue, MYPT1 associates with PP1 catalytic subunit PP1β and a small myosin phosphatase targeting subunit such as $M_{21}$ to form a trimeric complex which is the scaffold of the myosin phosphatase. Thus, the presence of MYPT1 is necessary for the relaxation of actomysoin fibers by regulating the dephosphorylation as it targets PP1 to myosin and determines the substrate specificity of the phosphatase (Hartshorne et *al.*, 1998; Cohen, P. T. W., 1997).

*Protein phosphatase 1, regulatory (inhibitor) subunit 15A, PPP1R15A*

*(NCBI reference sequence: NM_014330.2)*

PPP1R15A is encoded in human chromosome 19 which is located at 19q13.2. The whole gene contains 3 exons with a total length of about 2349 bp. PPP1R15A is also called growth arrest and DNA damage protein 34kDa, GADD34. GADD34 is a growth arrest and DNA damage inducible gene up-regulated in response to DNA damage, cell cycle arrest and apoptosis (Fornace et *al.*, 1989; Hollander et *al.*, 1997). It has been suggested that association of GADD34 with PP1 plays a crucial role in protein synthesis by targeting the PP1 to eukaryotic translation initiation factor eIF2α, in which the phosphorylated eIF2α represses the protein translation by sequestering eIF2B and thus inhibits the assembly of translation initiation complexes (Novoa et *al.*, 2001; Wu et *al.*, 2002). In this case, PP1 acts as eIF2α phosphatase to aid the recovery of the cell from stress. It has been found that reduced level of GADD34 will lead to hyperphosphorylation of eIF2α and hence the inhibiton of protein synthesis (Munoz et *al.*, 2000). For example, there is loss of

GADD34-associated PP1 complex in the brain of ground squirrels during hibernation. At the time of recovery from hibernation, the level of the complex will be restored to dephosphorylate eIF2α (Connor et *al.*, 2001). It is suggested that expression of GADD34 will be up-regulated in response to global ischemia in the human brain to regulate the protein synthesis regulation and DNA repair. Hence, the survival of the cells will be affected (White et *al.*, 2004). On the other hand, study from Shi et *al.* (Shi et *al.*, 2004) has found that Smad7 recruited GADD34-PP1 complex to dephosphorylate TbetaRI and inhibited TGFβ-induced cell cycle arrest which was responsible for mediating TGF-β resistance in response to UV light irradiation.

### 2.1.2 *Demethylating agent used in derepressing gene silenced by promoter hypermethylation*

DNA methyltransferases and histone deacytelases are the enzymes responsible for the *de novo* methylation and histone deacetylation, respectively. Although the underlying mechanism for the inappropriate DNA methylation or histone deacetylation has not yet been known, it is believed that inhibitors of these enzymes may give a good treatment response to those epigenetic diseases including cancer with tumor suppressor genes silenced through epigenetic mechanism. Yoo et *al.* (Yoo et al., 2006) proposed that epigenetic defects in cancer cells can be efficiently reverted by means of pharmacologic inhibitors of the enzymes that are responsible for establishing or maintaining the epigenetic marks.

DNMT family, including DNMT1, DNMT3a and DNMT3b are the enzymes mediating the de novo DNA methylation. Nucleoside analogues and non-nucleoside analogues include 5-azacytidine, 5-aza-2'-deoxycytidine (5-aza-CdR), 5-fluoro-2'-deoxycytidine, 5,6-dihydro-5-azacytidine and zebularine have been

proved to be successful in inhibiting DNMTs enzyme activities (Sigalotti et al., 2007). Nucleoside analogues of cytidine are relatively powerful DNMT inhibitors and the most widely used epigenetic drugs. 5-aza-cytidine and 5-aza-CdR are the well-known cytidine analogues that are widely used in anti-cancer treatment because of their high potency with low intrinsic toxicity (Pliml et *al.*, 1964; Sorm et *al.*, 1968; Yoo et *al.*, 2006). Although their half-lives in aqueous solution are short, which will complicate their delivery to target tissues (Lin et *al.*, 1981; Notari et *al.*, 1975), they are extremely potent in inhibiting DNA methylation as low as micromolar concentration. In fact, 5-azacytidine and 5-aza-CdR were found to be successful in treating haematological diseases and received FDA approval for the treatment of myelodysplasia (Kaminskas et *al.*, 2005). Reactivation of genes including apoptotic genes and cell-cycle regulators take place after DNA methylation inhibition, which then leads to cell death and cell cycle arrest, etc are the common phenomena that happened in cancer cells after administration of epigenetic drugs like 5-aza-cytidine and 5-aza-CdR.

As histone deacetylation is mediated by HDAC, histone deacetylase inhibitor appears as a good agent for treating epigenetic diseases. Generally, HDAC inhibitor can be divided into four groups: short chain fatty acids, hydroxamic acids, cyclic tetrapeptides and benzamides (Yoo et *al.*, 2006). Short chain fatty acid has been widely used in the clinic and has already shown promising results for treating leukaemia (Raffous et *al.*, 2005; Yang et *al.*, 2005). However, their relatively low potency limited their uses in studying the structure and mechanism of HDAC inhibitors. Hydroxamic acids are by far the most promising and most widely accepted HDAC inhibitor for clinical uses. Their potency can be as low as micromolar to subnanomolar concentrations. For instances, trichostatin A (TSA),

derived from streptomyces, was shown to be very effective against tumor when it was used synergistically with the demethylating agent 5-aza-CdR in a mouse cancer model by promoting the reactivation of TSG (Cameron et *al.*, 1999). Synthetic compound Suberoylanilide hydroxamic acid (SAHA) is another example of hydroxamic acid effective in inhibiting HDAC. It inhibits HDAC by binding zinc ion (co-factor of the HDAC) and thus halts the activity of the enzyme (Finnin et *al.*, 1999). SAHA has been found to be useful in treating solid tumor, haematological malignacies (Kelly et *al.*, 2003) or advanced cancer and is safe even for a chronic oral treatment course (Kelly et *al.*, 2005). Compounds like cyclic tetrapeptides and benzamides have all been proven to be effective in anti-cancer treatment in different extent. Many of them are currently under clinical trials (Yoo et *al.*, 2006).

### 2.1.3 *Gene silencing through promoter hypermethylation as therapeutics approaches, diagnostic and prognostic marker for cancer patients*

In spite of the use of epigenetic drugs is somehow beneficial to the cancer patients based on the idea of reactivating the silenced TSG, lack of specificity is still a concern for the application of epigenetic drugs especially for histone demethylase inhibitor. As mentioned in 2.12, methyl markers can either represent active (H3-K4) or inactive transcription (H3-K9). Inhibition of histone demtheylase such as LSD1 will non-discriminately remove the methyl marker on both H3-K4 and H3-K9, which can be beneficial or detrimental to the patients. In addition, the low selectivity of epigenetic drugs may also pose the risk for activating the oncogene transcription upon the demethylation treatment.

Epigenetics can be very useful in diagnostics and preventive measures for the disease caused by gene silencing through epigenetic mechanism. Presentation of the

symptoms for the occurrence of cancer is often not obvious in the early stages of cancer and only manifests itself at the late stage, at which the primary tumor will have metastasized. Besides, poor accessibility for some kinds of tumors may lead to the difficulty and delay detection of malignancy for the individuals that contribute to the high mortality rates for cancer. It has been reported that DNA methylation changes would occur early in carcinogenesis and therefore are potentially good early indicators of existing malignancy and the risk assessment for the future development of disease (Laird, P. W., 1997). It is believed that aberrant methylation of particular gene can be detected in the patients' serum or plasma samples (Laird, P. W., 2003). The prevalence of many methylation markers in tumor is considerably higher than that of genetic markers and the free floating DNA releases from the dead cancer cells or detached tumor cells that the normal cells do not release DNA into the bodily fluid such as blood stream in ordinary condition (Laird, P. W., 2003) are some of the advantages of using methylation markers for detecting malignancy. Individuals who are without the history of malignancy and acquired neoplastic lesions but with epigenetic alterations, such as epimutation, aberrant DNA methylation or histone-modification, can be used as indicators of the likehood of developing cancer (Yoo et *al.*, 2006; Holst et *al.*, 2003). It should be noted that different types and stages of cancer acquire distinct epigenetic patterns. Thus, having a detailed map of specific epigenetic patterns in each tissue type in both the normal and cancerous states would help improve the possibility of early diagnosis of cancer or intervention of pre-malignancy by chemo-preventive measure such as epigenetic drugs treatment (Yoo et *al.*, 2006). On the other hand, the presence of tumor-specific methylation markers in the serum or plasma of patients may have some indications to the prognosis and patients' responses from the treatment. For instance, the presence of

aberrant methylation of ASPP1 is associated with poor prognosis of acute lymphoblastic leukemia patients as mentioned in the previous chapter (Agirre et *al.*, 2006). Therefore, mapping the methylation markers to a specific type of cancer will be beneficial to the diagnosis, therapeutic design and predicting prognostic outcome of cancer patients.

### 2.1.4 *Objective of the study*

In the present study, gene expression profiles of multiple carcinoma cell lines were obtained to see whether PPP1R1B and PPP1R3C were frequently silenced across different kinds of carcinoma cell lines. Using a bioinformatic approach, the promoter regions of PPP1R1B and PPP1R3C were analyzed to determine the presence of CpG island upstream of the transcription start site for both genes. In addition, MSP analysis was performed to determine whether the PPP1R1B and PPP1R3C silencing was due to promoter hypermethylation that led to inhibition of gene transcription. BGS was also done so as to confirm the result of MSP analysis and gave a high resolution of the methylation pattern within the CpG island of the promoter region. Inhibition of *de novo* DNA methylation was achieved by the genetic knockout of DNMT1 and DNMT3b, and pharmacological inhibition of DNA methytransferases and histone deacetylases by 5'-azacytidine and TSA, respectively, in order to see if promoter CpG islands of PPP1R1B and PPP1R3C are demethylated and re-express PPP1R1B and PPP1R3C in the carcinoma cell lines. Finally, the methylation status of PPP1R1B and PPP1R3C in primary ESCC tumor was investigated so as to evaluate the diagnostic value for using the methylation analysis of PPP1R1B and PPP1R3C as a marker for cancer formation in an individual.

## 2.2 Materials

### 2.2.1 *Cell lines and normal tissues*

There were totally 80 different tumor cell lines used for the gene expression profiling study. They included 5 NPC (C666-1, CNE1, HK1, HNE1 and HONE1), 1 larynx cancer (Fadu), 17 ESCC (EC1, EC18, EC109, HKESC1, HKESC2, HKESC3, KYSE30, KYSE70, KYSE140, KYSE150, KYSE180, KYSE220, KYSE270, KYSE410, KYSE450, KYSE510, KYSE520 and SLMT1), 13 HCC (Hep3B, HepG2, Huh1, Huh4, Huh6, Huh7, Mahlava, PLC/PRF/5, SNU387, SNU398, SNU423, SNU449, SNU475), 4 colorectal carcinoma (HCT116, HT-29, LoVo and SW480), 7 lung carcinoma (A427, A549, H292, H358, H-1299, H1650 and H1975), 9 breast carcinoma (BT549, MB231, MB468, MCF-7, SK-BR-3, T47D, YCC-B1, YCC-B3 and ZR-75-1), 14 gastric carcinoma (KatoIII, YCC1, YCC2, YCC3, YCC6, YCC7, YCC9, YCC10, YCC11, YCC16, AGS, MKN45, SNU1, SNU16) and 4 RCC (A498, Caki, Caki-2, HH050, HH244, RCC52, RCC98 and 786-0), 4 cervical carcinoma (C33A, Caski, Hela and SiHA), 3 prostate carcinoma (DU145, Lncap and PC3) and 2 natural killer cell (NK-YS and YT) cell lines.

The immortalized epithelial cell lines used in this study have many features resembling the normal epithelial cells. They include an immortalized nasopharyngeal epithelial cell line NP69 (Tsao, S. W. et *al.*, 2002), 3 immortalized esophageal epithelial cell lines NE1, NE2 and Het-1A (Stoner, G. D. et *al.*, 1991), 2 human embryonic kidney epithelial cell line 293HEK and RHEK-1 and 2 human mammary epithelial cell lines HMEC purchased from Lonza Bioscience, Switzerland and HMEpC purchased from Applied Biosystems, Foster City, CA. HCT116 with double genetic knockout of *DNMT1* and *DNMT3B* (HCT116/DKO, gift of Bert Vogelstein,

Johns Hopkins) was included in the methylation study; HCT116/DKO was maintained in complete growth medium supplemented with either 0.4 mg/ml geneticin or 0.05 mg/ml hygromycin.

Total RNA samples from normal human adult tissue RNA samples were purchased from Stratagene, La Jolla, CA, USA.

### 2.2.2 *Chemicals*

RNAsecure™ resupension solution was purchased from Ambion Inc., Austin, TX, USA. TRI® Reagent was the product of Molecular Research Center, Cincinnati, OH. GoTaq® Flexi DNA polymerase is the product of Promega Corporation, Madison, USA. AmpliTaq Gold, BigDye v3.1, GeneAmp® RNA PCR kit and MuLV reverse transcriptase were purchased from Applied Biosystems, Foster City, CA, USA. RNase inhibitor was the product of Roche Applied Science, Germany. Costar® Spin-X® Centrifuged Tube Filter was from Cole-Parmer, Vernon Hills, IL. pCR® 4-TOPO® vector was the product of Invitrogen, Carlsbad, CA. Agarose powder was from Cambrex BioScience, USA. 5-aza-2-deoxycytidine (5-Aza-CdR) was the product of Sigma-Aldrich Corporation, St. Louis, MO. Trichostatin A (TSA) was from Cayman Chemical Company, Ann Arbor, MI. GIBCO® RPMI1640 and Dulbecco's modified Eagle's medium (DMEM) were the product of invitrogen. Sodium metabisulfite and mineral oil were purchased from Sigma-Aldrich Corporation, St. Louis, MO. QIAEX II DNA Clean-Up System was the product of Qiagen, Germany.

## 2.3 Methodology

### 2.3.1 *Cell culture*

Cell lines were maintained in the corresponding cell culture medium (RPMI1640/ Dulbecco's modified Eagle's medium, DMEM) supplemented with 10% FBS and 100 U/ml penicillin/streptomycin. Cells were sub-cultured when the cell confluency reached 70-80%. For each passage, cells were trypsinized and then resuspended in complete growth medium. Cell suspension was centrifuged at 1500 rpm for 3 min to pellet down the cells. Cell pellet was resuspended in complete culture medium while the supernatant was discarded. Appropriate proportion of the cell was added into fresh culture medium and incubated at 37°C with 5% $CO_2$ in humidified chamber.

### 2.3.2 *DNA and RNA quantification*

The amount of nucleic acids was determined by measuring the UV absorbance of the sample ($OD_{260nm}$). The $OD_{260}$ of the Tris-HCl diluted nucleic acids was measured by spectrophotometer (Beckman DU650). Ratio of $OD_{260nm}$ to $OD_{280nm}$ was also determined to check for the purity of the nucleic acids in which only samples with value greater than 1.8 would be used for further experiment.

### 2.3.3 *Total RNA extraction and Genomic DNA extraction*

Total RNA was extracted by TRI® Reagent. Cells were harvested for total RNA extraction whenever 70-80% cell confluency was reached. Harvested cells were lysed with 1 ml TRI® Reagent per 10 $cm^2$ culture dish area, in which the cell lysate was passed through a pipette for several times and then subjected to vortex until the

mixture became homogenous. The corresponding homogenate was kept at room temperature for 5 min. Samples were added with about 1:5 volume ratio of chloroform to TRI® Reagent and mixed vigorously for a further 15 sec. The resultant mixture was allowed to stand at room temperature for 3 min. After that, the mixture was centrifuged at 12,000g for 15 min at 4°C. At this point, the mixture was separated into three phases: lower phenol-chloroform phase, the interphase and the upper colourless aqueous phase (contains RNA). The upper aqueous phase was collected which the RNA inside was precipitated by adding 1:2 volume ratio of isopropanol to TRI® Reagent used initially and incubated at room temperature for 10 min. RNA pellet was subsequently obtained after centrifugation (12,000g for 15 min, 4°C). Thereafter, the supernatant was aspirated while the RNA pellet was resuspended with about 1 ml of chilled 75% ethanol and centrifuged again at 7500 g for 5 min at 4°C in order to remove small fragments of nucleic acid. Finally, the RNA pellet obtained was then air-dried in room temperature and subsequently dissolved in RNAsecure™ resupension solution which would be stored at -80°C. The amount of RNA extracted was quantified by spectrophotometric method as mentioned in section 2.3.2.

### 2.3.4 *Genomic DNA extraction*

The interphase and the lower phenol-chloroform organic phase obtained as mentioned in section 2.3.3 were used for genomic DNA extraction. The interphase and lower organic phase were collected and added with 0.3 ml of absolute ethanol for every 1 ml TRI® Reagent initially used. The mixture was incubated at room temperature for 3 min and subjected to centrifugation (2,000 g for 5 min at 4°C). The supernatant was aspirated gently while the DNA pellet was washed twice with equal

volume of 0.1 M sodium citrate-10% ethanol as the TRI® reagent used initially. For each wash, the DNA pellets were incubated in washing solution at room temperature for 30 min and then centrifugated at 2,000 g for 5 minutes at 4°C. After that, the DNA pellets were re-suspended in 75% ethanol (1.5-2 volume of TRI® reagent used) and left at room temperature for 20 min. After the incubation, samples were centrifuged again at 2,000 g for 5 minutes at 4°C. DNA pellets were then air-dried at room temperature. Subsequently, DNA pellets were added with 500 µl of 8 mM NaOH for every 50-70 mg of tissues or 10 x $10^6$ cells and allowed to dissolve at 4°C for overnight. The pH of the samples was corrected to 8.0 with HEPES buffer according to the manufacturer's protocol (Gibco BRL). Amount and purity of the DNA in the samples were determined by spectrophotometric method as mentioned in section 2.3.2 and then stored at 4°C.

### 2.3.5 *Reverse transcription (RT)*

Total RNA extracted from culture cells as described in section 2.3.3 was converted to complementary DNA (cDNA) by reverse transcription (RT) using the GeneAmp® RNA PCR kit according to the manufacturer's protocol. Briefly, 1 µg of total RNA was used for every 20 µl total reaction volume. Adequate amount of RNA was added in Diethyl pyrocarbonate (DEPC) -treated water to a final volume of 3.8 µl. Before the RT reaction, RNA samples were first denatured at 90°C for 5 min. The RNA samples were then mixed with other RT reaction components including 4 µl of 25 mM $MgCl_2$, 2 µl of 10x PCR Buffer II, 8 µl of 10 mM dNTP mix, 1 µl of 50 µM random hexamer, 0.2 µl of RNase inhibitor (40U/µl) and 1 µl of MuLV reverse transcriptase (50U/µl). The final RT mixtures were kept at room temperature for 10 min and then subjected to RT reaction. Mixtures were incubated at 42°C for 1 hr for

cDNA synthesis. Finally, cDNA samples were incubated at 99°C for 5 min and would be stored at -80°C.

### 2.3.6 *Semi-quantitative RT-PCR*

Pairs of gene specific primers spanning across two different exons (~ 200bp-300bp) of PPP1R1B [viz. 5' -ACCGCAAGAAGATCCAGTTC- 3' (forward primer) and 5' -TTCAGCGAAGGTGGTGTGTA- 3' (reverse primer)] and PPP1R3C [viz. 5' -TCTCTGCCTAATGAGCTGCA- 3' (forward primer) and 5' –AGCAAACACAACGCGCTTCT- 3' (reverse primer)] were designed. Designs of the primers were based on the mRNA sequence obtained from NCBI Genbank database (*Homo Sapiens Genome - Build 35.1*). The DNA polymerase for the RT–PCR was GoTaq® Flexi DNA polymerase while the recipe for the PCR reaction mixture was shown in the following table:

| Components | Volume used (μl) | Final Concentration |
|---|---|---|
| 10x PCR buffer | 1 | 1x |
| $MgCl_2$ | 0.5 | 2 mM |
| GoTaq® Flexi DNA polymerase (5 U/μl) | 0.5 | 1.25 U/50μl |
| Forward primer (10 μM) | 0.75 | 0.6 μM |
| Reverse primer (10 μM) | 0.75 | 0.6 μM |
| cDNA template | 2.5 | about 0.5μg/50μl |
| Autoclave distilled water | 6.5 | |
| *Total volume* | 12.5 | |

Table 2.1 Components of each RT-PCR reaction mixture

The PCR reaction was performed by using MJ DNA Engine Dyad® Thermal Cycler (MJ research, Waltham, MA) with conditions described as the following: Firstly, DNA samples were initially denatured at 95°C for 10 min. Then, they were PCR-amplified by the following thermal cycle (denaturation: 94°C for 30 s, annealing: 55-60°C for 30 s, extension: 72°C for 30 s) for around 32 rounds. A 10-min final extension at 72°C was implemented immediately after the thermal

cycling. PCR products were electrophoresed in 1.8-2% agarose gel and visualized under UV illumination and were documented by the Bio-Rad digital gel documentation system using the Quantity One® 1-D analysis software (Bio-Rad, Hercules, CA). Linear amplification of DNA samples could be obtained if thermal cycling between 25 and 40 cycles was performed as shown by standard curves of RT–PCR cycle number versus product yield for cellular genes (data not shown). Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) was used as an internal control for RNA integrity and reference of sample loading, in which 25 cycles were used for the amplification.

### 2.3.7 *Agarose Gel electrophoresis*

PCR products and DNA plasmids were electrophoresed in 1.8 or 2.0% (w/v) agarose gels. In brief, agarose gel was prepared by adding proper amount of agarose powder to 1X TAE buffer and followed by heating in microwave oven until the powder melted and dissolved completely. The resultant solution was allowed to cool added with trace amount of ethidium bromide (EtBr). The mixture was poured into the tray with combs inserted and allowed to cool and solidify. DNA samples added with 6x loading dye were loaded into the wells accordingly and electrophoresed (120V, 25 min) in the prepared agarose gel which was completely immersed in 1x TAE buffer. Agarose gel with resolved DNA samples was viewed under UV illumination (Bio-Rad, Hercules, CA).

### 2.3.8 *CpG island analysis*

CpG island located within the region around the core promoter, exon 1 and intron 1 of the candidate gene was analyzed by the online CpG island searcher

(http://www.uscnorris.com/cpgislands/cpg.cgi). The criteria for defining CpG island are set as CG content > 55%, ratio of observed CpG to expected CpG > 0.65 and minimal length of 500bp.

### 2.3.9 *Sodium bisulfite modification of DNA*

Bisulfite modification of DNA using sodium metabisulfite was carried out as follows: Firstly, Genomic DNA (1-5 μg) was denatured by incubating with 3M NaOH for 15 min at 37°C. For the preparation of sodium metabisulphite solution, 2.8 ml of distilled water was added into the tube containing 1.82 g of sodium metabisulfite and then followed by the addition of 430 μl of 3 M NaOH to make up the final concentration of sodium metabisulite to 2.4 M. Sodium metabisulfite powder was allowed to dissolved and gently mixed. The pH value (5.0-5.2) was monitored during the preparation. When sodium metabisulfite was completely dissolved, 210 μl of freshly prepared 10 mM hydroquinone (prepared by adding 50 ml distilled water with 0.055 g hydroquinone) was immediately added to obtain 333 μl sodium metabisulfite/hydroquinone solution. The reaction mixture was overlaid with 3 drops of mineral oil and incubated at 55°C in dark for 4 hr. Denatured DNA beneath the mineral oil was then carefully transferred to a new centrifuge tube.

For post-bisulfite treatment, the free bisulphite was desalted by QIAEX II DNA Clean-Up System according to the manufacturer's protocol. Briefly, sample was added with 1ml QXI and subsequently mixed with QX II (16 μl for every 2 μg DNA). Samples were incubated at room temperature for 1 hr with agitation. After the incubation, samples were washed twice with Buffer PE. The bisulfite treated DNA was dissolved with 50 μl of TE buffer and incubated with 0.3 M NaOH at 37°C for 15 min. Samples were then neutralized by mixing with 3 M Ammonium acetate (pH

7.0) which the final pH value of the samples were adjusted to below 7.0 by using sodium acetate. The bisulfited DNA was extracted by using QIAEX II according to the manufacturer's protocol. The bisulfited DNA was eluted with TE (20 μl /μg DNA) and then stored at -20°C.

2.3.10 *Methylation-specific PCR (MSP)*

The methylation status of the CpG island within the promoter region of the candidate gene was assessed by MSP (Herman, J. G. et *al.*, 1996). For each MSP analysis, two sets of primers, methylated (M) [PPP1R1B: viz. 5' -GAGTTTAGT ATAGATCGTCGTC- 3' (forward primer) and 5' -ATCGAACTAACTAAAAAACGCG- 3' (reverse primer)] and PPP1R3C: viz. 5' -CGGAGTTTTAGAATTTTTTCGC- 3' (forward primer) and 5' -CCTCGAACCCCAAAACGCG- 3' (reverse primer)] and unmethylated (U) [PPP1R1B: viz. 5' - GGAGTTTAGTATAGATTG TTGTT- 3' (forward primer) and 5' - AAAATC AAACTAACTAAAAAACACA- 3' (reverse primer)] and PPP1R3C: viz. 5' -TGTGGAGTTTTAGAATTTTTTTGT- 3' (forward primer) and 5' -AACCTCAAACCCCAAAACACA- 3' (reverse primer)] were designed which could specifically anneal to methylated and unmethylated sequences of the bisulphite treated DNA, respectively. Unmethylated cytosine would be converted to uracil upon bisulphite treatment, while leaving the methylated cytosine unaffected and uracil would be subsequently converted to thymidine in the PCR reaction. The methylated and unmethylated DNA could then be distinguished by analyzing the PCR products.

The DNA polymerase for MSP was AmpliTaq Gold while the recipe for the PCR reaction mixture was shown in the following table:

| Components | Volume used (μl) | Final Concentration |
|---|---|---|
| 10x PCR buffer | 1.25 | 1x |
| dNTPs (2.5 mM each) | 1 | 0.2 mM |
| $MgCl_2$ (25 mM) | 1 | 2 mM |
| Forward M or U primer (10 μM) | 0.75 | 0.6 μM |
| Reverse M or U primer (10 μM) | 0.75 | 0.6 μM |
| AmpliTaq Gold (5 U/μl) | 0.0625 | 2.5 U/50μl |
| Bisulfite-treated DNA | 0.5 | 0.1 μg/100μl |
| Autoclaved distilled water | 7.1875 | |
| ***Total volume*** | 12.5μl | |

Table 2.2. Components of each reaction mixture for MSP

The PCR reaction was performed by using MJ DNA Engine Dyad® Thermal Cycler (MJ research, Waltham, MA) with conditions described as the follows: Firstly, DNA samples were initially denatured at 95°C for 10 min. Then, they were PCR-amplified by the following respective thermal cycle (denaturation: 94°C for 30 s, annealing: 58°C for the U primers and 60°C for the M primers for 30 s, extension: 72°C for 30 s) for around 40 rounds. A 10-min final extension at 72°C was implemented immediately after the thermal cycling. PCR products were analyzed with the method described in section 2.3.6. Specificity of the MSP primers towards the methylated or unmethylated sequences were all tested and confirmed prior to the actual MPS analysis.

### 2.3.11 *Bisulfite genomic sequencing (BGS)*

The position and the relative abundance of methylated CpG dinucleotide within interested region was revealed by BGS (Frommer et *al.*, 1992). The bisulfite-treated DNA was PCR-amplified with the use of a pair of BGS primers [PPP1R1B: viz. 5' - TATTTAGGAGGGGAGAGATAT- 3' (forward primer) and 5' - CCTTACCATCTCCACCTAAC- 3' (reverse primer)] and PPP1R3C [viz. 5' -TTA TATTTTTTGTGTGTAGATTTATT- 3' (forward primer) and 5' -AATACAACTC ATTAAACAAAAAAAC- 3' (reverse primer)] targeting the region of interest. The

procedures for the PCR reaction for BGS were similar to that of MSP except the final extension time was 1 hr instead 5 min in order to produce full length PCR products with adenylation at 3' end. The PCR product was resolved by agarose electrophoresis as described in section 2.3.6. PCR band of correct size was revealed and excised under UV illumination. The excised gel was purified with the use of Costar® Spin-X® Centrifuged Tube Filter. The purified PCR products was cloned into the pCR® 4-TOPO® vector according to the manufacturer's protocol. The ligated vector was transformed into *E. coli* strain DH5α by heat-shock method. At least six bacterial colonies were randomly chosen, cultured overnight, of which the plasmid DNA were extracted and prepared for DNA sequencing.

### 2.3.12 *Epigenetic Drug treatments*

In this study, 5-Aza-CdR and TSA were used as the demethylating agent and inhibitor of histone deacetylase, respectively. For demethylation treatment of cancer cell lines, 1 x $10^5$ of respective carcinoma cells were seeded in a 6-well plate and allowed to grow overnight. After the incubation, culture medium was aspirated. Fresh culture medium with 10 μM 5-Aza-CdR was added to the cell samples and allowed to grow for 3 days with refreshment of the culture medium containing 5-Aza-CdR every 24 hr. After that, cells were harvested for genomic DNA and total RNA extraction. For the synergistic treatment of 5-Aza-CdR and TSA, cells were first treated with 5-Aza-CdR as mentioned above and then subjected to TSA (100 ng/ml in DMSO) treatment for an additional 24 hr. Finally, cells were harvested for genomic DNA and total RNA extraction.

## 2.4 Results

### 2.4.1 *RT-PCR for the screening of putative tumor suppressor genes*

We selected 18 carcinoma cell lines, including NPC (C666-1, CNE1, HK1 and HONE1), ESCC (EC1, EC18, HKESC1, HKESC2, HKESC3, KYSE180, KYSE220, KYSE410, KYSE510, KYSE520, SLMT1) and lymphoma cells (NK-YS, Rael and L1236) for the first round screening of tumor suppressor genes (Fig. 2.1). From the result of semi-quantitative RT-PCR, it was shown that PPP1R12A and PPP1R15A expressed in all selected cell lines while PPP1R3B was found silenced only in NK-YS cells. For PPP1R1B, it was silenced in 14 out of 18 selected cells lines and weak expression could only be detected in the 4 cell lines (HKESC2, KYSE220, KYSE410 and SLMT1). For PPP1R3C, very weak or loss of expression was detected in 8 cell lines including C666-1, HKESC3, KYSE180, KYSE410, SLMT1, NK-YS, Rael and L1236. Comparatively, silencing of PPP1R1B and PPP1R3C were more frequently observed in cancer cells so that they were chosen for further study.

### 2.4.2 *Gene expression profiles of putative TSG among different normal adult tissues and cancer cell lines*

*PPP1R1B normally expresses in most of the adult tissue but frequently downregulated in carcinomas cells*

First of all, we would like to see PPP1R1B expression status in a set of major normal adult tissues including Liver, Brain, colon, etc. From the result of semi-quantitative RT-PCR, PPP1R1B expressed in most of the tissue we have chosen

except liver, spleen, placenta and bone morrow (Fig. 2.2a). It could also be observed that PPP1R1B did not express in Peripheral blood mononuclear cell (PBMC). For the gene expression profile of PPP1R1B in multiple cancer cell lines (Fig. 2.3a), normal expression of PPP1R1B gene could be detected in immortalized normal cell lines including nasopharyngeal (NP69), esophageal (HET1, NE1 and NE3) and breast epithelial cells (HMEC and HMEpC). In contrast, PPP1R1B was frequently downregulated or silenced in the tested carcinoma cells including nasopharyngeal, esophageal, lung, breast, cervix, renal, gastric and prostate.

*PPP1R3C normally express in most of the adult tissue but frequently downregulated in carcinomas cells*

The expression status of PPP1R3C was studied by a similar fashion in a set of major normal adult tissue including Liver, Brain, colon, etc (Fig. 2.2b). From the result of semi-quantitative RT-PCR, it could be seen that PPP1R3C expressed in almost all of the tissue we have chosen except bone morrow. In addition, PPP1R3C did not express in PBMC. It could also be observed that PPP1R3C express strongly among the immortalized normal cell lines such as nasopharyngeal (NP69), esophageal (HET1, NE1 and NE3) and breast epithelial cells (HMEC and HMEpC). For the expression status of PPP1R3C in multiple cancer cell lines (Fig. 2.3b), down-regulation or silencing of PPP1R3C could be seen in the carcinoma cell lines like C666-1 (nasopharyngeal), KYSE150 (esophageal), breast, Caski (cervix), YCC-9 (gastric) and HCT116 (colon).

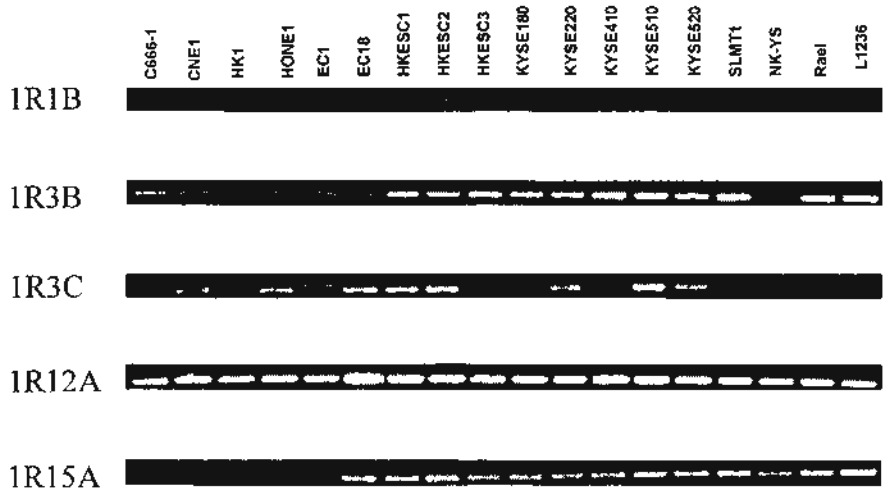



Fig. 2.1 Screening for the putative TSGs among the chosen protein phosphatase-1 regulatory subunits. Expression status of protein phosphatase-1 regulatory subunits in the carcinoma cell lines were determined by means of semi-quantitative RT-PCR.

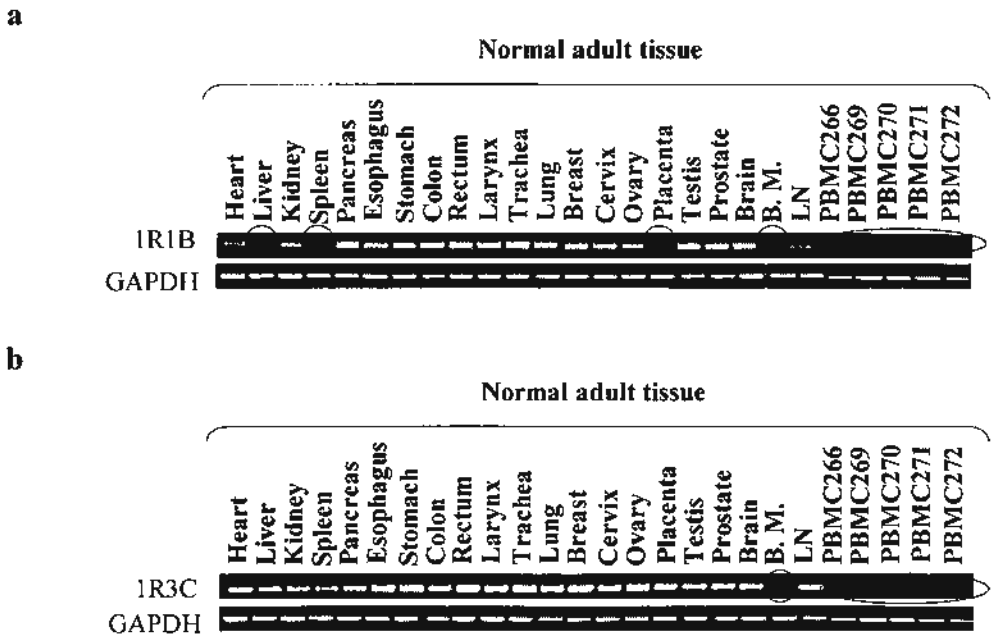



Fig. 2.2 Determination of (a) PPP1R1B and (b) PPP1R3C expression status in human major tissues by means of Reverse transcriptase PCR (RT-PCR).

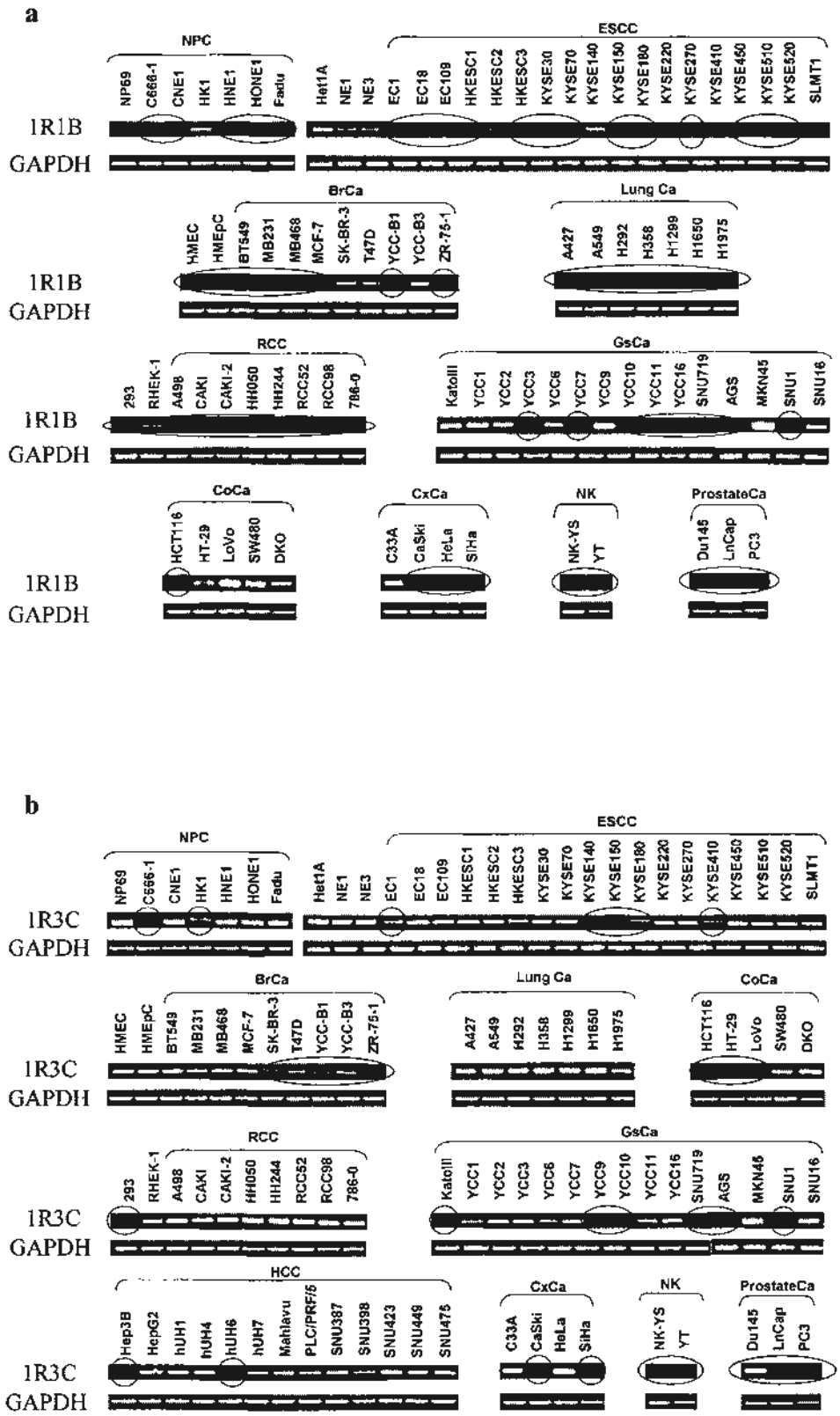


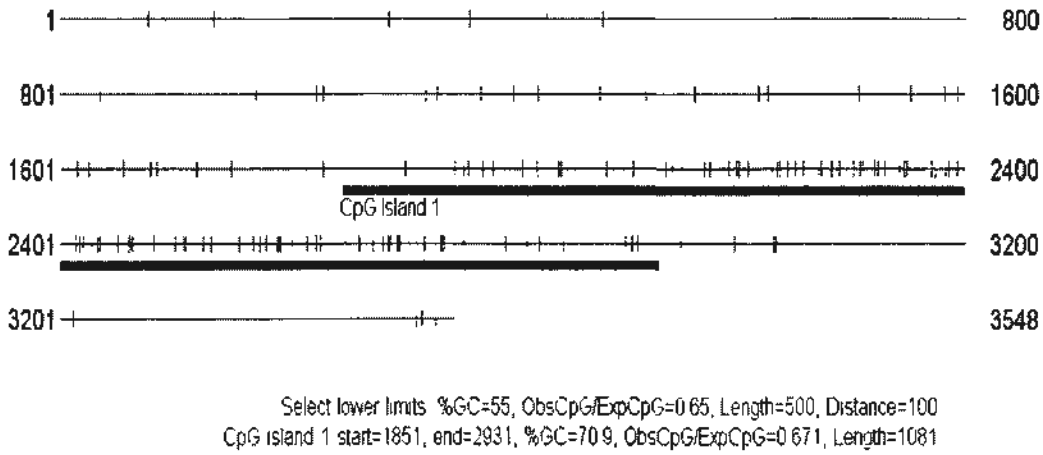

Fig. 2.3 Gene expression profiles of (a) PPP1R1B and (b) PPP1R3C across different carcinoma cell lines.

### 2.4.3 *Bioinformatic search for CpG island in the promoter region of PPP1R1B and analysis of its methylation status by MSP and high resolution BGS*

Using an online CpG island finder (http://cpgislands.usc.edu/), a CpG island spanning across the promoter region and transcription start site could be located (Fig. 2.4a). MSP was performed to determine whether the mechanism of PPP1R1B silencing is through an epigenetic mechanism. For the immortalized normal epithelial cells with PPP1R1B mRNA normally transcribed, CpG islands of PPP1R1B in promoter region were unmethylated as indicated by *U* band in the PCR result. In the meantime, PCR bands of M were detected in carcinomas cell lines (e.g. C666-1, HCT116, Caski, H1299) with silenced PPP1R1B. It indicated that PPP1R1B silencing was due to hypermethylation of CpG in their promoter region (Fig. 2.4b).

BGS was performed in which the methylation status of 50 individual CpG sites were examined (- 352 to + 87, relative to transcription start site) in order to reveal the degree of methylation status with higher resolution in the CpG island of promoter of PPP1R1B gene. Results of BGS analysis revealed that CpG islands were unmethylated in immortalized normal nasopharyngeal epithelial cells (NP69) but were densely methylated in those carcinomas cell lines (C666-1, Caski, HCT116, HONE1) with silenced PPP1R1B (Fig. 2.5). Such result was consistent with those of MSP which further confirmed transcriptional silencing of PPP1R1B is most likely due to promoter hypermethylation.

a)




b)

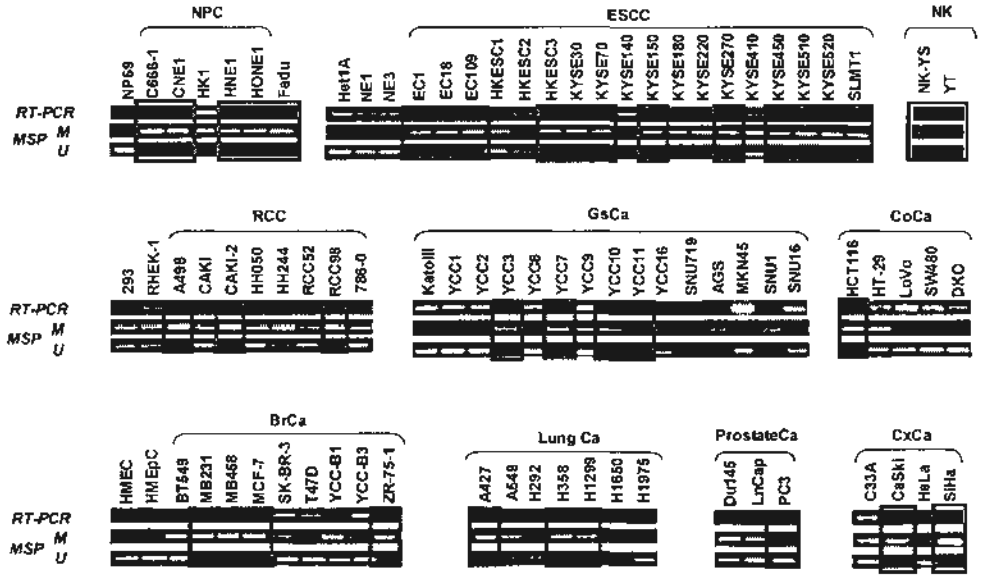



Fig. 2.4 Determination of the methylation status of the CpG island around the promoter region of PPP1R1B gene. a) CpG island around the promoter region of PPP1R1B gene was identified by bioinformatic approaches b) PPP1R1B expression silencing in multiple carcinoma cells was mostly due to promoter CpG island hypermethylation as shown by the result of MSP. Methylation status was denoted by M (methylated) or U (unmethylated).

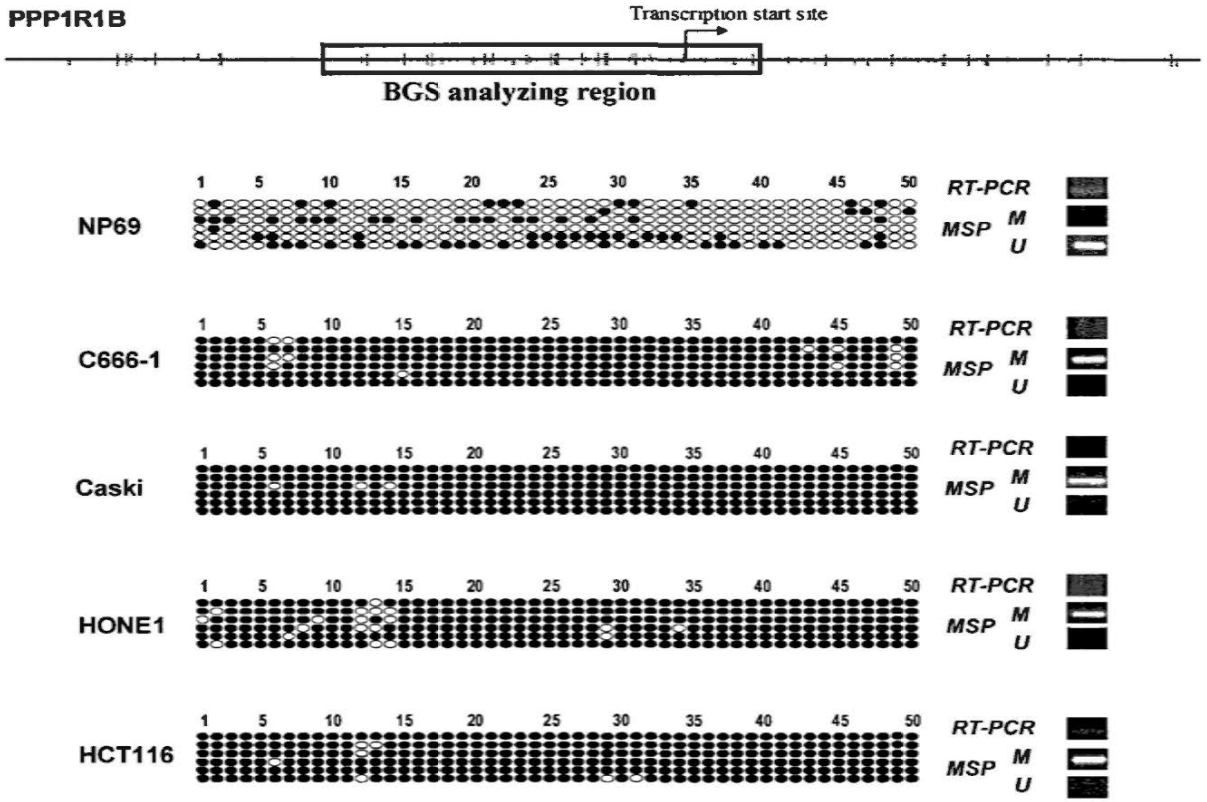


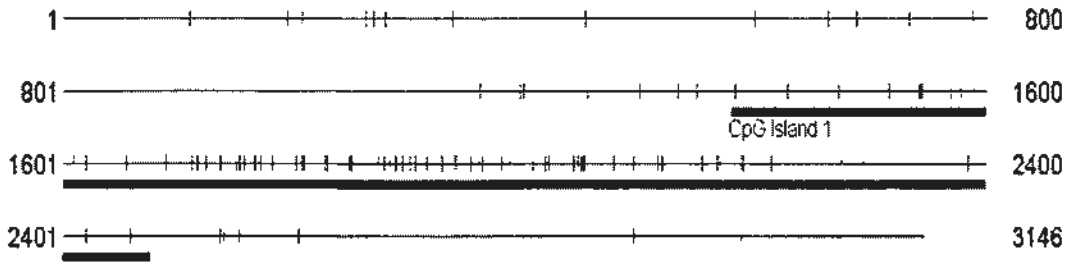

Fig. 2.5 High-resolution bisulphite genomic sequencing revealing the details methylation pattern around the selected promoter region of PPP1R1B gene. Results of the BGS were well-correlated with the result of RT-PCR and MSP. Methylated CpG dinucleotides were denoted by filled-circles while unmethylated CpG dinucleotides were denoted by open-cirlces.

### 2.4.4 *Bioinformatic search for CpG island in the promoter region of PPP1R3C and analysis of its methylation status by MSP and high resolution BGS*

Using an online CpG island finder (http://cpgislands.usc.edu/), a CpG island spanning across the promoter region and transcription start site could be located (Fig. 2.6a). MSP was performed to determine whether the mechanism of PPP1R3C silencing through an epigenetic mechanism. For the immortalized normal epithelial cells with PPP1R3C mRNA normally transcribed, CpG islands of PPP1R3C in promoter region were unmethylated as indicated by *U* band in the PCR result. In the meantime, PCR bands of M were detected in carcinomas cell lines (e.g. C666-1, HCT116, KYSE150, Caski) with silenced PPP1R3C. It indicated that PPP1R1B silencing was due to hypermethylation of CpG in their promoter region (Fig. 2.6b).

BGS was performed in which the methylation status of 46 individual CpG sites were examined (- 504 to + 14, relative to transcription start site) in order to reveal the degree of methylation status with higher resolution in the CpG island of promoter of PPP1R3C gene. Results of BGS analysis revealed that CpG islands were densely methylated in those carcinomas cell lines with silenced PPP1R3C. Results of BGS analysis revealed that CpG islands were unmethylated in immortalized normal nasopharyngeal epithelial cells (NP69) but were densely methylated in those carcinomas cell lines (C666-1, Caski, HCT116, KYSE150) were densely methylated in those carcinomas cell lines with silenced PPP1R3C (Fig. 2.7). Such result was consistent with those of MSP. It indicated that promoter hypermethylation is the most likely cause for the transcriptional silencing of PPP1R3C.

**a)**




**b)**

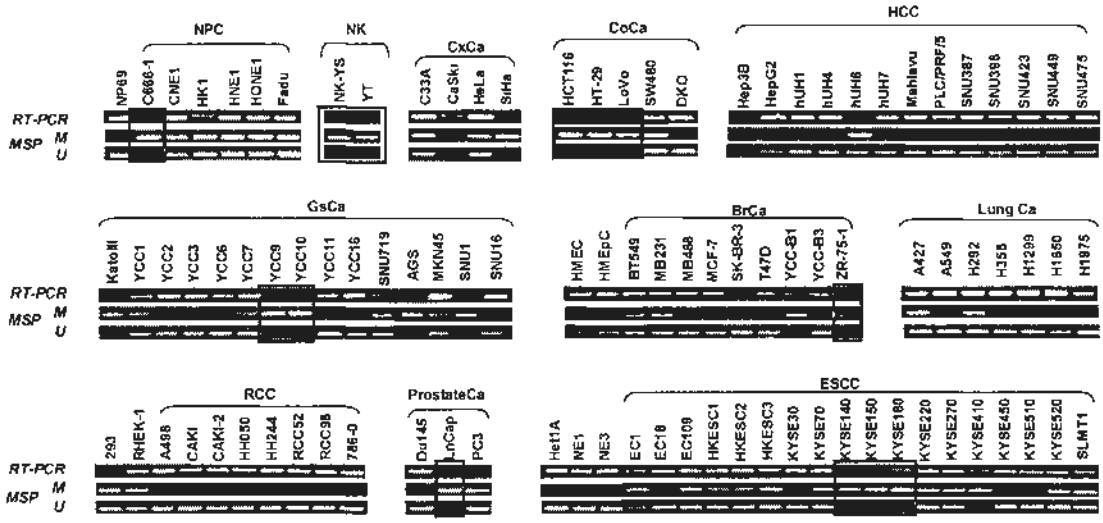



Fig. 2.6 Determination of the methylation status of the CpG island around the promoter region of PPP1R3C gene. a) CpG island around the promoter region of PPP1R3C gene was identified by bioinformatic approaches b) PPP1R3C expression silencing in multiple carcinoma cells was mostly due to promoter CpG island hypermethylation as shown by the result of MSP. Methylation status was denoted by M (methylated) or U (unmethylated).

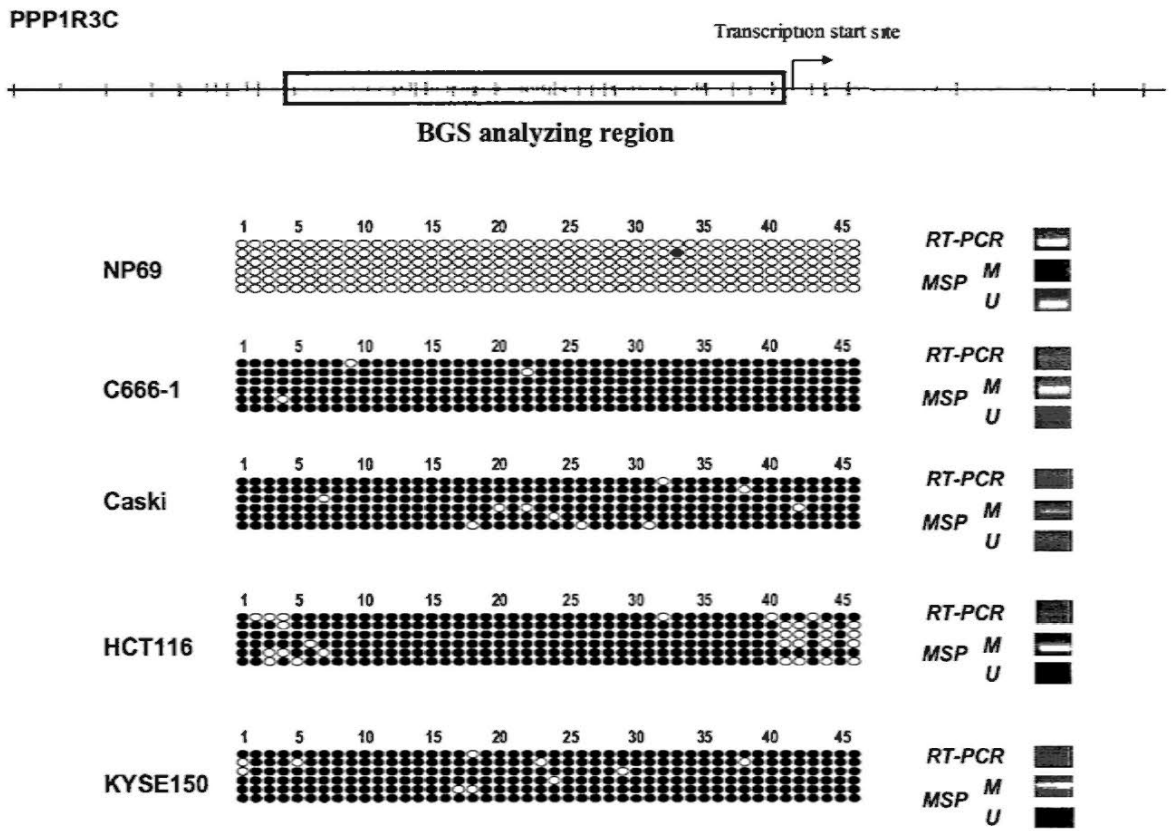



Fig. 2.7 High-resolution bisulphite genomic sequencing revealing the details methylation pattern around the selected promoter region of PPP1R3C gene. Results of the BGS were well-correlated with the result of RT-PCR and MSP. Methylated CpG dinucleotides were denoted by filled-circles while unmethylated CpG dinucleotides were denoted by open-cirlces.

### 2.4.5 *Demethylation treatment reduced promoter methylation and restore silenced gene expression*

Two different approaches were employed in the investigation of TSG expression upon demethylation treatment. On one hand, we would like to see the effect of pharmacological treatment on the expression of PPP1R1B (Fig. 2.8A) and PPP1R3C (Fig. 2.9A). On the other hand, we would like to see whether similar effect could be seen if a genetic approach was used. For the pharmacological treatment, cancer cell lines included C666-1 (NPC), HCT116 (colorectal carcinoma) and KYSE150 (ESCC) were co-treated with DNA methyltransferase inhibitor 5'aza-cytidine and histone deacetylase inhibitor TSA. Result of the RT-PCR revealed that expressions of PPP1R1B and PPP1R3C were restored after the treatment of 5' aza-cytidine and TSA. Furthermore, such restoration of the TSG expression was likely due to the promoter CpG demethylation according to the result of MSP (Fig. 2.8A & Fig. 2.9A). High-resolution BGS confirmed that a significant amount of CpG dinucleotides in the promoter region were demethylated (Fig. 2.8A & Fig. 2.9A). For the genetic approach, we found that PPP1R1B and PPP1R3C could be activated in the colorectal cell line HCT116 with double knockout of both DNMT1 and DNMT3B (DKO cell line) in which both genes were hypermethylated in wild type HCT116 cells (Fig. 2.8A & 2.9B). The results of MSP and BGS analysis indicated that the promoter of PPP1R1B (Fig. 2.8) and PPP1R3C (Fig. 2.9B & Fig. 2.9C) were unmethylated in HCT116 DKO cells. All the above results suggested the possible involvement of DNMT1 and DNMT3B in the maintenance of promoter methylation for PPP1R1B and PPP1R3C genes.

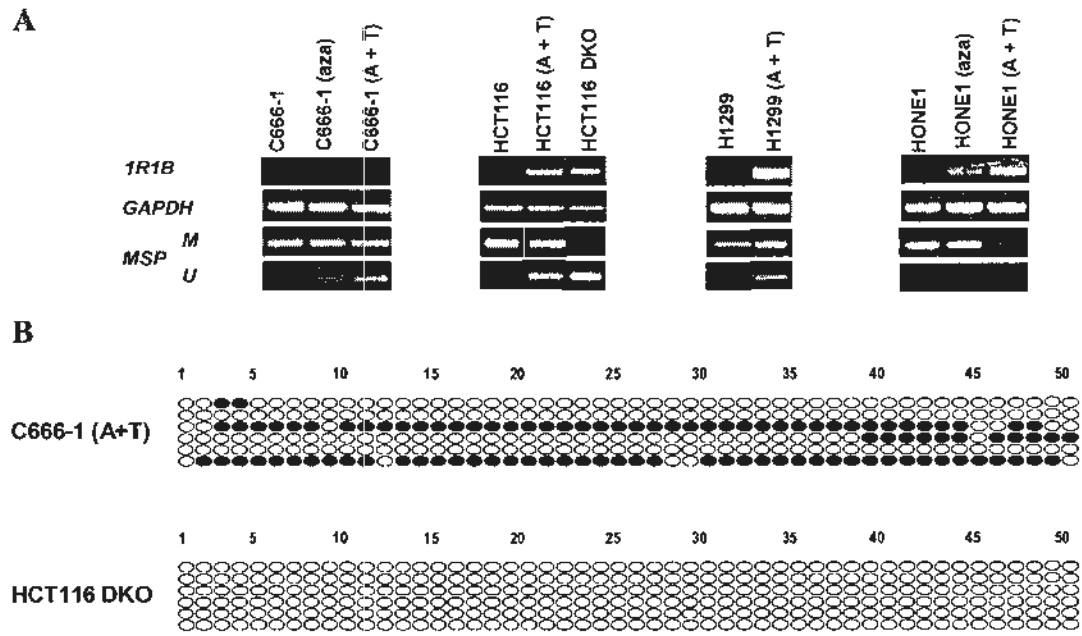



Fig. 2.8 Restoration of PPP1R1B expression upon inhibition of *de novo* methylation co-treatment of 5'aza-cytidine and Trichostatin and Genetic knockout of *DNMT1* and *DNMT3B* restored PPP1R1B expression in C666-1, HCT116 cells, H1299 and HONE1 cells. CpG island within PPP1R1B promoter region were demethylated by pharmacological inhibition or knockout genetic knockout of DNA methyltransferases as shown by (A) MSP and (B) high resolution BGS analysis.

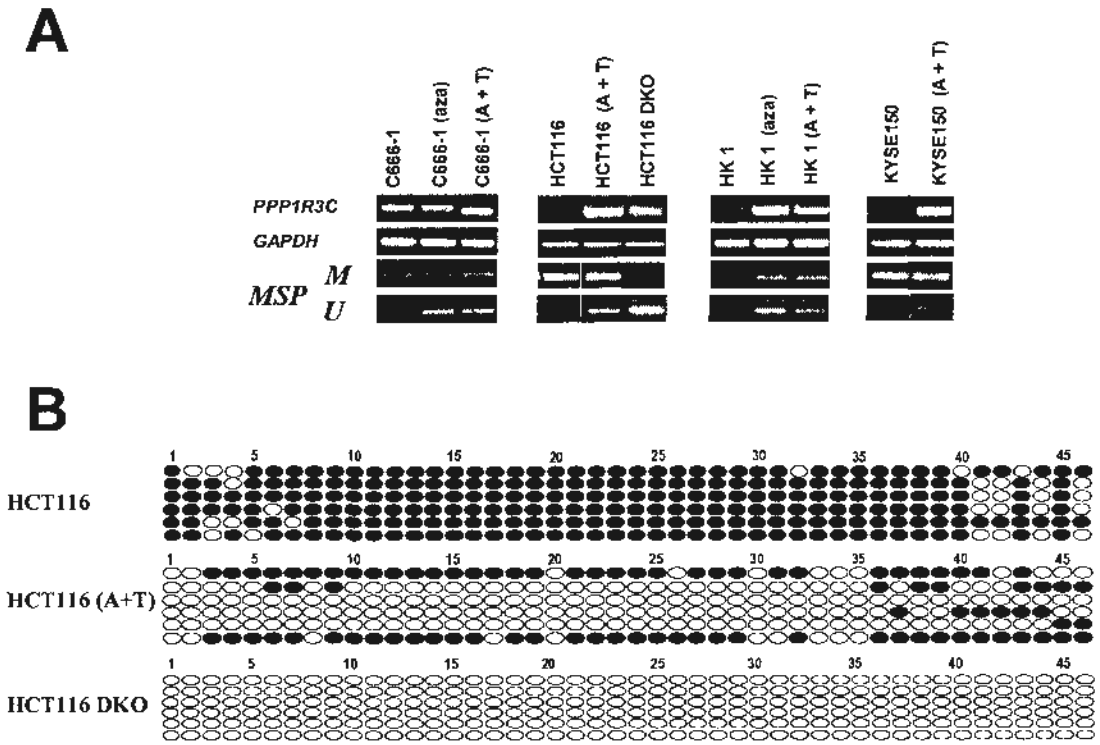



Fig. 2.9 Restoration of PPP1R3C expression upon inhibition of *de novo* methylation Co-treatment of 5'aza-cytidine and Trichostatin and Genetic knockout of *DNMT1* and *DNMT3B* restored PPP1R3C expression in C666-1, HCT116, HK1 and KYSE150 cells. CpG island within PPP1R3C promoter region were demethylated by pharmacological inhibition or knockout genctic knockout of DNA methyltransferases as shown by (A) MSP and (B) high resolution BGS analysis.

### 2.4.6 *Methylation status of PPP1R1B and PPP1R3C in primary NPC tumor*

We have further investigated the presence of methylation in a sample of primary ESCC tumors with their corresponding normal epithelial tissues as controls (Fig. 2.10). PPP1R1B methylation was detected in most of the ESCC primary tumor samples, except sample 33 (12/13). Aberrant methylation was also detected in about half of the surgical marginal esophageal tissues from patients with esophageal carcinoma, which could be due to the appearance of premalignant lesions or the presence of small number of tumors cells disseminated into the non-tumorous region. For PPP1R3C, methylation was detected in more than a half of the ESCC tumor samples (7/13) while none of the 13 normal epithelial tissues had promoter hypermethylation. These results indicated that hypermethylations of PPP1R1B and PPP1R3C promoters could be important in ESCC tumorigenesis. In particular, hypermethylation of PPP1R3C promoter is tumor specific.

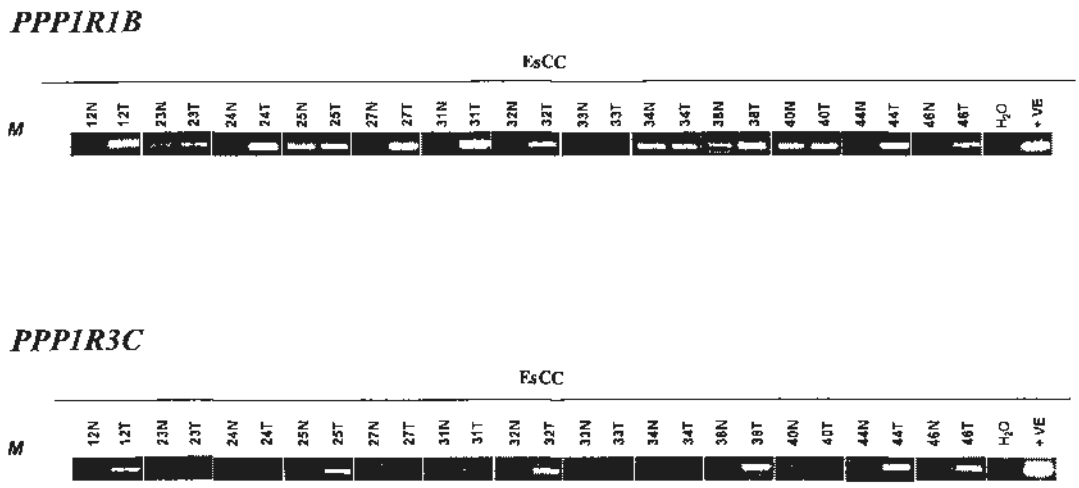



Fig. 2.10 Determination of methylation status for PPP1R1B and PPP1R3C in primary ESCC tumor.

## 2.5 Discussion

### *2.5.1 Epigenetic silencing of PPP1R1B and PPP1R3C are important events in caricinogenesis*

The discovery of ASPP family protein (PPP1R13A and PPP1R13B) and some other protein phosphatase-1 regulatory subunits member such as PPP1R10 involvement in tumor suppression prompt us to hypothesize that there are still other regulatory subunits of PP1 with tumor suppressor functions which have yet to be discovered. In the present study, we have used the gene expression analysis by means of RT-PCR to screen the putative tumor suppressor genes from the chosen five candidates among the family of protein phosphatase-1 regulatory subunits in which PPP1R1B and PPP1R3C have been identified. However, it should be noted that the choice of the carcinoma cell lines for the first round screening were mainly confined to NPC and ESCC. Therefore, it does not necessary mean that the remaining candidates do not possess tumor suppressor functions. Under our experimental setup, PPP1R1B and PPP1R3C were found to be silenced more frequently compared to other candidates. Thus, they were chosen for investigating their potential tumor suppressor functions. In addition, the larger scale of gene expression profile study indicated that silencing of PPP1R1B and PPP1R3C expression were not only limited to NPC and ESCC but were also found in other types of carcinoma cells such as colorectal carcinoma and cervical carcinoma. Thus, it is possible that both PPP1R1B and PPP1R3C are the targets which are often commonly silenced in different kinds of tumors during the development of cancer.

Loss of gene expression can be either due to genetic inactivation such as mutation and chromosome translocation or inactivation through epigenetic

mechanism. It has been accepted that epigenetic silencing of TSG is closely linked to with the onset and progression of various cancers. There is an increasing numbers of reports showing that TSGs are inactivated through epigenetic silencing or a combination of aberrant genetic and epigenetic changes more frequently than genetic inactivation alone (Jones et *al.*, 2002). Histone deacetylation and promoter CpG island hypermethylation are the mechanism frequently employed in TSG inactivation. In several previous reports, genes like ADAMTS18, PCDH10 and interferon regulatory factor 8 (IRF8) were identified as putative tumor suppressor genes using functional epigenetics approach (Ying et *al.*, 2006; Jin et *al.*, 2007; Lee et *al.*, 2008). By means of MSP analysis and BGS, we showed that PPP1R1B and PPP1R3C were silenced in multiple carcinomas through promoter CpG island hypermethyaltion. As normal PPP1R1B and PPP1R3C expressions were detected in immortalized normal epithelial cells such as NP69 (nasopharyngeal), HET1, NE1 and NE3 (esophageal), in which there was no detectable promoter CpG island hypermethylation found in these cell lines. For HCT116 cells, weak expressions of PPP1R1B and PPP1R3C were detected in the gene expression profile study, even though nearly completed methylation of the promoter CpG island could be observed in the methylation status analysis. It could be due to the presence of hemi-methylated or unmethylated in the DNA samples (only 6 bacterial clones were randomly drawn for the BGS analysis). Mechanisms other than promoter CpG island hypermethylation (e.g. acetylation of the histone) can also play a part, but in a lesser extent for the transcription of the genes. Therefore, a low gene transcription activity and hence weak expression of the gene is allowed. Nevertheless, we believe that epigenetic inactivation of PPP1R1B and PPP1R3C are the frequent and important events in the series of steps in tumor formation.

Investigation about the Gene expression status of PPP1R1B and PPP1R3C in cancer cells has been very limited. For PPP1R1B, expression analysis was only mentioned in the study of gastric cancer development (overexpression of PPP1R1B was the cause of gastric cancer formation). In addition, there was no study about the molecular events that lead to the aberrant expression of PPP1R1B. The present study was the first to report that the silencing of PPP1R1B could be a generalized phenomenon for various cancers which might have very different intrinsic properties. Also, we were first to demonstrate the silencing of PPP1R1B through an epigenetic mechanism (promoter hypermethylation). Until now, genetic mutation of PPP1R1B was rarely reported. It would be interesting to shed light on why silencing through epigenetic mechanism seems to be the major way causing inactivation of PPP1R1B and unveiling the kind of implication about this phenomenon. Though, we still could not rule out the possible existence of genetic mutation of PPP1R1B as one of the steps for cancer formation as downregulation of PPP1R1B gene was also found in some carcinoma cell lines while their promoter regions were not hypermethylated (HKESC1, KYSE180, H292 and Hela).

PPP1R3C belongs to the group of glycogen targeting subunits (including PPP1R3 or PPP1R3A, PPP1R3B, PPP1R3C, PPP1R3D, PPP1R3E, PPP1R3F and PPP1R3G). PPP1R3A was the only member from the same groups that had been reported with genetic alterations in human cancer. It has been reported that nonsense and missense mutations of the PPP1R3A gene were found in primary non-small cell lung carcinoma and the cell lines from lung carcinoma, ovarian carcinoma, colorectal carcinoma and gastric carcinoma (Kohno et *al.*, 1999; Takakura et *al.*, 2000). Among those genetic mutations, two single nucleotide polymorphisms in the coding region of the PPP1R3 gene were identified that were suggested to be the cause of

substitutions of evolutionarily conserved amino acids. After that, none of the other members of the same group has been reported to have association with cancer development and progression. Until recently, it has been reported that PPP1R3C was significantly downregulated in oral tongue squamous cell carcinoma (Ye, H. et *al.*, 2008). Later, Bonazzi et *al.* (Bonazzi et *al.*, 2009) has suggested that PPP1R3C may contribute to the development or progression of melanoma as they found that PPP1R3C was silenced by promoter CpG island hypermethylation in cell lines derived from melanoma. Consistent with these findings, PPP1R3C was found to be epigenetically silenced in multiple carcinomas by promoter CpG island hypermethylation. PPP1R3A and PPP1R3C are the examples that the group of glycogen targeting subunits may be important in carcinogenesis in addition to the function of cooperating with PP1 in glycogen metabolism. We believed that aberrant genetic or epigenetic changes of other members in this group in various cancers are waiting to be discovered, despite being found that no frequent downregulation of PPP1R3B could be detected in cancer cell lines from the result of RT-PCR analysis.

It should also be noted that gene expression profile and epigenetic study in the present study only aimed at discovering the abnormal changes of PPP1R1B and PPP1R3C in various cancers. There is still plenty of room for further study about the contribution of PPP1R1B and PPP1R3C in cancers. For example, doing similar gene expression and epigenetic analysis by using the tumor samples from different stages, origins and aggressive phenotype in order to locate the emergence of epigenetic silencing of PPP1R1B and PPP1R3C as an early event causing cancer formation or contributing to the metastatic potential of cancer cells. It can also help find out if there is any association between epigenetic silencing of PPP1R1B and PPP1R3C with the aggressiveness of the tumor. In fact, phosphorylation of the PPP1R1B

protein was suggested to be a critical process in impairing the migration activity of breast cancer cell line MDA-MB-231 (Hansena et *al.*, 2006).

### 2.5.2 *Reversal of promoter hypermethylation restoring PPP1R1B and PPP1R3C expression*

Demethylation treatment for restoring expression of the silenced gene was performed to further prove that loss of PPP1R1B and PPP1R3C expression was a consequence of silencing through epigenetic mechanism rather than dysregulation of gene expression through genetic mutation. As mentioned, promoter CpG island hypermethylation and deacetylation of histone tails in the chromatin are the two mechanisms leading to epigenetic silencing. These two epigenetic events are closely linked and affect each other through the interaction of DNMT with the HDAC complex at methylated CpG sites (Robertson et *al.*, 2000; Rountree et *al.*, 2000). Pharmacological demethylation by aza treatment or inhibition of histone deacetylation by histone deacetylase inhibitor TSA and genetic demethylation by "knock-out" of DNA methyltransferase DNMT1 and DNMT3B would result in hypomethylation of genomic DNA. These methods are the common strategies employed to restore the expression of the methylation-silenced genes (Rhee et *al.*, 2002).

DNMT1 and DNMT3B are DNA methyltransferases that are responsible for mediating *De novo* DNA methylation. In our experiment, it was shown that PPP1R1B and PPP1R3C could properly express in HCT116 cells with DNMT1 and DNMT3B gene knocked out. Moreover, the results of MSP and BGS analysis revealed that the particular sequences within the promoter region were completely unmethylated, which indicated that *de novo* methylation was responsible for the

promoter CpG island hypermethylation of PPP1R1B and PPP1R3C. Based on this argument, it has been thought that inhibition of DNA methyltransferases would be possible to demethylate the promoter CpG island and hence restore the expression of both genes. Pharmacological inhibition of DNA methylatransferase by aza treatment again showed that expressions of PPP1R1B and PPP1R3C were restored where their promoter sequences were also demethylated as shown in the results of MSP and BGS analysis. Treating the carcinoma cells with Histone deacetylase inhibitor TSA was to pose a synergistic effect on restoring epigenetic silenced gene in combination with aza treatment. The present result not only indicated that the expressions loss of PPP1R1B and PPP1R3C were mainly through epigenetic mechanism instead of genetic mutation, but also paved the way of the possibility of using pharmacological demethylation and histone deacetylase inhibition treatment to restore TSG expression, as a therapeutic strategy for treating cancer, regarding to this case, by restoring PPP1R1B and PPP1R3C expressions. It has been suggested that this method could be a novel cancer therapy by reactivating TSG functions and inducing tumor cell apoptosis or silenced viral immunodominant proteins (Chan et al., 2004; Szyf et al., 2004). However, we should be cautious that demethylation and histone deacetylase inhibition treatment would indiscriminately re-activate the TSG and also the oncogene. In theory, reactivation of some oncogenes might result in producing tumor with increased aggressiveness, which makes the outcome of the epigenetic treatment of cancer unpredicatable. Nevertheless, the combination of pharmacological demethylating treatment and methylation status analysis (MSP and BGS) is still useful to identify aberrant epigenetic alterations across the whole tumor cell genome and to search for candidate TSGs that are important in carcinogenesis (Ying et *al.*,

2006), in which PPP1R1B and PPP1R3C are suggested to be the TSGs using this approach in our study.

### 2.5.3 *Epigenetic silencing of PPP1R1B and PPP1R3C as tumor markers for diagnosis and predicating prognosis of cancer patients*

Now that epigenetic alterations of tumor suppressor genes have been known as the causes of neoplastic transformation or carcinogenesis, it is recognized as a useful tool in cancer prevention and detection, in addition to the therapeutic purposes which have mentioned previously. For many years, scientists have identified many genes that are persistently altered by epigenetic mechanism in specific type of cancer (Table 2.3). With the growing number of epigenetic silenced genes identified in contributing to cancer development and progression, we were able to produce a more precise diagnosis for the cancer patients.

| Cancer type | Genes |
|---|---|
| Breast | *p16, BRCA1, GSTP1, DAPK, CDH1, TIMP-3* |
| Brain | *p16, p14ARF, MGMT, TIMP-3* |
| Bladder | *p16, DAPK, APC* |
| Colon | *p16, p14ARF, MGMT, hMLH1, DAPK, TIMP-3, APC* |
| Oesophagus | *p16, p14ARF, GSTP1, CDH1, APC* |
| Head and neck | *p16, MGMT, DAPK* |
| Kidney | *p16, p14ARF, MGMT, GSTP1, TIMP-3, APC* |
| Leukaemia | *p15, MGMT, DAPK1, CDH1, p73* |
| Liver | *p16, GSTP1, APC* |
| Lymphoma | *p16, p15, MGMT, DAPK, p73* |
| Lung | *p16, p14ARF, MGMT, GSTP1, DAPK, FHIT, TIMP-3, RARb, RASSF1A* |
| Ovary | *p16, BRCA1, DAPK,* |
| Pancreas | *p16, MGMT, APC* |
| Stomach | *p16, MGMT, APC* |
| Uterus | *p16, p14ARF, hMLH1* |

Table 2.3 Cancer types and genes regulated by epigenetic mechanisms (adapted from Verma et *al.*, 2002)

It is known that aberrant epigenetic alterations could occur prior to the acquirement of neoplastic lesion. In addition, it is suggested that aberrant DNA

methylation is observed in individuals with no history of malignancy (Holst et *al.*, 2003) and can be used as an indicator of the likelihood of developing cancer (Laird, P. W. 2003). It will be interesting to know whether hypermethylation of PPP1R1B and PPP1R3C genes could be detected in pre-neoplastic tissue, which may aid in designing early intervention for those individuals with tendency in developing cancer of specific types provided that we have more information about the methylation status of PPP1R1B and PPP1R3C in regard to particular kinds of tumor. Our data showed that epigenetic silencing of PPP1R1B and/or PPP1R3C through promoter CpG island hypermethylation could be relevant events in cancer development or progression as hypermethylation of both genes were detected in the selected ESCC primary tumor samples in which hypermethylation of PPP1R3C promoter is tumor specific in ESCC tumor samples. However, it will be premature to state that detecting epigenetic silenced PPP1R1B and/or PPP1R3C gene in the biopsy samples is a significant diagnostic marker for the presence of cancerous tissues. Analysis of the methylation status for other kinds of primary carcinoma, for examples, NPC, breast carcinoma, hepatocellular carcinoma, etc, should be done in order to evaluate the potential use of PPP1R1B and PPP1R3C methylation in diagnosis of cancer.

Discovery of accurate and non-invasive method for cancer detection is always a top priority in cancer research. There is a growing tendency in using the detection of aberrant epigenetic changes as a diagnostic marker for cancer because it is a relatively non-invasiveness approach. It has been suggested that accurate diagnosis of cancer can be made even for patients with blood sample only as hypermethylated DNA originated from the detached cancer cells can be purified and amplified by PCR method that make detection of abnormal epigenetic changes feasible (Yoo et *al.*, 2006). It is now accepted that having a detailed map of specific

epigenetic patterns in each tissue type of their normal and cancerous states is at the top of the agenda in epigenetic research. All in all, the findings of the present study can contribute to a more comprehensive knowledge of the epigenome which will facilitate the development of early detection method and various drugs designed for cancer treatment.

# Chapter 3

# Functional consequences for the epigenetic silencing of PPP1R1B

## 3.1 Introduction

### 3.1.1 *Genomic and protein structure of PPP1R1B*

There are totally 8 exons in PPP1R1B gene. According to the study of El-Rifai et *al.* (El-Rifai et *al.*, 2002), there are two isoforms for PPP1R1B, which are DARPP-32 and t-DARPP. DARPP-32 and t-DARPP share an identical sequence from their exon 2 to the 3' end. The only difference between DARPP-32 and t-DARPP is that exon 1 of t-DARPP is alternatively spliced from the intron 1 of PPP1R1B. Such difference in mRNA structure of DARPP-32 and t-DARPP ultimately lead to the difference in their product. DARPP-32 mRNA encodes a protein of 204 amino acids while t-DARPP mRNA encodes a protein consisting 168 amino acids. The full-length DARPP-32 protein contains four phosphorylation sites. They are Thr-34, Thr-75, Ser-102, and Ser-137 (Greengard, P., 2001). It is known that DARPP-32 can function either as a kinase inhibitor or a protein phosphatase inhibitor depending on which amino acid is phosphorylated (Bibb et *al.*, 1999). If DARPP-32 is phosphorylated by Protein kinase A (PKA) at Thr-34, it will be a PP1 inhibitor. If DARPP-32 is phosphorylated by cyclin dependent kinase 5 (cdk-5) at Thr-75, it will be a PKA inhibitor. In fact, such property may have some implications for the tumor suppressor role of PPP1R1B. For t-DARPP, the shorter isoform of PPP1R1B, it lacks the Thr-34 phosphorylation

site of DARPP-32. t-DARPP has been suggested to be an anti-apoptotic protein which may contribute to the carcinogenesis (Belkhiri et *al.*, 2005; El-Rifai et *al.*, 2002). However, there is still only limited information about the function of t-DARPP.

### 3.1.2 *Oncogenic role of DARPP-32 and t-DARPP*

In the past, most of the studies about PPP1R1B or DARPP-32 precisely focus on its neurological roles like the function in dopaminergic signaling and the drug abuse behaviour. The role of DARPP-32 in carcinogenesis has only been recently realized in this decade. El-Rifai et *al.* (El-Rifai et *al.*, 2002) first reported that both DARPP-32 and t-DARPP consistently overexpress in Gastric cancer. In their study, they identified that DARPP-32 and t-DARPP mRNA transcripts and proteins were overexpressed in gastric tumor samples by means of quantitative real-time PCR and western blots analysis. In addition, there was abundant protein overexpression in neoplastic but not in normal gastric epithelial cells according to the staining of tumor tissue arrays. Later, it was suggested that DARPP-32 contributed to carcinogenesis by exhibiting its anti-apoptotic role in cancer cells. In the study of Belkhiri et *al.* (Belkhiri, et *al.*, 2005), they found that there was about 68% of the upper gastrointestinal adenocarcinomas (UGS) tumor samples with concomitant overexpression of mRNA for DARPP-32 and t-DARPP. The anti-apoptotic roles of DARPP-32 and t-DARPP were revealed in the experiment that AGS and RKO gastrointestinal cells were more resistant to camptothecin, sodium butyrate and ceramide treatment under the ectopic DARPP-32 and t-DARPP overexpression. In particular, it had been observed that there was up to 4-fold reduction in the rate of apoptosis. They suggested that DARPP-32 and t-DARPP enhanced the apoptotic

resistance of the gastric cancer cells by increasing the protein level of Bcl-2 which has a significant role in preserving the mitochondrial transmembrane potential. Such postulation was further confirmed by the experiment that small interfering RNA for DARPP-32 and t-DARPP could reverse the up-regulated Bcl-2 protein level. In a further study by Belkhiri et *al.* (Belkhiri, et *al.*, 2008), they suggested that t-DARPP increased the Bcl-2 protein levels by increasing the level of pSer-473 Akt, which would enhance the Akt kinase activity and hence the phosphorylation of CREB and ATF-1 transcription factors, where the t-DARPP/Akt axis was likely to contribute to gastric carcinogensis and resistance to drug-induced apoptosis.

### 3.1.3 *Tumor suppressor role for DARPP-32*

Despite the suggested anti-apoptotic role of DARPP-32 and t-DARPP, debate about the function of PPP1R1B in carcinogenesis continues. There are studies that suggested that DARPP-32 may actually serve as a tumor suppressor gene. In Hansena et *al.*'s study (Hansena et *al.*, 2006), they believed that DARPP-32 has an effect on the metastatic behaviour of breast cancer cells. They found that ectopic expression of DARPP-32 in DARPP-32 deficient MCF-7 breast carcinoma cell lines could inhibit the cell migration activity. However, such effect was absence in Discoidin Domain Receptor 1 (DDR1) deficient breast cancer cell line MDA-MB-231 cells while co-expression of both DARPP-32 and DDR1 could restore the ability of inhibiting cell migration. Furthermore, alanine mutation of Thr-34 but not for Thr-75 would abolish the anti-migratory effect of DARPP-32. This suggests that Thr-34 phosphorylated DARPP-32 is critical for inhibiting cell migration and hence the metastasis of cancer cells. Thr-34 of DARPP-32 is phosphorylated by PKA where its activity will be activated if there is detachment of

cells in the extracellular matrixes (Andersson et *al.*, 2005; Howe et *al.*, 2002; Howe et *al.*, 2000). They suggested that inhibition of PP1 by Thr-34 phosphorylated DARPP-32 could be a critical event for anti-migratory effect through altering the phosphorylation statues of motor or motor-associated proteins (Hansena et *al.*, 2006).

On top of this, DARPP-32 was suggested to be possibly involved in cell differentiation. It has been found that DARPP-32 expression was often absence in those dedifferentiated, tumoral or Ras-transformed thyrocytes (Garcia-Jimenez et *al.*, 2005). They found that TSH and IGF-I, the thyroid hormones necessary for maintaining differentiated state of thyroid cells during their proliferation, would target to DARPP-32 protein. It was also found that DARPP-32 silencing by siRNA would lead to the loss of differentiation markers such as thyroid transcription factor 1, Pax 8, thyroglobulin and the Na/I symporter, while reexpression of DARPP-32 in Ras-transformed cells would reactivate the thyroglobulin and thyroperoxidase promoter. Thus, they have suggested that DARPP-32 is important for the maintenance of thyroid differentiation induced by TSH and IGF-I. Loss of DARPP-32 expression might be a possible cause for the thyroid cell carcinogenesis.

Based on the above information, it was likely that DARPP-32 was a tumor suppressor protein. In fact, it has been reported that patients with primary esophageal cancer samples expressing DARPP-32 will have a better prognosis than those who are DARPP-32 negative (Ebihara et *al.*, 2004). Also, patients with esophageal squamous carcinoma have reduced survival outcome when their tumors are negative for DARPP-32 expression (Lundell, L., 2006).

The idea of whether PPP1R1B is an oncogene or tumor suppressor gene is still contradictory. Further studies should be done to gather more information about the function of PPP1R1B protein products.

### 3.1.4 *Objective of the study*

In this chapter, we are going to present the results that reveal the tumor suppressor function of PPP1R1B and the effect of ectopic PPP1R1B expression on the growth ability of carcinomas cell lines in anchorage-dependent condition. Western blot analysis was done to investigate the downstream signaling pathway regulated by PPP1R1B. In particular, the level of Ser-473 phosphorylated Akt and Ser-552 phosphorylated β-catenin were studied. In addition, the activity of AP-1, a transcription factor necessary for cell proliferation was determined by Dual-luciferase Reporter Assay in order to understand whether PPP1R1B expression has an inhibitory effect on the signal for cell proliferation on the transcriptional level. Finally, the subcellular localization of PPP1R1B was revealed by means of fluorescence microscopy.

## 3.2 Materials

pcDNA™ 3.1 directional TOPO® expression kit with mammalian expression vector pcDNA ™3.1D/V5-His-TOPO was the product of Invitrogen, Carlsbad, CA. while pEGFP-C1 vector was the product of Clontech, Palo Alto, CA. PfuUltra@ high-fidelity DNA Polymerase was the product of stratagene, U.S.A. Costar® Spin-X® Centrifuged Tube Filter was the product of Cole-Parmer, Vernon Hills, IL. HindIII and XhoII were purchased from the New England Biolabs, USA. FuGENE 6 transfection reagent, T4 DNA ligase was purchased from Roche Applied Science, Germany. QIAGEN EndoFree® Plasmid Midi Kits was purchased from QIAGEN, Germany. EDTA, RPMI-1640 medium, 4',6-diamidine-2'-phenylindole dihydrochloride (DAPI) and monoclonal mouse anti-Flag antibodies were purchased from Sigma-Aldrich Corporation, U.S.A. DNase was the product of Ambion Inc., Austin, TX, USA. Genetian Violet was the product of ICM Pharma, Singapore. Full-Range Rainbow Molecular Weight Markers, Amersham Hybond-P blotting membrane and Amersham ECL western blotting detection reagents were purchased from GE healthcare. Mouse anti-α-tublin, rabbit anti-human p-Ser473 Akt, rabbit anti-human total Akt, rabbit anti-human p-Ser552 β-catenin were the product of Cell Signaling, Beverly, MA, while mouse anti-human β-catenin, polyclonal Goat anti-rabbit IgG, polyclonal rabbit anti-mouse IgG were the product of Dako company, Denmark. Total mRNA from normal adult brain and AP1-Luc with seven AP1 binding site were purchased from Stratagene, La Jolla, CA, USA. Dual-luciferase@ reporter assay system was the product of Promega corporation, U.S.A.

## 3.3 Methodology

### 3.3.1 *Molecular cloning of full length human PPP1R1B*

Mammalian expression vector pcDNA3.1 (+) was selected as the vector to carry the full-length cDNA of *PPP1R1B* insert. Full length cDNA of PPP1R1B was obtained by reverse transcription reaction described in section 2.3.5. in which 1 μg of total mRNA from normal adult brain tissue was reverse-transcribed into cDNA using random hexamer for priming. The resultant cDNA was used as the template for PCR amplification. The 5' end and 3' end of the full-length cDNA sequence with Flag-tag sequence at 5' end was amplified by using a pair of gene specific primers, viz. 5'-CACCATGGATTACAAGGATGACGACGATAAGATGGACCCCAAGGACCGC -3' (forward primer) and 5'- TGTGCCAGGCTCAGAGGG-3' (reverse primer), designed according to the nucleotide sequence retrieved from NCBI Genbank. The DNA polymerase for PCR of cloning reaction was PfuUltra@ high-fidelity DNA Polymerase while the recipe for the PCR reaction mixture was shown in the following table:

| Components | Volume used (μl) |
|---|---|
| 10x PfuUltra@ HF reaction Buffer | 2.5 |
| PPP1R1B-cloningF (Forward, 10μM) | 0.5 |
| PPP1R1B-cloningR (Reverse, 10μM) | 0.5 |
| dNTPs (2.5mM for each kind of dNTP) | 0.2 |
| PfuUltra@ high-fidelity DNA Polymerase (2.5U/μl) | 1 |
| cDNA template (100ng/μl) | 2 |
| Autoclaved distilled water | 18.3 |
| **Total volume** | 25μl |

Table 3.1 Components of the reaction mixture for the cloning PCR of PPP1R1B

The PCR was carried out as follows: DNA samples were initially denatured at 95°C for 2 min. Then, they were PCR-amplified by the following thermal cycle: denaturation: 94°C for 30 s, annealing: 58°C for 30 s, extension: 72°C for 60 s, for around 37 rounds. A 10-min final extension at 72°C was implemented immediately after the thermal cycling.

The PCR product was resolved by agarose gel electrophoresis in which PCR band of the correct size was excised under UV illumination. The PCR product in the gel slice was purified by using Costar® Spin-X® Centrifuged Tube Filter. Then, the purified product was cloned into the pcDNA ™3.1D/V5-His-TOPO vector, according to the manufacturer's protocol. The *PPP1R1B protein* coding region was inserted into the mammalian expression vector pcDNA ™3.1D/V5-His-TOPO which contained a neomycin resistance gene. Such Plasmid was constructed to allow the expression of PPP1R1B with a Flag-tag at the N-terminus in mammalian cells. After sequence verification, Endotoxin-free pcDNA3.1/Flag-PPP1R1B-V5-His plasmids for subsequent experiments were prepared by using QIAGEN EndoFree® Plasmid Midi Kits according to the manufacturer's protocol (section 3.3.2).

### 3.3.2 *Plasmid extraction*

The Original pcDNA3.1/Flag-PPP1R1B-V5-His plasmid was transformed into chemically competent DH5α E.coli cells by heat-shock transformation. The transformed cells were recovered and cultured in LB broth for an hour at 37°C. After that, the transformed E.coli cells were transferred and spread on Agar plate containing appropriate antibiotics (100 μg/ml ampicillin or 50 μg/ml kanamycin) where plasmid transformed E.coli were selected and grown overnight at 37°C.

*Mini-scale preparation of plasmid DNA*

Bacterial colony picked from agar plates was inoculated into 1.5 ml LB medium with appropropriate antibiotic and allowed to culture for overnight at 37°C with agitation at 250 rpm. The resultant solutions of bacteria culture were transferred into a new centrifuge tube and centrifuged at 8,000 rpm for 1 min. Supernatant was aspirated after centrifugation while the bacterial pellet was resuspended in 150 µl of Solution I (50 mM glucose, 25 mM Tris-Cl, 10 mM EDTA and 0.5 mg/mL RNase A) to obtain a homogenous solution and allowed to incubate at room temperature for 10 min. Then, bacteria were lysed with 300 µl of freshly prepared Solution II [200 mM NaOH, 1% (w/v) SDS]. The mixture was kept on ice from 3 to 5 min after the gentle mixing. Mixtures were then neutralized with 255 µl chilled Solution III (60 ml of 5M glacial acetic acid added with 11.5 ml of absolute glacial acetic acid and 28.5 ml $H_2O$; pH5.2) and incubated at room temperature for 10 min. Subsequently, the mixture was subjected to centrifugation (14,000 rpm, 15 minutes) at room temperature. After centrifugation, about 550 µl of supernatant containing the plasmid DNA was carefully extracted and precipitated with 330 µl isopropanol. Samples were then incubated for at least 15 min at room temperature. After the incubation, samples were centrifuged at 14,000 rpm for 10 min at room temperature. After centrifugation, supernatant was discarded while the remaining plasmid DNA pellets were washed with 800 µl of 70% ethanol thoroughly and subjected to another round of centrifugation (10,000 rpm for 5 min) at room temperature. Supernatant was discared and the plasmid DNA pellets were air-dried for a least 15 min at room temperature. Finally, the plasmid DNA pellets were dissolved in nuclease-free Tris-EDTA buffer and stored at 4°C prior to subsequent experiments. Integrity of the plasmid was checked by agar gel electrophoresis as described in section 2.3.7.

*Midi-scale preparation of endotoxin-free plasmid DNA*

Midi-scale preparation of endotoxin-free plasmid DNA was performed by using QIAGEN EndoFree® Plasmid Midi Kit (QIAGEN, Germany) according to the manufacturer's protocol.

### 3.3.3 *Plasmid DNA transfection*

Cell lines (*Caski* cervical carcinoma cell line, *H1299* lung carcinoma cell line, *HCT116* colorectal carcinomas cell line, *HONE1* NPC cell line) were maintained in the corresponding cell culture medium (RPMI1640/ Dulbecco's modified Eagle's medium, DMEM) supplemented with 10% FBS and 100U/ml penicillin/streptomycin. Around 0.75 to 1.5 x $10^5$ Cells were seeded in the 6-well cell culture plate. Cell culture samples were allowed to grow overnight at 37°C with 5% $CO_2$ in a humidified chamber. After overnight incubation, cells were transfected with the empty-vector or plasmid with insert using the FuGENE 6 transfection reagent according to the manufacturer's protocol. Briefly, about 3:1 (v/w) ratio of Fugene 6 reagent to amount of DNA (μg) samples were mixed with serum free cell culture medium and incubated for at least 15 min at room temperature. Subsequent to the incubation, plasmid DNA mixed with transfection reagent was added dropwisely into the cell with gentle shaking. Finally, samples were allowed to grow at 37°C with 5% $CO_2$ in a humidified chamber.

*Colony formation assay*

At 48 hr after transfection, cells were harvested by trypsinization and seeded in a new 6-well plates at a density ranging from 1-3×$10^4$ per well. Plasmid transfected cells were selected with antibiotic G418 (0.4 mg/ml) for 1-2 weeks. Total

RNA from the transfected cells was extracted as described in section 2.3.3 and treated with DNase. Western blot analysis was used to confirm successful transfection and proper ectopic expression PPP1R1B. The G418-containing cell culture medium would be refreshed every 3 days. On the day of colony staining, cell culture medium were discarded and the cells were rinsed with 1x PBS. Cells were then fixed with methanol for around 10 min and rinsed with 1x PBS again. After that, the surviving colonies (≥ 50 cells/colony) were stained with Genetian Violet for around 10 min. The staining solution was subsequently removed while the samples were gently rinsed with distilled water. Results were photographed for record and the number of colonies was counted. Data were presented as Mean ± SEM from three independent experiments in which each assay was performed in triplicate.

### 3.3.4 *Preparation of protein samples, Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot anaylsis*

Transfected cells were lysed in freshly prepared ice-cold cell lysis buffer (10 mM Tris, pH7.4, 1% SDS, 5 mM $MgCl_2$ with protease inhibitor crocktail containing 1 mM PMSF, 1 mM sodium orthovandate, 5 μg leupeptin and 21 μg aprotinin). The protein lysates were then separated by SDS-PAGE.

Electrophoresis on SDS-polyacrylamide gel was performed according to the method of Laemmli and Favre (Laemmli et *al.*, 1973) using 12% separating gel and 5% stacking gel. Samples were diluted with sample loading buffer (10% glycerol (v/v), 0.4% SDS (w/v), 0.005% bromophenol blue (w/v), 20 mM EDTA and 5% β-mercaptoethanol (v/v) in 0.5 M Tris-HCl, pH 7.5). The samples were boiled for 10 min and cooled to room temperature before being loaded onto the polyacrylamide gel. Electrophoresis was performed at a constant voltage of 140 V at room temperature.

The subunit molecular mass of the protein was estimated by using Full-Range Rainbow Molecular Weight Markers. After the electrophoresis, protein samples resolved in polyacrylamide gel were electro-blotted onto Hybond-PVDF membranes with voltage at 18V for 40 min. Then, PVDF membrane blotted with protein samples were submerged in Tris-buffered saline with 5% (w/v) non-fat milk and 0.1% (v/v) Tween 20 to block all the non-specific binding site. After blocking, membranes were incubated with designated primary anti-body [1:2000 (v/v) mouse anti-α-tublin, 1:1000 (v/v) rabbit anti-human p-Ser473 Akt, 1:1000 (v/v) rabbit anti-human total Akt, 1:1000 (v/v) rabbit anti-human p-Ser552 β-catenin, 1:2000 (v/v) mouse anti-Flag monoclonal antibodies, 1:2000 (v/v) mouse anti-human β-catenin] at $4^0$C for overnight with agitation. After the overnight incubation with primary antibody, membranes were washed 3 times with Tris-buffered saline with 0.1% (v/v) Tween 20 before incubating with suitable secondary antibody [1:2000 (v/v) polyclonal Goat anti-rabbit IgG or polyclonal rabbit anti-mouse IgG] for 1 hr at room temperature with agitation. Membranes were again washed 3 times with Tris-buffered saline with 0.1% (v/v) Tween 20 before being visualized by enhanced chemiluminescence methods according to manufacturer's protocol (Amersham Biosciences).

### 3.3.5 *Renilla-Firefly Dual-luciferase Reporter assay (AP-1)*

Caski, HCT116 and H1299 cells (5 x$10^4$) were seeded in 24-well and incubated with complete RPMI 1640 medium for 24 hr. AP-1 Luciferase reporter plasmid with seven AP-1 binding site and pcDNA3.1-Flag-PPP1R1B were co-transfected into respective carcinoma cell lines (Caski, HCT116 and H1299) by the transfection protocol described in section 3.3.3 and incubated for 48 hr at 37°C with 5% $CO_2$ in a humidified chamber. At 48 hr after transfection, the luciferase

activities of the samples were measured by using the Dual-luciferase@ reporter assay system with the following procedures: Firstly, cell samples were lysed by adding 80 µl *passive lysis buffer* and 15 min incubation at room temperature with agitation. Cell lysates were collected and transferred into a new centrifuge tube in which the cell debris were separated by centrifugation (12000rpm, 4 min at $4^0$C). About 20 µl supernatant from each sample was mixed with 40 µl of *Luciferase Assay Reagent II* and added to 96-well optical plate where the firefly luciferase activity was measured. Forty µl of *Stop & Glo@ reagent* was dispensed to each sample immediately after firefly luciferase activity measurement. The Luciferase activities were recorded. Relative luciferase activities were calculated (Firefly luciferase activities / Renilla luciferase activities) with normalization versus Renilla luciferase activities. Fold-induction of the promoter activity was determined by the ratio of the relative luciferease activities of sample transfected with gene of interest to the sample transfected with empty vector.

All the reagents used in this assay were provided by the Dual-luciferase@ reporter assay kit and prepared according to the manufacturer's recommendation. All the readings were recorded by the PerkinElmer Multilabel Counter. Data were presented as Mean ± SEM from three independent experiments in which each assay was performed in duplicate.

### 3.3.6 *Subcellular localization study*

Flag-PPP1R1B-V5-His inserts were obtained by digesting the pcDNA3.1/Flag-PPP1R1B-V5-His plasmids with restriction enzyme HIND III and Sac II. Flag-PPP1R1B-V5-His inserts were sub-cloned into pEGFP-N1 vector to form Flag-PPP1R1B-V5-His-EGFP that encoded for full-length PPP1R1B proteins

tagged with GFP at its C-terminus. At 48 hr after transfection, H1299 cells were washed with PBS and fixed with 4% paraformaldehyde for 10 min. After fixing, cells were rinsed with PBS. Then, sufficient amount of DAPI (1 mg/ml) was incubated with the fixed cell in dark for 5 min at room temperature. Slides with cell samples were observed directly under fluorescence microscope - Axiovert 40 CFL - HBO100 microscope (Carl Zeiss, Jena, Germany) which the subcellular localization of PPP1R1B would be revealed.

### 3.3.7 *Statistical analysis*

Statistical analysis was carried out by means of Student's t-test. All the statistical comparison was accomplished by using the SPSS 14.0 for windows software with $p < 0.01$ was considered as statistically significant.

## 3.4 Results

### 3.4.1 *Ectopic PPP1R1B expression inhibits anchorage-dependent growth of carcinoma cells*

From the result of gene-expression profile, MSP and BGS analysis, we have found that PPP1R1B normally expressed in adult normal tissue and in immortalized epithelial cell lines but was frequently silenced in multiple carcinoma cells through promoter hypermethylation. To determine whether silencing of PPP1R1B is contributed to carcinogenesis, the ability of PPP1R1B in suppressing tumor growth has to be investigated in which the assay of colony formation in anchorage-dependent condition was performed. From fig. 3.2, it could be seen that there was a significant reduction in the number of colonies formed in all the corresponding carcinoma cell lines [Caski (Cervical), H1299 (Lung), HCT116 (Colon) and HONE1 (NPC)] with ectopic PPP1R1B expression. The quantitative result of the colony formation assay showed that ectopic PPP1R1B expression in carcinoma cell lines would significantly decrease the colony formation efficiency by 50-60% when comparing with that of the carcinomas cell lines transfected with pcDNA3.1 empty vector only (Fig. 3.3). In particular, the growth inhibition by ectopic PPP1R1B expression was the most prominent in HCT116 cells, in which approximately 62% of inhibition could be achieved (52% for Caski, 55% for HONE1 and 47% for H1299) (Fig. 3.3). The ability of PPP1R1B in inhibiting colony formation of carcinoma cells indicates the possible involvement of PPP1R1B in controlling the cell proliferation. Thus, dysregulation in PPP1R1B expression will contribute to carcinogenesis.

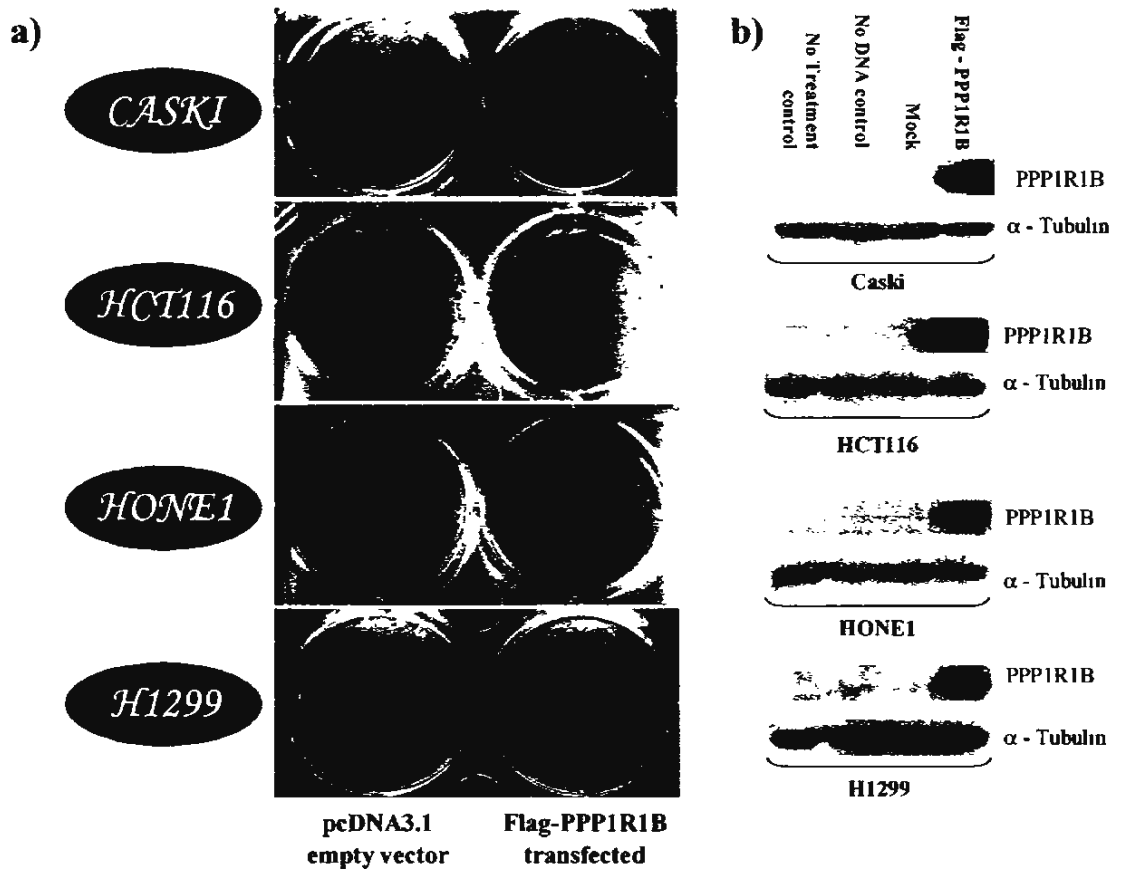



Fig. 3.1 Ectopic expression of PPP1R1B inhibits the colongenicity of carcinoma cell as shown by the colony formation assay. (a) Cells were transfected with pcDNA3.1-PPP1R1B-Flag or empty vector. At 48 hr after transfection, antibiotics G418 was used to select the transfected cells for 7-10 days and stained with Gentian violet. (b) Western blots analysis showed successful PPP1R1B expression after transfection.

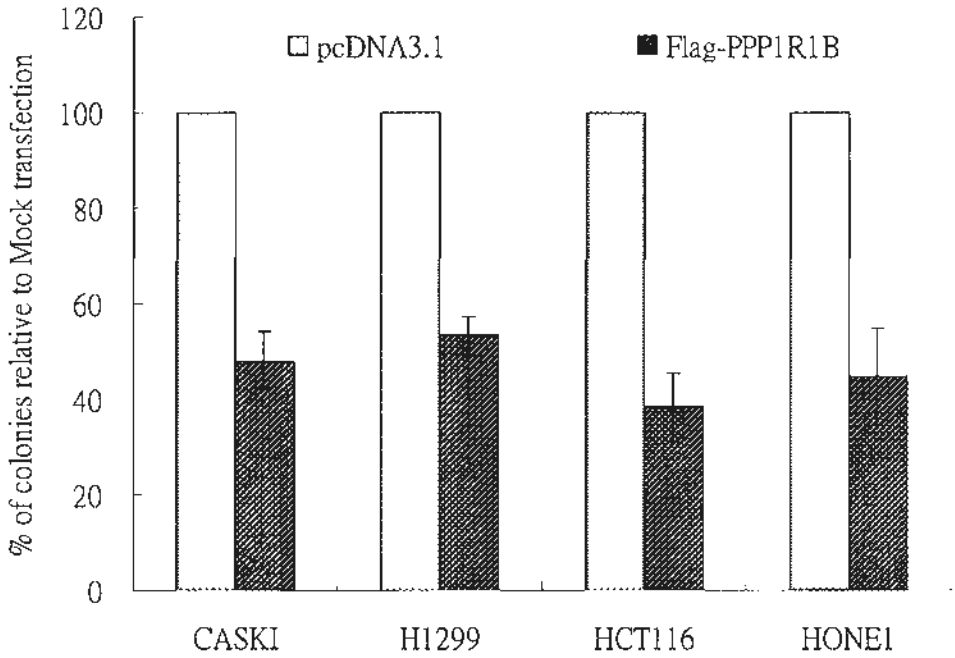
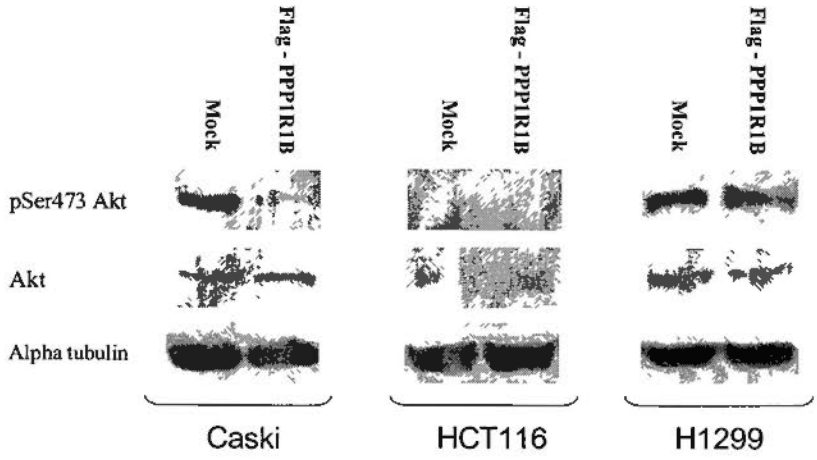



Fig. 3.2 Quantitative analysis of colony formation. Relative colony forming ability was determined in which the empty vector-transfected control was referred as 100%. Data were represented by Mean ± S.D. from three independent experiments.

### 3.4.2 *Effect of ectopic PPP1R1B expression on the level of p-Ser473 Akt, p-Ser552 β-catenin*

Since p-Thr75 DARPP32 is a potent PKA inhibitor, we would like to study whether ectopic PPP1R1B expression has an inhibitory effect to the intracellular PKA activity in the carcinoma cell lines that could be a possible signaling pathway controlling the proliferation of cancer cells. Akt and β-catenin are the downstream targets of PKA. Akt is phosporylated at ser-473 and β-catenin is phosphorylated at ser-552 by PKA. Inhibition of PKA by ectopic PPP1R1B expression was studied by investigating the level of p-ser473 Akt and p-ser552 β-catenin. From the result of the western blot analysis, ectopic PPP1R1B expression could significantly down-regulate the level of p-ser473 Akt in Caski cells with no observable change in the level of total Akt. On the other hand, only a slightly decrease in the level of p-ser473 Akt could be observed in H1299 cells (Fig. 3.4a). Quantitatively, the down-regulation of p-ser473 Akt in Caski cells was about 35% (Fig 3.5a). For HCT116, the basal level of Akt in it was very low where only a very weak band could be detected. The data was thus ignored. Similar to that of p-ser473 Akt, the level of p-ser552 β-catenin was significantly reduced in all the tested carcinoma cell lines without observable change in the level of the amount of β-catenin after re-expressing PPP1R1B in them (Fig. 3.4b). The reductions in the levels of p-ser552 β-catenin were approximately 24%, 33% and 34% in Caski, H1299 and HCT116 cells, respectively (Fig. 3.5b). The significant downregulation of p-ser473 Akt and p-ser552 β-catenin indicated that the kinase activity of PKA was successfully inhibited upon the ectopic PPP1R1B expression.

**a)**




**b)**

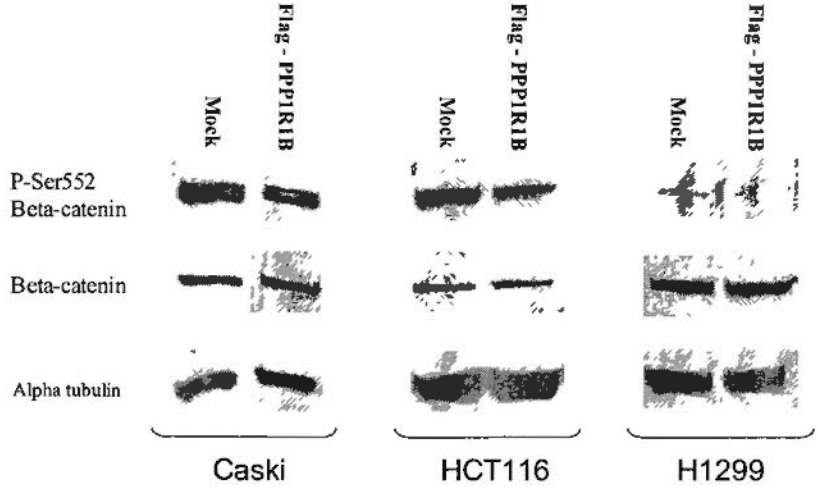
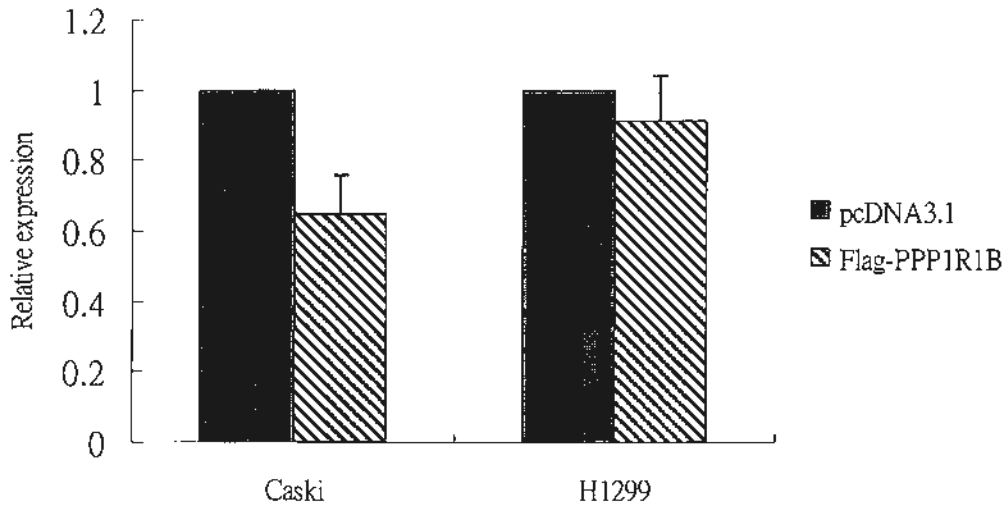



Fig. 3.3 Western blot analysis for the protein level of a) p-ser473 Akt and b) p-ser552 β-catenin. PPP1R1B was re-expressed in the respective carcinoma cell lines (Caski, HCT116 and H1299 cells) using the transfection protocol stated in section 3.3.2. All the protein samples were harvested at 48 hr after plasmid transfection. Data showing the representative figures of the study from three independent experiments.

**a)**




**b)**

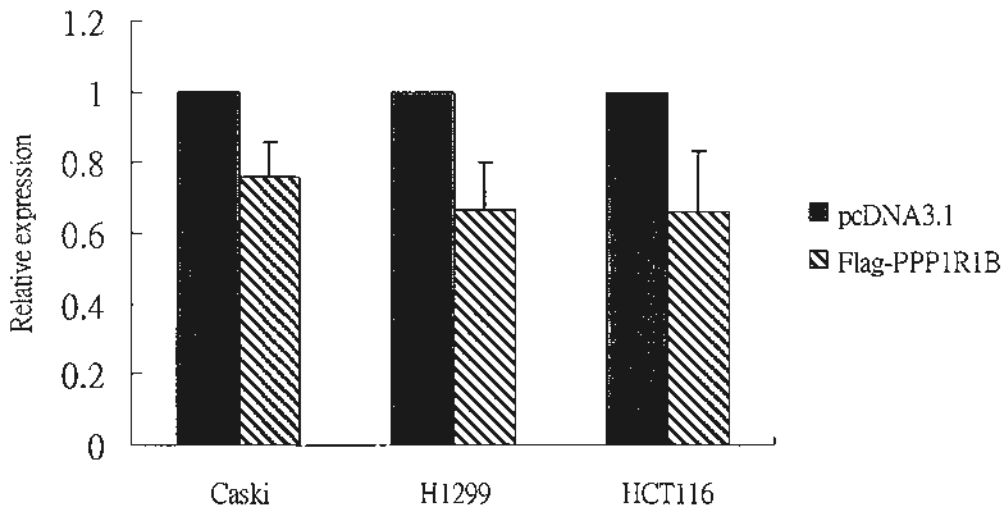



Fig. 3.4 Histograms for the relative expression of a) p-ser473 Akt and b) p-ser552 β-catenin. The expressions of the protein in the PPP1R1B transfected cells were normalized against that of the mock transfected cells, i.e. relative expression = protein expression in PPP1R1B transfected cells / protein expression in mock transfected cells. Data were represented by Mean ± S.D. determined from three independent experiments.

### 3.4.3 *Effect of ectopic PPP1R1B expression on AP-1 transcriptional activity*

AP-1 is an important transcription factor that induces the gene transcription for triggering the downstream signaling for cell proliferation. The activity of AP-1 will be turned on when it is phosphorylated. As ectopic expression of PPP1R1B was found to be able to inhibit the growth of cancer cell (section 3.4.1), we would like to investigated whether re-expressing PPP1R1B in carcinoma cell lines could inhibit the transcriptional activity of AP-1 and hence the downstream target gene transcription for cell proliferation. From fig. 3.6, ectopic expression of PPP1R1B could significantly inhibit the transcription activity of AP-1 by approximately 58% and 54% in H1299 and HCT116 carcinoma cell lines, respectively, (Table 3.2) in accord with the result of Renilla-firefly Dual Luciferase Reporter Assay. AP-1 is the downstream target of phosphorylated CREB and the phosphorylation of CREB is positively regulated by PKA. This indicated that the role of PPP1R1B in suppressing cancer cell growth could possibly involve the CREB/AP-1 axis in which the transcriptional activities of AP-1 and CREB were reduced due to the inhibition of PKA under the influence of PPP1R1B expression.

### 3.4.4 *Subcellular localization of PPP1R1B revealed by GFP-tagged fusion protein*

Fluorescence microscopic study for GFP-tagged PPP1R1B fusion protein was performed in order to have a concise idea about the subcellular localization of the protein encoded by PPP1R1B (DARPP-32). DAPI staining was used to reveal the nucleus of the H1299 cells. From fig. 3.7a, the nucleus of the H1299 remained intact upon re-expression of PPP1R1B under our transfection protocol. It could be

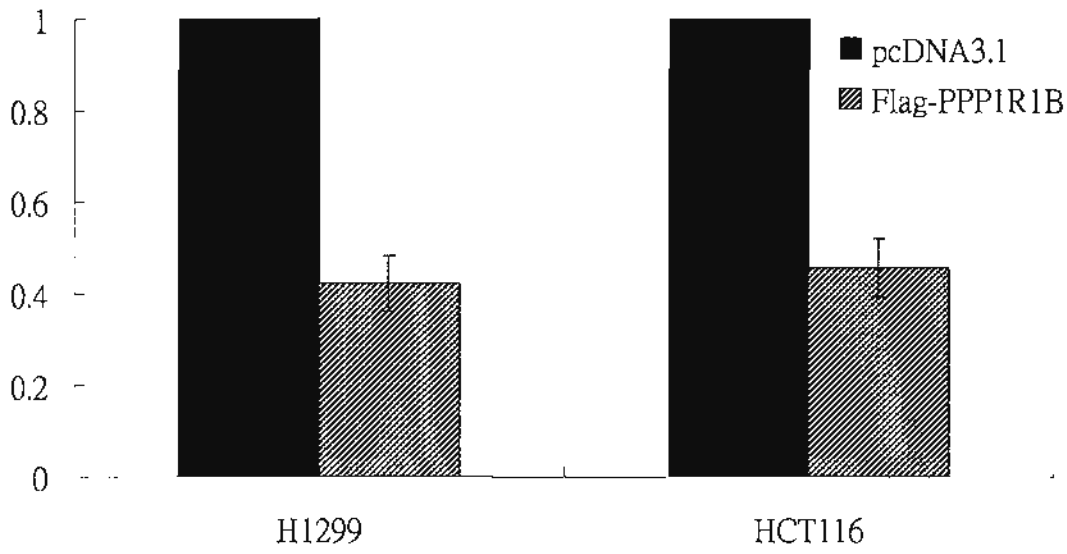



Fig. 3.5 Determination of AP-1 transcription activity in by Renilla-Firefly Dual Luciferase Reporter Assay. Briefly, PcDNA3.1-Flag-PPP1R3C, Renila-luciferase vector and AP-1-Lucifease reporter plasmid were co-transfected into the corresponding cancer cell lines H1299 and HCT116 cells. The luciferase activities of the corresponding samples were measured at 48 hr after plasmid transfection. Data were represented by Mean ± S.E.M from three independent experiments.

| **Relative transcriptional activity** | | |
|---|---|---|
| | **Mock** | **Flag-PPP1R1B** |
| **H1299** | **1** | **0.423±0.064** |
| **HCT116** | **1** | **0.458±0.127** |

Table. 3.2 Relative transcriptional activity of AP-1 for PPP1R1B and Mock transfected samples.

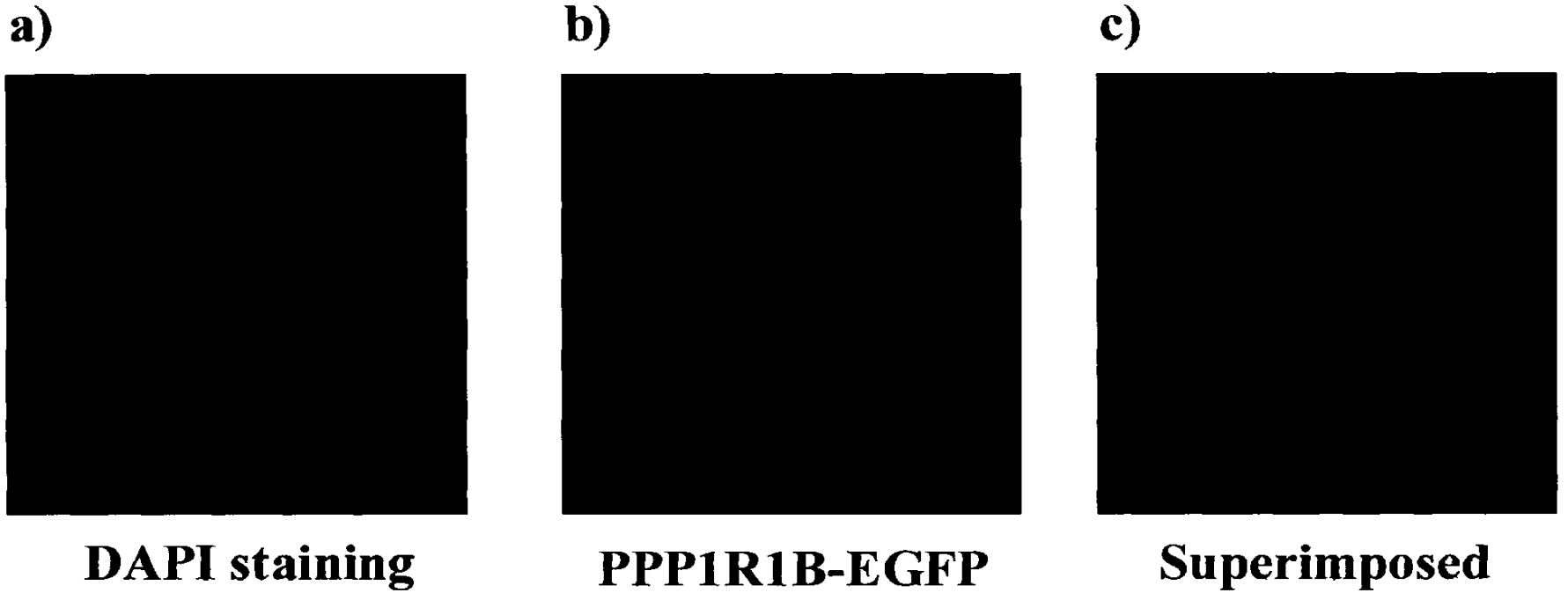



Fig. 3.6 Subcellular localization study of PPP1R1B using GFP-tagged fusion protein. a) Nuclei of H1299 cells were stained by DAPI while b) the subcellular localization of PPP1R1B was revealed by the green fluorescence of the GFP-tagged fusion protein. c) The superimposed image indicated that protein products of PPP1R1B were likely to be a cytoplasmic protein. Data shown was the representative figure from three independent experiments.

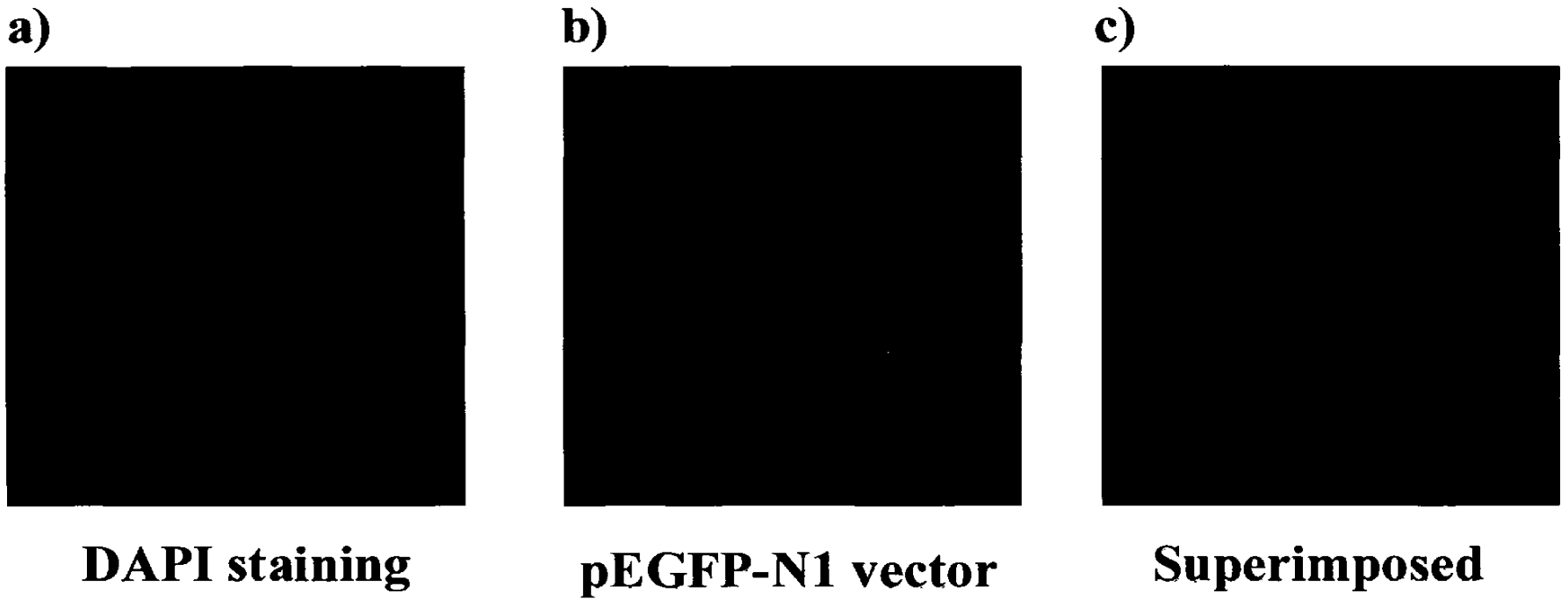



Fig. 3.7 Fluorescence microscopic image of H1299 cells expressing pEGFP-N1 vector. a) Nuclei of H1299 cells were stained by DAPI while b) expression of GFP as revealed by fluorescence microscope. c) Superimpose of the image of DAPI staining and GFP fluorsence image. Data shown was the representative figure from three independent experiments.

observed that the green fluorescence was indiscriminately distributed all over the cells including the nucleus upon the transfection of pEGFP-N1 vector plasmid only (Fig. 3.8b). The fluorescence image of the GFP-tagged fusion protein revealed that PPP1R1B did not likely localize in a specific organelle to exert its intracellular function (Fig. 3.7b). From the superimposed image, PPP1R1B was more likely a cytoplasmic protein as the green fluorescence of the PPP1R1B GFP-tagged fusion protein dispersed around the cytosol (Fig. 3.7c).

## 3.5 Discussion

### 3.5.1 *DARPP-32 encoded by PPP1R1B gene is responsible for inhibiting cancer cell growth*

As there is contradictory evidence about the functional roles of PPP1R1B (DARPP-32 and t-DARPP) in carcinogenesis, it is necessary to study the PPP1R1B signaling pathway so as to gain insight into how PPP1R1B contributes to cancer formation. There were reports suggested that PPP1R1B could be an oncogene as overexpression of its encoded protein, DARPP-32 and t-DARPP were both detected in primary gastric adenocarcinoma and the gastric cancer cell lines, AGS cells (El-Rifai et *al.*, 2002; Belkhiri et *al.*, 2005). There was another study reported that expression of both t-DARPP and DARPP-32 in primary breast cancers was the cause for the resistance to trastuzumab or so called Herceptin™ (a humanized antibody against the HER2 growth factor receptor) in breast cancer cells (Hamel et *al.*, 2009). In contrast to these suggestions, we herein proposed that PPP1R1B was a tumor suppressor gene and exhibited inhibitory function to cancer cell proliferation.

In the previous chapter, PPP1R1B was found to be frequently silenced in multiple carcinomas. PPP1R1B silencing in multiple carcinoma was likely due to transcription inhibition by promoter hypermethylation as shown by the result of MSP and BGS analysis. Among the carcinoma cell lines silenced by promoter hypermethylation, Caski (cervical), HCT116 (colon), HONE1 (NPC) and H1299 (Lung) were chosen for further study to investigate the tumor suppressor function of PPP1R1B. Based on the result of anchorage-dependent colony formation assay, we found that re-expressing PPP1R1B in PPP1R1B silenced cancer cells could inhibit the colony formation ability of the cancer cells. Thus, we believed that PPP1R1B

expression is important in proliferation control of the cells. The key to explaining our contradictory findings with those previous reports about the oncogenic function of PPP1R1B may lie in the understanding about the function of DARPP-32 and t-DARPP. In the present study, the full-length PPP1R1B protein coding sequence was successfully cloned into pcDNA3.1 mammalian expression vector. We were also able to ectopically express protein of PPP1R1B in carcinoma cell lines with PPP1R1B expression silencing as shown by the image of the western blot analysis (Fig. 3.1). The sequence length of isoform t-DARPP is about 108 bp shorter than full length DARPP-32 sequence, of which t-DARPP protein is about 36 amino acids less than DARPP-32. As the cDNA for the cloning came from the total mRNA of the brain tissue, the protein re-expressed in the carcinoma cell lines was likely to be DARPP-32 rather than t-DARPP, which was reflected in the size of protein as shown in the western blotting result (~ 36 kDa including Flag-tag peptide). We speculate that DARPP-32 is a tumor suppressor protein while t-DARPP is an oncoprotein that will counteract the tumor suppressor function of DARPP-32 and allow the cells to proliferate. Normally, the presence of DARPP-32 is essential for the cell growth control and proper differentiation of the cells (Garcia-Jimenez et *al.*, 2005). Consistent with the findings by El-Rifai et *al.*, (El-Rifai et *al.*, 2002) we also detected PPP1R1B (or DARPP-32) expression in MKN45 gastric cancer cells while PPP1R1B was silenced in AGS cells. Based on our rationale, silencing of PPP1R1B and hence the absence of DARPP-32 expression is more likely the cause of malignant transformation and tumor formation rather than PPP1R1B overexpression. Thus, we believe that t-DARPP expression is the culprit for the carcinogenesis in transformed or malignant cells. t-DARPP expression is induced (not express in normal cells) in which its influence will overhaul the growth inhibitory function of

DARPP-32 and lead to uncontrolled cell proliferation. For the gastric cancer cells expressing both DARPP-32 and t-DARPP, we speculate that the influence of t-DARPP is dominant over DARPP-32. DARPP-32 was found to be possible in maintaining the differentiation by inducing the expression of differentiation markers such as Pax 8 in thyrocytes (Garcia-Jimenez et *al.*, 2005). It will be interesting to study whether overexpressing t-DARPP would cause the downregulation of those differentiation markers and the proteins essential for cell cycle control. In the present study, we focused on investigating the tumor suppressor function of PPP1R1B.

### 3.5.2 *Ectopic expression of PPP1R1B regulates the cell proliferation by inhibiting PKA kinase activity*

DARPP-32 possesses the dual role of serine/ threonine phosphatase and kinase inhibitor (Thr-34 phosphorylated DARPP-32 is a PP1 inhibitor; Thr-75 phosphorylated DARPP-32 is a PKA inhibitor). Undoubtedly, it affects the signal transduction pathway by regulating the phosphorylation status of its target protein. It is known that stability of many proteins or activity of enzymes is regulated by the phosphorylation status of the target in many circumstances. For example, the kinase activity of Akt will be switched on when it is phosphorylated by PKA at ser-473, while β-catenin will be stabilized and prevented from ubiquitination and proteosomal degradation when it is phosphorylated by PKA at ser-552. Activated Akt is an oncoprotein. Akt and the phosphorylation of the downstream targets of Akt by its kinase activity are often associated with promoting the cell proliferation pathway. For instances, MDM2 can be phosphorylated by Akt which in turn will destabilize p53 by ubiquitination and subject to proteosomal degradation (Osaki et *al.*, 2004). On the other hand, it has been reported that activation of Akt would allow the G1 or

G2 phase cell cycle progression and eventually permit the cell growth and proliferation (Ramaswamy et *al.*, 1999; Kandel et *al.*, 2002). For β-catenin, it is an important component in Wnt signaling pathway. In Wnt signaling pathway, accumulation of β-catenin and its nuclear translocation will positively regulate the transcription factors for triggering the cell proliferation. Therefore, the level of p-ser473 Akt and p-ser552 might directly affect the rate of the cancer cell growth. As Thr-75 phosphorylated DARPP-32 can effectively inhibit the kinase activity of PKA, expressing PPP1R1B and hence increasing the amount of Thr-75 phosphorylated DARPP-32 might also negatively regulate the level of both p-ser473 Akt and p-ser552 β-catenin. Our results showed that ectopic expression of PPP1R1B could significantly reduce the level of p-ser473 Akt and p-ser552 β-catenin in Caski cells and H1299 cells. It indicated that the kinase activity of PKA was inhibited. For HCT116 cells, owing to the low basal level of Akt, changes in the level of p-ser473 Akt was undetectable. However, downregulation of p-ser552 β-catenin could also be observed in HCT116 after re-expressing PPP1R1B. Therefore, kinase activity of PKA should also be inhibited in HCT116 upon ectopic PPP1R1B expression. We believe that if we overexpress PPP1R1B in other colorectal carcinoma cells such as HT29 or deliberately upregulate the level of Akt in HCT116 cells in concomitant with the ectopic PPP1R1B expression, downregulation of p-ser473 Akt could also be observed. In a study about the effect of DARPP-32 and truncated isoform t-DARPP expression in breast cancer cells, the authors suggested that DARPP-32 and t-DARPP had antagonistic effects on breast cancer cell growth and herceptin resistance (Gu et *al.*, 2009). In the same study, it was found that overexpression of t-DARPP would confer Herceptin resistance. The authors pointed out that herceptin treatment would normally induce complete dephosphorylation of Akt in breast cancer

cells (SK-Br-3 cells in that study) where stable transfection of t-DARPP in breast cancer cells would help maintain a significant level of p-ser473 phosphorylated Akt even in the present of Herceptin. The presence of full-length DARPP-32 would exhibit negative effect on the cell proliferation and survival in breast cancer cells such as SK-Br-3 cells (Gu et *al.*, 2009). As mentioned, DARPP-32 and t-DARPP could antagonize with each other in which expressing high level of DARPP-32 could reverse the Herceptin resistance conferred by t-DARPP. This was confirmed by their experiment that nearly a complete dephosphorylation of Akt in SK-Br-3 cells could be detected when both DARPP-32 and t-DARPP were stably transfected into the SK-Br-3 cells. Consistent with their findings, we were also able to prove that ectopic expression of PPP1R1B could downregulate p-ser473 Akt level. Thus, regulation of PI3K/Akt pathway could be one of the important tumor suppressor functions of PPP1R1B. Gu et *al.* (Gu et *al.*, 2009) suggested that t-DARPP-32 may act as an activator of PKA or has a dominant-negative effect on DARPP-32's inhibitory activity. Although further experiments have to be done to verify this notion, it is possible that t-DARPP and DARPP-32 are two counteracting forces inside the cells to regulate cell proliferation. This can explain why the presence of both t-DARPP and DARPP-32 expression was found in Herceptin resistance breast cancer cells (Hamel et *al.*, 2009) or in some primary tumors so that DARPP-32 was mistakenly interpreted as an oncoprotein where the cause for the drug resistance or carcinogenesis was actually due to the dominant influence of t-DARPP.

### 3.5.3 *Ectopic expression of PPP1R1B negatively regulate the transcriptional activity signaling for cell proliferation*

In addition to the investigation of the effect of ectopic PPP1R1B expression on the Akt phosphoyrlation status, we were also able to demonstrate the effect of re-expressing PPP1R1B in PPP1R1B silenced carcinoma cells on AP-1 transcription activity. Gu et *al.* (Gu et *al.*, 2009) has showed that DARPP-32 expression could reverse the effect of t-DARPP induced CREB activation (phosphorylation of CREB). In our study, it has been showed that AP-1 transcription activity was decreased nearly by half in the presence of PPP1R1B expression in both HCT116 cells and H1299 cells. As AP-1 is an downstream effector of CREB which is also activated by phosphorylation in the prescence of phosphorylated CREB. The result of the study suggested that transcriptional activity and also the phosphorylation level of CREB should also be decreased after re-expressing of PPP1R1B similar to the findings of Gu et *al.*'s study (Gu et *al.*, 2009). Belkhiri et *al.* (Belkhiri et *al.*, 2008) found that t-DARPP could increase the CREB/ATF-1-CRE binding activity. This further confirms the antagonistic effect of DARPP-32 and t-DARPP. Moreover, they have found that t-DARPP displays anti-apoptotic activity in which the cell survival of AGS gastric cancer cells was promoted by upregulation of Bcl-2 protein and hence the maintenance of mitochondrial transmembrane potential through Akt and CREB dependent mechanism. It would be interesting to see if DARPP-32 could down-regulate the level of Bcl-2 protein expression.

It should be noted that PKA is the common upstream protein of p-ser473 Akt, p-ser552 β-catenin and CREB. As both DARPP-32 and t-DARPP possess Thr-75 residue in their amino acid sequences that could bind to the enzyme PKA, further experiments should be done to find out the reason for the contrasting effect of

DARPP-32 and t-DARPP on PKA, for example, different intrinsic properties or mechanism controlling the activity of DARPP-32 and t-DARPP on PKA.

### 3.5.4 *Subcellular localization study indicated protein encoded by PPP1R1B is cytosolic protein*

The subcellular localization of a protein may have a significant implication on its intracellular function. For instance, cytochrome C is a mitochondrial protein which is proved to be an important component for induction of apoptosis. Another example is p53. Nuclear p53 is essential for triggering the DNA repairing activity inside the nucleus. As mentioned, different protein phosphatase regulatory subunits locate in different subcellular compartments to exhibit its function. For example, the PP1 nuclear targeting subunit (PPP1R10) is a nuclear protein which plays a pivotal role in the post-translational modification of p53 and MDM2 (Lee et al., 2007). It has been reported that the subcellular localization of full-length DARPP-32 is mainly in the cytosol (Cohen, P. T. W., 2002). We herein repeated the subcellular localization study of full-length DARPP-32 in H1299 cells to gain more information about whether cytosol is the sole subcellular localization of DARPP-32, or it exhibited its function in a distinct cellular compartment. Akt, PKA, un-stimulated β-catenin and Bcl-2 are all cytosolic proteins. By determining the subcellular localization of DARPP-32, we would be more confident in claiming whether DARPP-32 directly affects those cytosolic proteins, or affect their activity indirectly through other mechanism such as by recruiting other interacting partner or by conveying the intracellular signal from different cellular compartment such as nucleus. In the present study, H1299 cells with ectopic PPP1R1B expression is used as the model. Consistent with Cohen's reports (Cohen, P. T. W., 2002), we found that the green

fluorescence disperse around the cytosol without distinct fluorescence pattern. Also, the green fluorescence did not overlap with the blue DAPI stain, indicated that DARPP-32 was not a nuclear protein or had any nuclear translocation. In fact, DARPP-32 is also not likely to be a mitochondrial protein as no mitochondrial localization signal peptide was found by means of the bioinformatics approach (data not shown). Thus, we believed that DARPP-32 produced upon ectopic PPP1R1B expression directly interacted and regulate Akt, PKA, un-stimulated β-catenin and Bcl-2 in the cytoplasm.

# Chapter 4

# Functional consequences related to the epigenetic silencing of PPP1R3C

## 4.1 Introduction

### 4.1.1 *Genomic and protein structure of PPP1R3C*

PPP1R3C DNA contains two exons. According to the NCBI database, ptg (protein encoded by PPP1R3C) possesses a carbohydrate binding motif. It has also been found that the amino acid sequence of PPP1R3C has 42% identity and about 60% similarity compared to that of PPP1R3B. This could be the reason why studies about PPP1R3C were focused on its glycogen binding ability and the glycogen metabolism. Until recently, a gene expression profiling study for melanoma has indicated that PPP1R3C was silenced in melanoma cell line (derived from primary cutaneous melanomas or their metastases) by promoter hypermethylation (Bonazzi, V. et *al.*, 2009). The study by Ye et *al.* (Ye et *al.*, 2008) showed that PPP1R3C was one of the genes that was significantly downregulated in oral tongue squamous cell carcinoma. Findings from these studies implied that PPP1R3C might have tumor suppressor function in normal cells. However, study about the intracellular signaling pathways regulated by PPP1R3C is still very limited.

### 4.1.2 *Role of GSK-3β in carcinogenesis*

Glycogen synthase kinase 3β (GSK-3β) is a multi-functional serine/threonine kinase. It was initially identified as an important regulator for the

insulin dependent glycogen synthesis. At present, there are many reports about the role of GSK-3β in carcinogensis. It has been known that GSK-3β is an important regulator of Wnt/β-catenin signaling pathway (Grimes et *al.*, 2001). Activity of GSK-3β depends on its site-specific phosphorylation, for example, Ser9 phosphorylation of GSK-3β is an inactive form while unphosphoryalted GSK-3β is the active form. On the other hand, phosphorylation at Tyr216 will allow the full activity of GSK-3β. In the canonical pathway of Wnt/β-catenin signaling, β-catenin will form complex with Tcf/Lef transcription factor family. It will then promote nuclear translocation of β-catenin which subsequently bind to DNA and initiate cell proliferation (Behrens et *al.*, 1996; Huber et *al.*, 1996; Molenaar et *al.*, 1996). It has been suggested that active GSK-3β, in concert with the scaffolding protein axin, is able to phosphorylate β-catenin, in which the phosphoprylated β-catenin will be targeted for ubiquitination and proteosomal degradation. Hence the canonical Wnt-signaling pathway for cell proliferation will be inactivated (Rubinfeld et *al.*, 1996; Yost et *al.*, 1996; Sakanaka et *al.*, 1998). In regard to this, the role of GSK-3β is more likely to involve in suppressing tumor growth. However, the contribution of GSK-3β in carcinogenesis seems more complicated than it is expected.

In contrast to the tumor suppressor role of GSK-3β, there are studies suggested that GSK-3β promotes carcinogenesis in some circumstances. One of the findings supporting this notion is that overexpression of GSK-3β has been detected in human ovarian, colon and pancreatic carcinomas (Ougolkov et *al.*, 2006). In ovarian cancer, overexpression of active GSK-3β increases the cyclin D1 expression and induces cell cycle progression (Cao et *al.*, 2006). It has also been suggested that

nuclear accumulation of GSK-3β is significantly correlated with human pancreatic cancer dedifferentiation in which nuclear accumulation of active GSK-3β is found in some pancreatic adenocarcinomas (Ougolkov et *al.*, 2006; Luo, J., 2008). Reduction in pancreatic cancer cell survival and proliferation, and arrest of pancreatic tumor growth in established tumor xenografts could be observed after the inhibition of GSK-3β (Ougolkov et *al.*, 2005; Ougolkov et *al.*, 2006). It seems that whether GSK-3β suppresses or promotes the tumor growth depends on the physiological condition of the microenvironment and the type of the cancer cells.

### 4.1.3 *NF-κB (Nuclear factor-κB)*

NF-κB has an important role in regulating normal immune and inflammatory responses. It is also critical in controlling cell proliferation, differentiation and apoptosis. NF-κB or precisely called Rel/NF-κB transcription factors family consists of c-Rel, RelA, RelB, p105/ NF-κB1 and p100/ NF-κB2. These transcription factors share a very high homology with Rel homology domain. Such homology allows these transcription factors to dimerize or interact with IκB inhibitory proteins, which may ultimately affect their nuclear translocating activity and the NF-κB DNA binding for the induction of intracellular response such as cell proliferation (Kucharczak et *al.*, 2003). In basal condition, NF-κB is confined in the cytoplasm because the binding of IκB will mask its nuclear localization signal (Beg et *al.*, 1992; Ganchi et *al.*, 1992). In response to external stimuli, phosphorylation and ubiquintin dependent proteosomal degradation of IκB is induced, which will release the NF-κB for nuclear translocation and hence the transcription of NF-κB regulated genes (Beg et *al.*, 1993; Henkel et *al.*, 1993; Palombella, et *al.*, 1994).

NF-κb is a transcription factor that can either promote cell survival or mediates the cell death. The decision of NF-κB's influence on cell survival or cell death is different from one kind of cell to the other. Upon bacterial or viral infection, a cascade of inflammatory responses will be induced including the secretion of inflammatory cytokine. The inflammatory cytokines such as tumor-necrosis factor α, interleukin 1 and the necrotic cell products trigger the NF-κb activation. The activated NF-κb is responsible for further inducing the release of inflammatory cytokine, upregulation of angiogenic factors and induction of the gene transcription for cell growth and survival. In regards to the transcription of the genes activated by NF-κb, they include cyclin D1 (which facilitates the cell cycle progress and hence increases cell proliferation), Bcl-$X_L$ (which inhibits apoptosis) and matrix metalloproteinases (which is responsible for matrix degradation and associated with cancer cell invasion and metastatsis) (Karin, M., 2006). This is the reason why chronic inflammation and sustained activation of NF-κb is linked with cancer development and progression (Karin et *al.*, 2005; Karin, M., 2006).

Paradoxically, NF-κb can also mediate the cell death in some circumstances. It has been reported that c-Rel induces the expression of TRAIL (tumor-necrosis factor-related apoptosis-inducing ligand) receptors DR4, DR5 and the proapoptotic Bcl-2 family member Bcl-xS to senstize cells to TRAIL-induced apoptosis (Chen et *al.*, 2003). It has also been suggested that NF-κb could work with p53, IRF-1 to stimulate production of the death-promoting inducible nitric oxide synthase after ischemic injury (Kucharczak et *al.*, 2003). It appears that whether NF-κb behaves as an oncoprotein or pro-apoptoic protein is context dependent. Thus, it is difficult to tell the upstream effector of NF-κb is a tumor suppressor protein or oncoprotein by

only following the upregulation and downregulation of the transcription level of NF-κb.

### 4.1.4 *Objective of the study*

In this chapter, I am going to present the result that reveals the tumor suppressor function of PPP1R3C, effect of ectopic PPP1R3C expression on inhibiting the growth of carcinomas cell lines in anchorage-dependent condition. Western blot analysis was done to investigate the downstream signaling pathway regulated by PPP1R3C. In particular, the level of Ser-9 phosphorylated GSK-3β and p53 were studied. In addition, the activity of NF-κB was determined by Dual-luciferase Reporter Assay to observe how the ectopic PPP1R3C expression affected the NF-κB activity that was supposed to be regulated by GSK-3β activity. Finally, the subcellular localization of PPP1R3C has also been revealed by means of fluorescence microscopy.

## 4.2 Materials

pcDNA™ 3.1 directional TOPO® expression kit with mammalian expression vector pcDNA ™3.1D/V5-His-TOPO was the product of Invitrogen, Carlsbad, CA while pEGFP-C1 vector was the product of Clontech, Palo Alto, CA. PfuUltra@ high-fidelity DNA Polymerase was the product of stratagene, U.S.A. Costar® Spin-X® Centrifuged Tube Filter was the product of Cole-Parmer, Vernon Hills, IL. HindIII and XhoII were purchased from the New England Biolabs, USA. FuGENE 6 transfection reagent, T4 DNA ligase was purchased from Roche Applied Science, Germany. QIAGEN EndoFree® Plasmid Midi Kits was purchased from QIAGEN, Germany. EDTA, RPMI-1640 medium and DAPI were purchased from Sigma-Aldrich Corporation, U.S.A.. DNase was the product of Ambion Inc., Austin, TX, USA. Genetian Violet was the product of ICM Pharma, Singapore. Amersham Hybond-P blotting membrane and Amersham ECL western blotting detection reagents were purchased from GE healthcare. Mouse anti-α-tublin, rabbit anti-human p-Ser9 GSK-3β, mouse anti-human p53 were the product of Cell Signaling, Beverly, MA while polyclonal Goat anti-rabbit IgG, polyclonal rabbit anti-mouse IgG were the product of Dako Company, Denmark. Total mRNA from normal adult oesophagus and NF-κB-Luc with five NF-κB binding site and p53-Luc with fourteen p53 binding site were purchased from Stratagene, La Jolla, CA, USA. Antibiotics G418 were the product of Calbiochem, Germany. Dual-luciferase@ reporter assay system was the product of Promega corporation, U.S.A.

## 4.3 Methodology

### 4.3.1 *Molecular cloning of human full length PPP1R3C*

Mammalian expression vector pcDNA3.1 (+) was used to carry the full-length cDNA of *PPP1R3C* insert. Full-length PPP1R3C cDNA was obtained by reverse transcription reaction described in section 2.3.5 in which 1 μg of total mRNA from normal adult esophagus was reverse-transcribed into cDNA using random hexamer for priming. The resultant cDNA was used as the template for PCR amplification. The 5' end and 3' end of the full-length cDNA sequence with Flag-tag sequence at 5' end was amplified by using a pair of gene specific primers, viz. 5'-CACCATGGATTACAAGGATGACGACGATAAGATGAGCTGCACCAGAATG ATC-3' (forward primer) and 5'- TCGATAAGAGGCCAAGTTCTC-3' (reverse primer), designed according to the nucleotide sequence retrieved from NCBI Genbank. The DNA polymerase for PCR of cloning reaction was PfuUltra@ high-fidelity DNA Polymerase while the recipe for the PCR reaction mixture was shown in the following table:

| Components | Volume used (μl) |
|---|---|
| 10x PfuUltra@ HF reaction Buffer | 2.5 |
| PPP1R3C-cloningF (Forward, 10μM) | 0.5 |
| PPP1R3C-cloningR (Reverse, 10μM) | 0.5 |
| dNTPs (2.5mM for each kind of dNTP) | 0.2 |
| PfuUltra@ high-fidelity DNA Polymerase (2.5U/μl) | 1 |
| cDNA template (100ng/μl) | 2 |
| Autoclaved distilled water | 18.3 |
| **Total volume** | 25μl |

Table 4.1 Components of the reaction mixture for the cloning PCR of PPP1R3C

The PCR was performed under the following conditions: Firstly, DNA samples were initially denatured at 95°C for 2 min. Then, they were PCR-amplified by the following thermal cycle: denaturation: 94°C for 30 s, annealing: 58°C for 30 s,

extension: 72°C for 2 min, for around 37 rounds. A 10-min final extension at 72°C was implemented immediately after the thermal cycling.

The PCR products were resolved by agarose gel electrophoresis in which PCR band of the correct size was excised under UV illumination. The PCR product in the gel slice was purified by using Costar® Spin-X® Centrifuged Tube Filter. Then, the purified product was cloned into the pcDNA™ 3.1 Directional TOPO® vector, according to the manufacturer's protocol. The *PPP1R13C protein* coding sequence was inserted into the mammalian expression vector pcDNA ™3.1D/V5-His-TOPO, which contains a neomycin resistance gene. Such Plasmid was constructed to allow the expression of PPP1R3C with a Flag tag at the N-terminus in mammalian cells. Endotoxin-free pcDNA ™3.1D/Flag-PPP1R3C-V5-His-TOPO plasmid for subsequent experiments was prepared by using QIAGEN EndoFree® Plasmid Midi Kits according to the manufacturer's protocol (section 3.3.2).

### 4.3.2 *Plasmid extraction*

Plasmid were extracted by the method described in Section 3.3.2

### 4.3.3 *Plasmid transfection*

The Original pCR®4.0-TOPO® - PPP1R3C plasmid was transformed into chemically competent DH5α E.coli cells by heat-shock transformation. The transformed bacteria were recovered and cultured in LB broth for an hour at 37°C. After that, the transformed E.coli were transferred and spread on Agar plate containing respective antibiotics (100 μg/ml ampicillin or 50 μg/ml kanamycin), in which plasmid transformed E.coli were selected and grown for overnight at 37°C.

Carcinoma cell lines (*Caski* cervical carcinoma cell line, *HCT116* colorectal

carcinomas cell line, *KYSE150* ESCC cell line) were maintained in the corresponding cell culture medium (RPMI1640/ Dulbecco's modified Eagle's medium, DMEM) supplemented with 10% FBS and 100U/ml penicillin/streptomycin. Plasmid DNA transfection and Colony formation assay was performed as described in section 3.3.4 where the successful transfection and proper ectopic expression PPP1R3C with Flag tag was confirmed by semi-quantitative RT-PCR. Results were photographed for record and the number of colonies was counted. Data were presented as Mean ± SEM from three independent experiments in which each assay was performed in triplicate.

### 4.3.4 *Preparation of protein samples, SDS-PAGE and Western blot anaylsis*

Protein samples were prepared and subjected to SDS-PAGE and western blot analysis as described in section 3.3.4. The primary antibodies used in this study were mouse anti-α-tublin [1:2000 (v/v)], rabbit anti-human p-Ser9 GSK-3β [1:1000 (v/v)], mouse anti-human p53 [1:2000 (v/v)] while 1:2000 (v/v) polyclonal Goat anti-rabbit IgG or polyclonal rabbit anti-mouse IgG were the secondary antibodies being used.

### 4.3.5 *Renilla-firefly Dual-luciferase Reporter assay (p53 and NF-κB)*

Caski, HCT116 and H1299 cells (5 x$10^4$) were seeded in 24-well and incubated with complete RPMI 1640 medium for 24 hr. NF-κB-Luciferase reporter plasmid with five binding site or p53-Lucifease reporter plasmid with fourteen p53 binding site and pcDNA3.1-Flag-PPP1R3C were co-transfected into respective carcinoma cell lines (Caski, HCT116 and KYSE150) by the transfection protocol described in section 3.3.3 and incubated for 48 hr at 37°C with 5% $CO_2$ in a

humidified chamber. At 48 hr after transfection, the relative luciferase activities of the samples were measured by following the procedures as described in section 3.3.5. Data were presented as Mean ± SEM from three independent experiments in which each assay was performed in duplicate.

### 4.3.6 *Subcellular localization study*

Flag-PPP1R3C-V5-His inserts were obtained by digesting the pcDNA3.1/Flag-PPP1R3C-V5-His plasmids with restriction enzyme HIND III and Sac II. Flag-PPP1R3C-V5-His inserts were sub-cloned into pEGFP-N1 vector to form Flag-PPP1R3C-V5-His-EGFP that was encoded for full-length PPP1R1B proteins tagged with GFP at its C-terminus. At 48 hr after transfection, HCT116 cells were washed with PBS and fixed with 4% paraformaldehyde for 10 min. After fixing, cells were rinsed with PBS. Then, sufficient amount of DAPI (1 mg/ml) was incubated with the fixed cell in darkness for 5 min at room temperature. Slides with cell samples were observed directly under fluorescence microscope - Axiovert 40 CFL - HBO100 microscope (Carl Zeiss, Jena, Germany) - in which the subcellular localization of PPP1R3C would be revealed.

### 4.3.7 *Statistical analysis*

Statistical analysis was carried out by using Student's t-test. All the statistical comparison was accomplished by using the SPSS 14.0 for windows software with $p < 0.01$ was considered as statistically significant.

## 4.4 Results

### 4.4.1 *Ectopic PPP1R3C expression inhibit anchorage-dependent growth of carcinoma cells*

From the result of gene-expression profile, MSP and BGS analysis, we found that PPP1R3C normally expressed in adult normal tissue and in immortalized epithelial cell lines but was frequently silenced in multiple carcinoma cells through promoter hypermethylation. In order to determine whether silencing of PPP1R3C is contributed to carcinogenesis, the ability of PPP1R3C in suppressing tumor growth has to be investigated where the assay of colony formation in anchorage-dependent condition was performed. From fig. 4.1, it could be seen that ectopic PPP1R3C expression significantly reduced the number of colonies formed in all the corresponding carcinoma cell lines [Caski (Cervical), HCT116 (Colon) and KYSE150 (ESCC)]. The quantitative data of the colony formation assay showed that ectopic PPP1R3C expression in carcinoma cell lines would significantly decrease the colony formation efficiency by 40-50% when compared to the carcinomas cell lines transfected with pcDNA3.1 empty vector only (Fig. 4.2). In particular, the growth inhibition by ectopic PPP1R3C expression was the most prominent in KYSE150 cell, in which approximately 47% of inhibition could be achieved (37% for Caski, 34% for HCT116) (Fig. 4.2). This indicated that PPP1R3C could be important in suppressing cell proliferation. If there is any disruption in the intracellular signaling pathway related to PPP1R3C, it will lead to carcinogenesis.

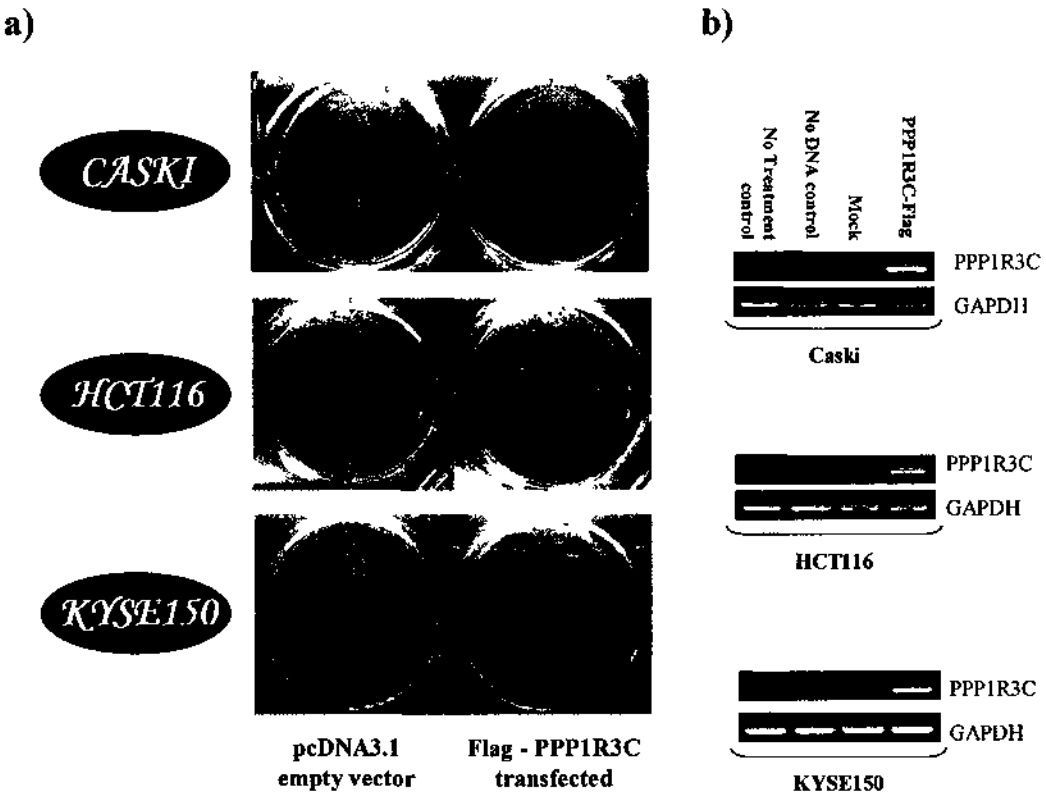



Fig. 4.1 Ectopic expression of PPP1R3C inhibits the clonogenicity of carcinoma cell as shown by the colony formation assay. (a) Cells were transfected with pcDNA3.1-PPP1R3C-Flag or empty vector. At 48 hr after transfection, antibiotics G418 was used to select the transfected cells for 7-10 days and stained with Gentian violet. (b) Reverse-transcription PCR analysis showed successful PPP1R3C expression after transfection with GAPDH served as internal control.

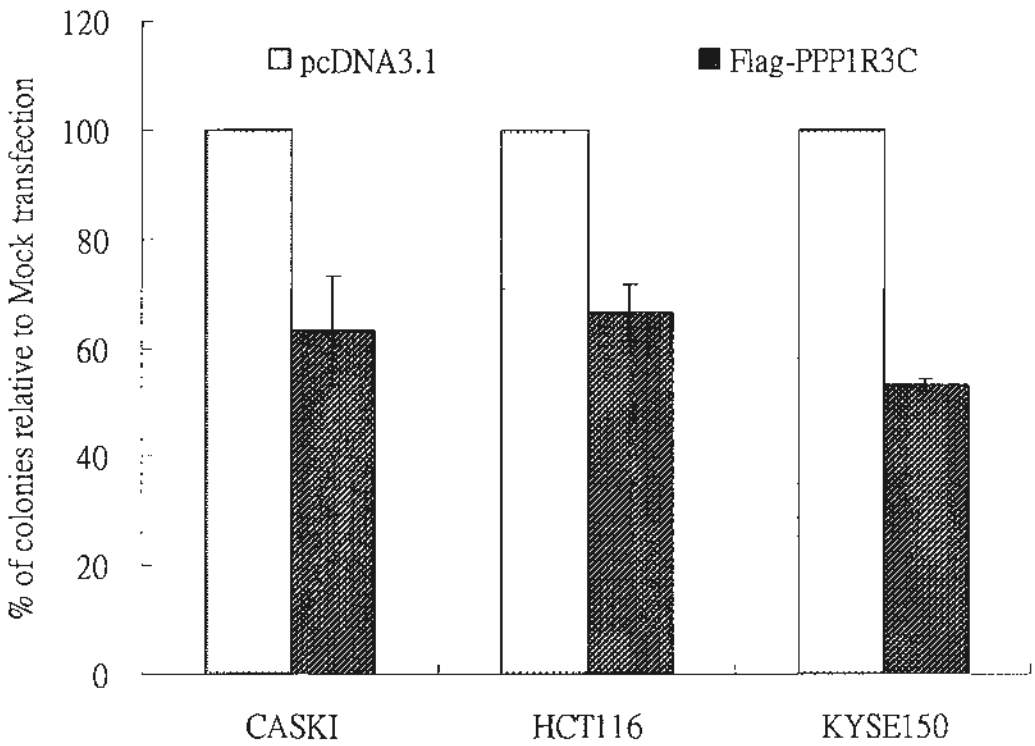



Fig. 4.2 Quantitative data of colony formation of carcinoma cells upon ectopic PPP1R3C expression. Relative colony forming ability was determined in which the empty vector-transfected control was referred to as 100%. Data were shown by Mean ± S.E.M from three independent experiments.

### 4.4.2 *Effect of ectopic PPP1R3C expression on the level of p-Ser9 GSK-3β and p53*

As mentioned, GSK-3β plays an important role in controlling the cancer cell growth (either promoting or suppressing cell proliferation). As an important regulator in canonical pathway of Wnt signaling, studying the phosphorylation status of GSK-3β and hence its kinase activity under the influence of ectopic expression of PPP1R3C could give us insight into the functional role of PPP1R3C in tumor suppression. Result of the western blot analysis revealed that a dramatic increase in the level of p-ser9 GSK-3β was seen in Caski, HCT116 and KYSE150 cells after the ectopic expression of PPP1R3C (Fig. 4.3). The relative expression levels of phosphorylation of GSK-3β at ser-9 position were 2.24, 2.01 and 2.54 in Caski, HCT116 and KYSE150 cells, respectively, i.e. about 1- 1.5 fold increased could be observed when PPP1R3C was re-expressed in the cancer cells (Fig 4.4a). On the other hand, there was a slight increase in p53 level in HCT116 cells but no detectable change in KYSE150 cells under the effect of ectopic expression of PPP1R3C (Fig. 4.3). For the p53 level of Caski cells, the absence of p53 expression was likely due to the influence of HPV-16 virus present in the Caski cells. Based on the result, it could be concluded that ectopic expression of PPP1R3C was able to increase the phosphorylation level of GSK-3β which would switch off the kinase activity of GSK-3β. The enhancement of p-ser9 GSK-3β could be due to the inhibition of phosphatase of GSK-3β and hence the reduction in its dephosphorylation activity.

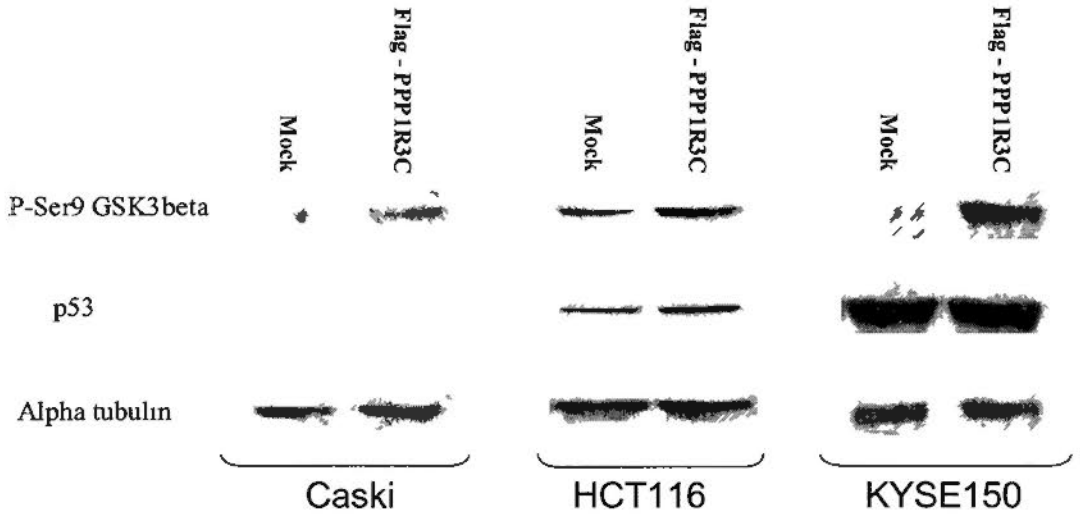
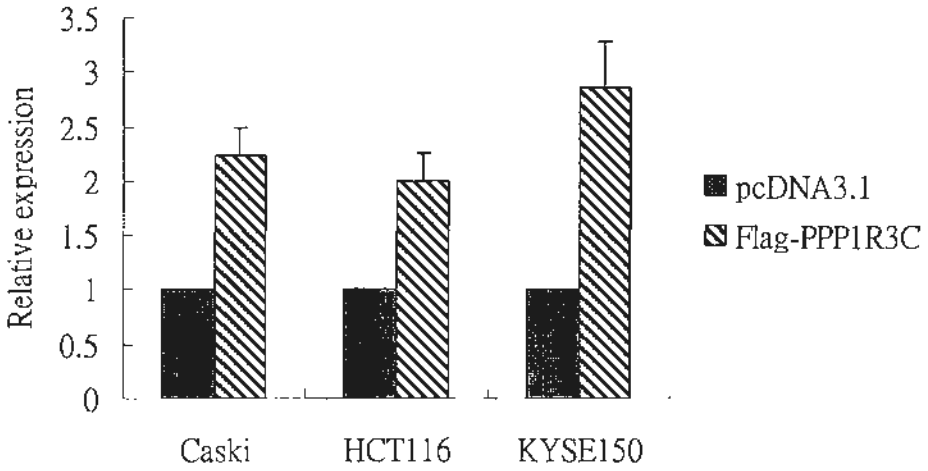



Fig. 4.3 Western blot analysis of the level of p53 protein and ser-9 phosphorylated GSK-3beta. PPP1R3C was re-expressed in the respective carcinoma cell lines (Caski, HCT116 and KYSE150 cells) using the trasnfection protocol stated in section 3.3.2. All the protein samples were harvested at 48 hr after plasmid transfection. Data showing the representative figures of the study from three independent experiments.

**a)**




**b)**

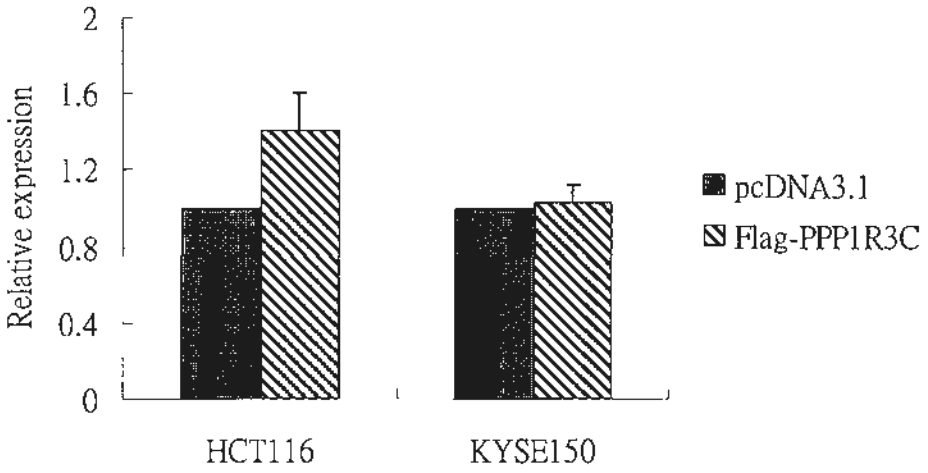



Fig. 4.4 Histograms for the relative expression of a) p-ser9 GSK-3β and b) p53. The expressions of the protein in the PPP1R3C transfected cells were normalized against that of the mock transfected cells, i.e. relative expression = protein expression in PPP1R3C transfected cells / protein expression in mock transfected cells. Data were represented by Mean ± S.D. determined from three independent experiments.

### 4.4.3 *Effect of ectopic PPP1R3C expression on p53 transcriptional activity*

From the result of the western blot analysis, the level of p53 protein was shown to have increased slightly in HCT116 cells upon ectopic expression of PPP1R3C. Renilla-firefly Dual Luciferase Reporter Assay on the p53 transcriptional activity was performed to investigate the mechanism that induce the enhancement of p53 protein level. From Fig. 4.5, it could be seen that there was no significant difference in the p53 transcription activity between the Flag-PPP1R3C trasnfected samples and the mock transfected control. Thus, increase in p53 level was not likely to be due to the induction of p53 transcription. In this experiment, a positive control sample was included in which pcDNA3.1 plasmid with p53 was transfected into HCT116 cells in order to overexpress p53 protein in the cells. It could be observed that the p53 transcription activity increased dramatically, which was more than 26-fold of induction (Table 4.2). The data of the positive control verified the validity of the present experimental setup and the conclusion that p53 transcriptional activity was not affected by ectopic PPP1R3C expression.

### 4.4.4 *Effect of ectopic PPP1R3C expression on NF-κB transcriptional activity*

As mentioned in section 4.1.3, activity of GSK-3β is regulated by the phosphorylation status at ser9 position. GSK-3β can either positively or negatively affect the NF-κB transcriptional activity which is context dependent, such as the type of the cancer cells. All three carcinoma cell lines tested in this study were found to have increased in p-ser9 GSK-3β (Fig. 4.2). We would also like to study whether increase in p-ser9 GSK-3β would alter the level of NF-κB transcriptional activity. Result of Renilla-firefly Dual Luciferase Reporter Assay showed that there was no significant difference in NF-κB transcriptional activity between the Flag-PPP1R3C

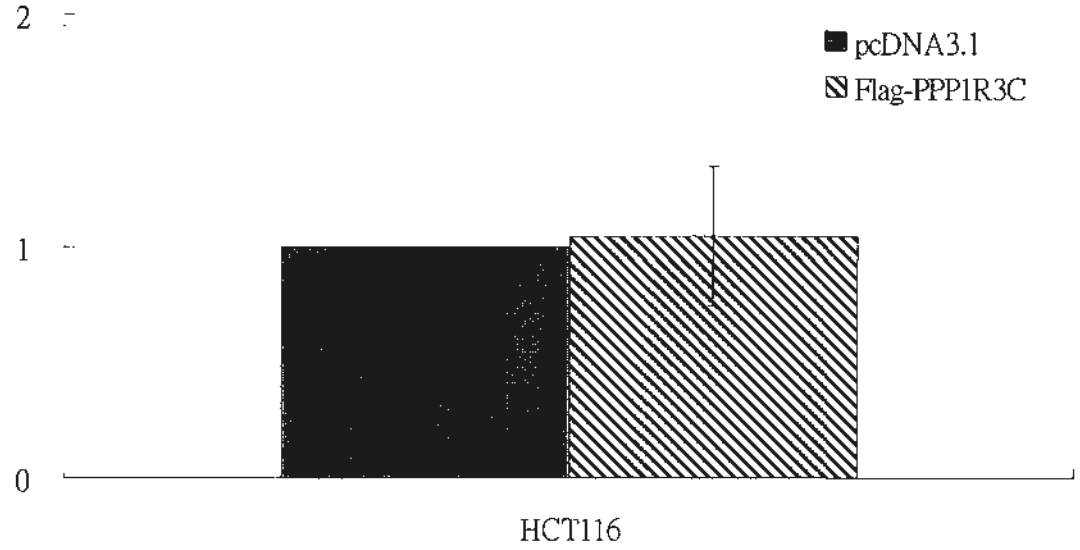



Fig. 4.5 Determination of p53 transcription activity by Renilla-Firefly Dual Luciferase Reporter Assay. Briefly, PcDNA3.1-Flag-PPP1R3C, Renila-luciferase vector and p53-Lucifease reporter plasmid were co-transfected into the corresponding cancer cell lines (Caski, HCT116 and KYSE150 cells). The luciferase activities of the corresponding samples were measured at 48 hr after plasmid transfection. Data were represented by Mean ± S.E.M from three independent experiments.

| | **Relative transcriptional activity** |
|---|---|
| Mock | 1 |
| PPP1R3C | 1.040 ± 0.154 |
| ***P53*** | ***26.693 ± 3.194*** |

Table. 4.2 Quantitative data of p53 transcription activity.

transfected sample and the mock-transfected control in Caski cells (Fig 4.6). For HCT116 cells, there was a nearly 1.7-fold induction of NF-κB transcriptional activity when PPP1R3C was re-expressed in the cells. In contrast, ectopic expression of PPP1R3C significantly down-regulated NF-κB transcriptional activity in KYSE150, which was about 40% reduction relative to that of the mock-transfected cells (Table 4.3).

### 4.4.5 *Subcellular localization of PPP1R3C revealed by GFP-fusion protein*

Similar to PPP1R1B, the subcellular localization of the protein encoded by PPP1R3C was revealed by fluorescence microscopy with the use of GFP-tagged PPP1R3C fusion protein. Nuclei staining by DAPI revealed that the nucleus of HCT116 cells remained intact upon re-expression of PPP1R3C under our transfection protocol (Fig. 4.6a). It can be observed that the green fluorescence indiscriminately distributed all over the cells upon the transfection of pEGFP-N1 vector plasmid only (Fig. 4.7b). From the fluorescence image of the GFP-tagged fusion protein, it could be observed that the green fluorescence formed a discrete circular/eclipse structure (Fig. 4.6b). When DAPI stained image is superimposed on the green fluorescence image, it could be clearly seen that the green fluorescence from the GFP-tagged fusion protein almost completely engulfed the nucleus of HCT116 cells (Fig. 4.6c). Based on the result, we speculated that protein encoded by PPP1R3C might exhibit its function around the surface of nuclear membrane, in which it could be a nuclear membrane protein.

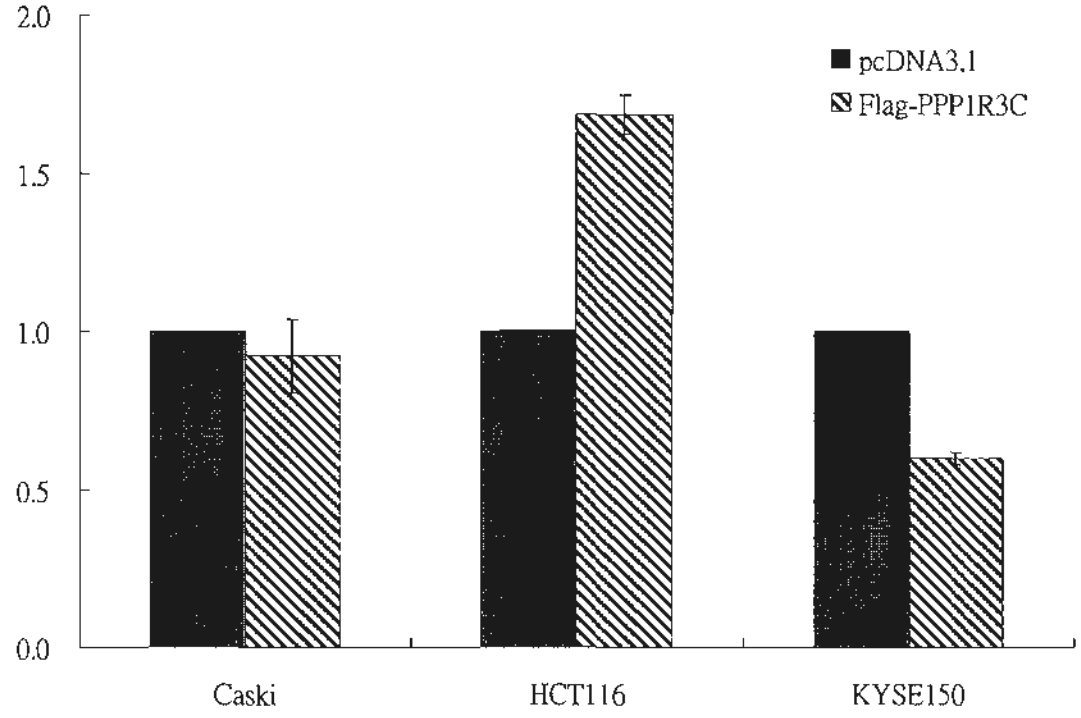



Fig. 4.6 Determination of NF-κB transcription activity by Renilla-Firefly Dual Luciferase Reporter Assay. Briefly, PcDNA3.1-Flag-PPP1R3C, Renila-luciferase vector and NF-κB-Lucifease reporter plasmid were co-transfected into the corresponding cancer cell lines (Caski, HCT116 and KYSE150 cells). The luciferase activities of the corresponding samples were measured at 48 hr after plasmid transfection. Data were represented by Mean ± S.E.M from three independent experiments.

| **Relative transcriptional activity of NF-κB** | | |
|---|---|---|
| | **Mock** | **Flag-PPP1R3C** |
| **Caski** | 1 | 0.921±0.118 |
| **HCT116** | 1 | 1.684±0.062 |
| **KYSE150** | 1 | 0.599±0.019 |

Table. 4.3 Quantitative data of NF-κB transcription activity.

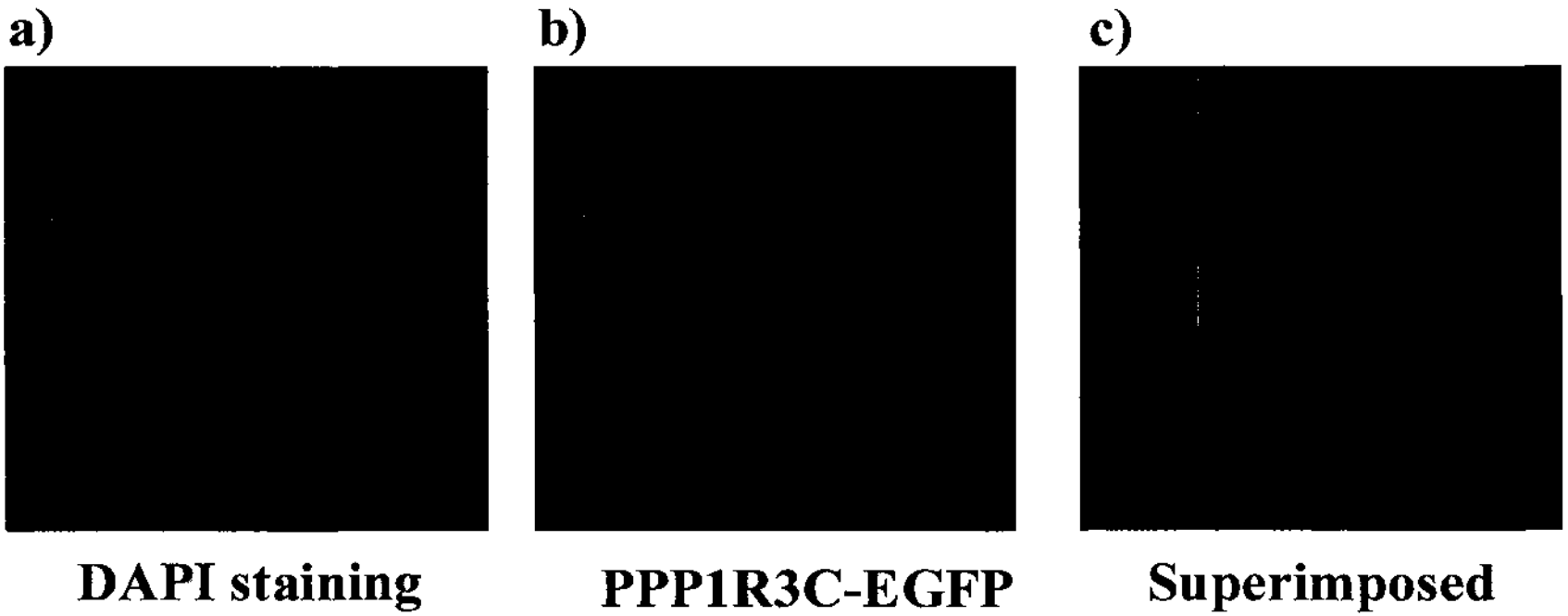



Fig. 4.7 Subcellular localization study of PPP1R3C using GFP-tagged fusion protein. a) Nuclei of HCT116 cells were stained by DAPI while b) the subcellular localization of PPP1R3C was revealed by the green fluorescence of the GFP-tagged fusion protein. c) The superimposed image indicated that protein products of PPP1R3C were likely to reside around the nuclear membrane. Data shown was the representative figure from three independent experiments.

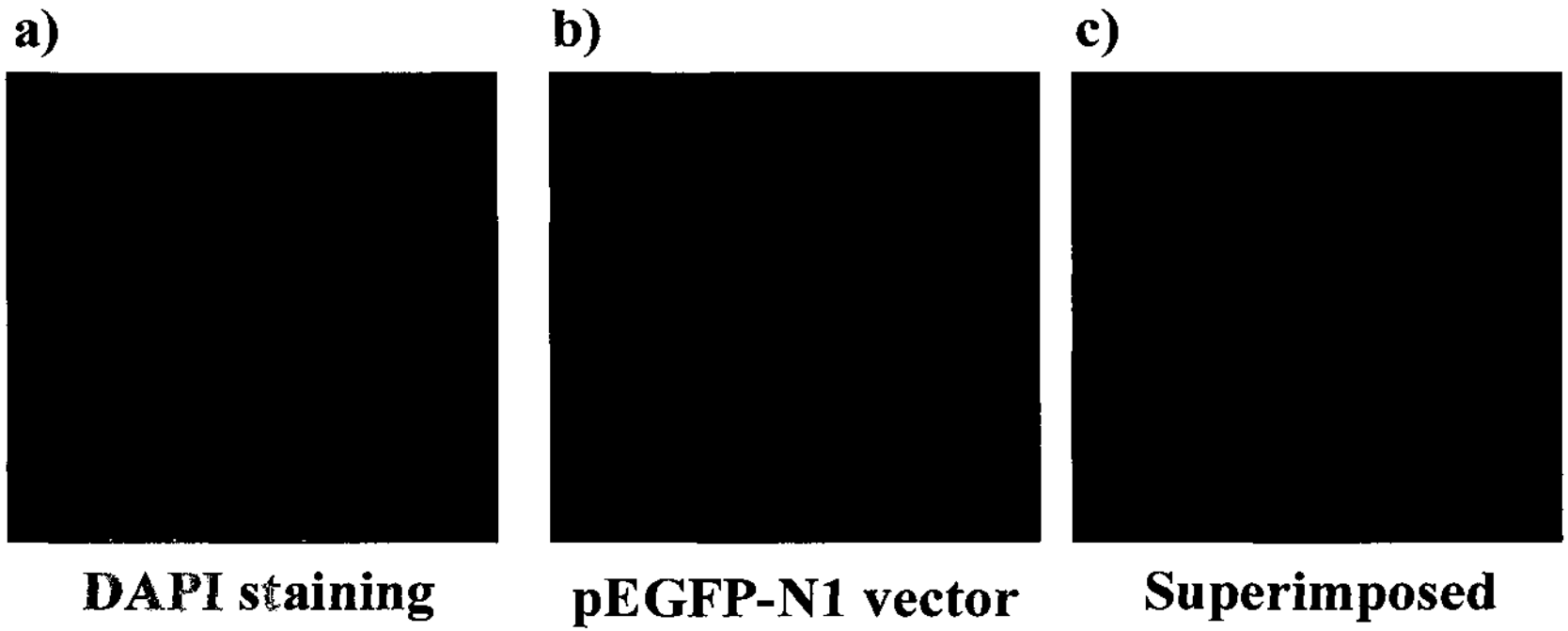



Fig. 4.8 Fluorescence microscopic image of HCT116 cells expressing pEGFP-N1 vector. a) Nuclei of HCT116 cells were stained by DAPI while b) expression of GFP as revealed by fluorescence microscope. c) Superimpose of the DAPI staining image and GFP fluorescence image. Data shown was the representative figure from three independent experiments.

## 4.5 Discussion

### 4.5.1 *Glycogen metabolism regulator, PPP1R3C involves in tumor suppression*

As the regulatory subunits of protein phosphatase 1 regulate many important physiological functions, their discovery and further investigation are focused on how they regulate those physiological functions which sometimes will mask their functional role in carcinogensis. PPP1R1B is one of the examples. For many years, studies related to PPP1R1B were about its role in dopamine signaling in neurons. Reports about its role in tumor suppression have only started to increase in the past decade. Similarly, research efforts on PPP1R3C were placed on the investigation about the regulation of glycogen metabolism by PPP1R3C because of its interaction with Laforin and PP1, which is associated with the autosomal recessive disorder, Lafora disease. PTEN as a lipid phosphatase, was once known for its involvement with lipid metabolism but was later reported to be a tumor suppressor gene. The lipid phosphatase activity of PTEN regulates cell prolieferation by dephosphorylating the secondary signal transduction messenger, $PIP_3$ so as to prevent it from triggering the downstream signaling pathway. It is the reason why epigenetic silencing of PTEN would lead to carcinogenesis. Similar to PTEN, we have found that PPP1R3C is not only necessary in regulating the glycogen storage but also possesses tumor suppressor functions. Although genome wide expression studies in melanoma oral tongue squamous cell carcinoma were able to detect downregulation of PPP1R3C in both cancer cells (Bonazzi et *al.*, 2009; Ye et *al.*, 2008), detailed investigation about the tumor suppressor role of PPP1R3C has not yet been carried out.

In chapter 2, we were able to show that expressions of PPP1R3C were silenced by promoter hypermethylation in Caski (cervical), HCT116 (colon) and

KYSE150 (ESCC). Herein, we were able to show that PPP1R3C was successfully expressed in all three cancer cell lines under our transfection protocol as shown by the result of semi-quantitvative RT-PCR (Fig. 4.1b). Ectopic expression of PPP1R3C revealed that PPP1R3C could significantly inhibit the growth of these three different types of cancer cells (Fig. 4a). Although it is premature to state that PPP1R3C has a generalized growth suppression effect regardless of the type of the cells, we could at least demonstrate the potential tumor suppressor functions of PPP1R3C where epigenetic silencing of PPP1R3C could be one of the series aberrant changes leading to carcinogenesis besides its function in glycogen metabolism. Unfortunately, only the carbohydrate binding motif has been discovered in the structural analysis of PPP1R3C which gives little insight of how PPP1R3C suppresses the cancer cell growth.

### 4.5.2 *PPP1R3C did not suppress tumor growth through Wnt/β-catenin signaling as it upregulated the ser-9 phosphorylated GSK-3β*

PTG is an important regulator of glycogen metabolism; it interacts with Laforin and PP1, which subsequently affects the activity of glycogen synthase. As GSK-3β is critical in regulating Wnt signaling pathway, we would like to know whether ectopic expression of PPP1R3C also regulates the Wnt signaling by controlling the activity of GSK-3β. Dephosphorylated GSK-3β is an active form of the kinase that phosphorylates β-catenin and leads to its ubiqutination and proteosomal degradation of β-catenin. Phosphorylation of GSK-3β at ser-9 position will lead to the inactivation of the kinase activity. Thus, intracellular β-catenin will accumulate and trigger downstream signaling for cell proliferation. We hypothesized that phosphatase activity of PP1 would be activated in the presence of PPP1R3C

expression. It might be essential for maintaining GSK-3β in a dephosphorylated state and halt the signal transduction in Wnt/β-catenin signaling. Surprisingly, dramatic increase in the inactive, ser-9 phosphorylated form of GSK-3β was detected in all the tested carcinoma cell lines upon ectopic expression of PPP1R3C. Based on this finding, it seems that there is no association between PPP1R3C expression and the regulation of Wnt/β-catenin signaling pathway as the active, dephosphorylated GSK-3β is the one which is responsible for it. Many oncoproteins or tumor suppressor proteins are regulated by GSK-3β. Tumor suppressor protein P53 is one of those. Inactivation of GSK-3β has been associated with increasing the level of p53 or enhancing the cytotoxicity of p53 activating chemotherapeutic drug. It has been reported that abolishment of GSK-3β could sustained the activation of transcriptionally active p53 and p53-dependent apoptosis in colorectal cancer cells expressing wild-type p53 (Ghosh et *al.*, 2005). In a similar study in glioblastoma performed by Miyashita et *al.* (Miyashita et *al.*, 2009), higher expression levels of dephosphorylated GSK-3β and p-tyr216 GSK-3β (also an active kinase form) were frequently detected in glioblastoma samples compared with that of the non-neoplastic brain tissues. In the same study, an effective dose of their small-molecule GSK-3β inhibitor or inhibition of GSK-3β by RNA interference in glioblastoma cells was able to induce apoptosis and attenuate the survival and proliferation of glioblastoma cells in which wild-type p53 and p21 expression were increased. In the present study, there was a slight increase in p53 level in HCT116 cells (a wild type p53 expressing colon carcinoma cells) after re-expressing PPP1R3C in it with concomitant elevated level of p-ser9 GSK-3β, which was consistent to what has been suggested from those previous studies mentioned here.

Interestingly, Matsui et *al.* (Matsui et *al.*, 2008) has demonstrated that *in vitro* treatment of the urothelial cancer cell lines with triptolide, an oxygenated diterpene derived from a Chinese herb, would increase the inactive form of GSK-3β in which the p-ser9 phosphorylated GSK-3b would interact with p53 in nucleus and promote apoptosis under the existence of genotoxic stress of chemotherapeutic agents such as the cisplatin treatment used in that study. They have also found that pretreating the urothelial cancer cells with triptolide would slightly enhance the cisplatin-induced p53 expression. Based on our similar observation after the ectopic expression of PPP1R3C, it is worthwhile to further investigate whether inducing PPP1R3C expression in cancer cells will enhance the sensitivity of cancer cells towards cytotoxic drugs that trigger p53-mediated apoptosis or any correlation in the resistance to chemotherapeutic agents in PPP1R3C silenced tumors. Attention should be paid to such theory that will only account for the cells expressing the wild-type p53. In case of those cancer cells with completed abolishment of p53 expression or expressing mutant p53 like Caski (absence of p53 expression) and KYSE150 (point mutation in p53), respectively, the mechanism of the cell death could follow a very different context. In a study about the influence of GSK-3β activity on the growth of ovarian cancer cells, pharmacological inhibition of GSK-3β would suppress the proliferation of ovarian cancer cells while overexpressing the constitutively active form of GSK-3β would induce the entry into S-phase and facilitated the proliferation of ovarian cancer cells (Cao et *al.*, 2006). This suggested that GSK-3β was a positive regulator for the proliferation of ovarian cancer cells by promoting the cell cycle progression. As growth inhibition and significant enhancement of inactive form of GSK-3β were detected in Caski cells and KYSE150 cells in our study, it will be intrigued to investigate further about

whether cell cycle arrest in G1/S phase will be induced by re-expressing PPP1R3C in cancer cells without expressing wild-type p53 like that of Caski and KYSE150.

### 4.5.3 *PPP1R3C re-expression induced ser-9 phosphorylated GSK-3β did not increase the p53 transcription in HCT116 cells*

The p53 expression level is affected by the stability of the protein and the transcriptional activity of the gene. Matsui et *al.* (Matsui et *al.*, 2008) claimed that the intranuclear association of p53 with inactive p-ser9 GSK-3β would suppress p53 transcriptional activity. In contrast to what they have claimed, our data suggested that there was no change in the p53 transcription activity upon PPP1R3C re-expression and increased level of inactive p-ser9 GSK-3β. Thus, the modulation in the stability of p53 should be the possible explanation for the increased p53 protein level. Post-translational modifications such as phosphorylation, acetylation would prevent p53 from degradation and induce its transactivating activity. DNA damage (Gamma or UV iiradiation, alkylation of bases, depurination of DNA), genotoxic stress after the treatment of chemical agents, oxidative stress or even deregulation of oncogene expression are the factors that activate p53 protein.Activated p53 protein will mediate the DNA repairing activity or trigger cell cycle arrest and apoptosis whenever the cells suffer irreversible DNA damage (Harris, et *al.* 2005). Thus, it is essential to determine whether post-translational modification such as phosphorylation at ser-15 and ser-37 of p53 will be induced in the presence of PPP1R3C expression as these two modifications are important in stabilizing p53 protein and also the transactivating activity of p53. In fact, it has been reported that PP1 is able to dephosphorylate p53 at ser-15 and ser-37, which will promote cell survival (Li et *al.*, 2006). The presence of these two modifications upon PPP1R3C

re-expression may imply that the phosphatase activity of PP1 is inhibited by PPP1R3C. Inhibition of PP1 might also be a reason for the increased level of phosphorylated GSK-3β. Though, further experiments should be performed to verify such hypothesis.

### 4.5.4 *PPP1R3C re-expression induced GSK-3β phosphorylation at ser-9 increases NF-κb transcription activity in HCT116 cells but reduces that in KYSE150 cells*

Regulation of NF-κB activity by GSK-3β has been showed in several reports. However, it is complicated that GSK-3β can either positively or negative regulate the NF-κB activity. TNF-α is able to induce NF-κB DNA binding activity and promotes the cell survival while TNF-α is cytotoxic itself. Hoeflich et *al.*'s (Hoeflich et *al.*, 2000) discovered that sensitivity of TNF-α induced toxicity was higher in GSK-3β null mice and the TNF-α induced NF-kB DNA binding activity was significantly decreased in GSK-3β null embryonic fibroblasts compared to that of wild-type cells. They also found that inhibition of GSK-3β by lithium could reduce NF-kB activity in HEK293 cells. This indicated that GSK-3β is a positive regulator of NF-κB activity upon TNF-α induction. On the contrary, lithium inhibition of GSK-3β or expression of dominant negative GSK-3β resulted in increased NF-κB activity in Wnt-1 signlaing and promoted the survival of PC12 cells. It indicated the negative regulation of NF-κB activity by GSK-3β (Bournat et *al.*, 2000). However, it should also be noted that NF-κB can be either proapoptotic or anti-apoptotic. The above information does not necessary mean NF-κB would only promote cell survival or proliferation under the influence of GSK-3β while it only

echo the fact of the complexity about the interaction between GSK-3β and NF-κB. The outcome is unpredictable upon the effect of the differential activity of GSK-3β and NF-κB. In the present study, we have also demonstrated this contradictory phenomenon. Accumulation of inactive p-ser9 GSK-3β upregulated the NF-κB transcription activity in HCT116 cells but decreased significantly in KYSE150 cells in the presence of PPP1R3C expression despite using the same transfection protocol. Grimes et *al.* (Grimes et *al.*, 2001) suggested that the outcome of NF-κB activity regulated by GSK-3β might rely on the modulatory effect of GSK-3β on the signaling systems that lead to the activation of NF-κB. p53-induced apoptosis is one of the cases. Ryan et *al.* (Ryan et al., 2000) revealed that p53-induced NF-κB activity activated by p53 would mediate the p53 induced apoptosis with the facilitation of MEK1 and activated $pp90^{rsk}$ in which the inhibition or loss of NF-κB activity would abrogate p53 induced-apoptosis. Hence, the therapeutic response for those tumors with wild-type p53 will diminish. The findings of their study could explain the surge of NF-κB activity when inactive p-ser9 GSK-3β is upregulated in HCT116 cells in the presence of PPP1R3C expression as HCT116 cells express wild type p53.

It is interesting that stabilization of p53 has been reported to be one of the proapoptotic functions of NF-κB. Thus, we speculate that increased in NF-κB transcriptional activity also contributes to the increased p53 level in HCT116 upon ectopic PPP1R3C expression. For KYSE150, it is more likely that the transcription of anti-apoptotic and cell survival gene were suppressed along with the decreased NF-κB activity to cause the reduction in cell proliferation. Nevertheless, further study on searching any proapoptotic (in HCT116) and antiapoptotic (in KYSE150)

genes regulated by PPP1R3C induced/reduced NF-κB transcription activity is necessary to explain the difference in the response for PPP1R3C expression in carcinoma cells with wild-type p53 expression and mutant p53 expression like HCT116 cells and KYSE150 cells, respectively.

### 4.5.5 *PPP1R3C might be a nuclear membrane protein in exerting its tumor suppressor function*

By far, only the tissue distribution of PPP1R3C protein has been reported owing to the well-known function of its glycogen storage regulation. Study about the subcellular localization of PPP1R3C is limited. In the present study, the subcellular localization was unveiled in order to have a better understanding of how PPP1R3C protein links to GSK-3β, NF-κB and even the p53 protein. From the fluorescence microscopic image, we found that the PPP1R3C-GFP tagged fusion protein was located around the nucleus. It has been known that activated p53 is found inside the nucleus. In the resting state, *MDM2* will continuously bind and mono-ubiquitinate p53. Monoubiqutinated p53 will then be exported to the cytoplasm and subject to proteosomal degradation after the polyubiquitination. When the cells are under stress condition, kinases such as ATM (mutated in ataxia-telangiectasia) will phosphorylate p53, which will stabilize and retain p53 inside the nucleus for inducing gene transcription and DNA repairing activity. For NF-κB, it is a transcription factor located inside the nucleus that activated the transcription of proapoptotic gene or genes for cell survival and proliferation (Lavin et *al.*, 2006). It was once expected that PPP1R3C would disperse around the cytoplasm because of its interaction with PP1 and enzymes regulating glycogen metabolism. However, our data suggested that PPP1R3C protein localized around the surface of nuclear membrane where it might

bind to its interacting partner. If it is true for the intranuclear association of GSK-3β with p53 and p-ser9 GSK-3β mediated induction or repression of NF-κB, it is very likely that PPP1R3C protein serves as a mediator (when bind with its interacting partner) to relay the cytoplasmic signal to nucleus that is responsible for activating or repressing the NF-κB mediated by p-ser9 GSK-3β inside the nucleus. Thus, it is necessary to discover the identity of the interacting partner of PPP1R3C which might be a nuclear membrane protein and prove if PPP1R3C protein inhibits the phosphatase activity of such nuclear membrane protein that is necessary for dephosphorylating GSK-3β.

# Chapter 5

# General Discussion and Future Perspectives

## 5.1 *Epigenetic silencing of PPP1R1B and PPP1R13C is important in detection and treatment of cancer and understanding the molecular pathogenesis of cancer*

Carcinogenesis is a process caused by a series of mutations leading to loss or gain of functions for the TSG or oncogene respectively through both genetic and epigenetic mechanisms. Scientists strife to identify TSGs (silencing) and Oncogene (overexpression) with aberrant expression in order to gather information for better understanding of the molecular pathogenesis of cancer. In particular, identification of TSGs has been essential for delivering an earlier, more accurate diagnosis and discovery of novel therapeutics targets in tackling cancer. In searching for TSGs, one of the strategies is to spot the chromosome loci with frequent copy loss by techniques such as array comparative genome hybridization (aCGH) or identification of the loss of heterozygozity in specific types of cancer. Within the chromosome region, putative TSGs would be identified and its possible tumor suppressor functions would be studied. In the present study, putative TSGs were identified based on the expression studies previously done. In addition, their functional properties were studied.

Reversible phosphorylation is critical in the activation of many intracellular functions. Protein phoshphatase and protein kinase are the two large protein families that govern the phosphorylation status of the enormous intracellular protein. In terms of cancer, It is known that protein phosphatases often play a tumor suppressor role

such as controlling the cell cycle progression and inhibition of gene transcription for cell proliferation, while protein kinases often act as onco-protein in which it phosphorylates their target protein to activate the protein promoting the cell growth or inactivating those proteins responsible for triggering cell cycle arrest and apoptosis. As mentioned previously, the substrate specificity, activity and the subcellular localization of the Protein phosphatases such as PP1 are regulated by what regulatory subunits it interacts with. Hence, the regulatory subunits also have a critical role in tumor suppressor functions because of its influence on the protein phosphatase.

The functions of Protein phosphatase 1 regulatory subunits were initially thought to solely control the activity of PP1 as implied by its name. However, the studies about the functions of regulatory units such as GADD34 and ASPP protein family has inspired scientists to rethink that PP1 regulatory subunits might have functions not only limited to regulating PP1 activity. In the present study, PPP1R1B and PPP1R3C were the PP1 regulatory subunits identified with frequent loss of expression in multiple carcinomas. Based on the experiment of analyzing the methylation status (MSP and BGS) and the restoration of PPP1R1B and PPP1R3C expression by genetic and pharmacological demethylation in combination with histone deacetylase inhibitor TSA, we were convinced that epigenetic silencing of the gene expression through promoter CpG island hypermethylation is the main mechanism for the silencing of PPP1R1B and PPP1R3C. It has been suggested that epigenetic abnormalities could play a seminal role in the earliest step in cancer initiation (Baylin et *al.*, 2006; Feinberg et *al.*, 2006). As the abnormal gene imprinting and silencing are the catalyst for early aberrant clonal expansion of cells which provide the foundation for the subsequent genetic and epigenetic alterations

that further foster tumor progression, a better understanding on the correlation between the timing for the aberrant epigenetic gene silencing occurred and the cancer progression is beneficial to the intervention of cancer (Jones et *al.*, 2007; Baylin et *al.*, 2006; Feinberg et *al.*, 2006). Thus, it is important to know the time for the occurrence of epigenetic silecning of PPP1R1B and PPP1R3C, which might be some of the essential events in the early neoplastic transformation. Unfortunately, none of the above information could be provided in our study based our experimental setup. It has been suggested that transformation of a single normal cell to malignant cell may be affected by a series of abnormal epigenetic changes. These changes may function as networks in which multiple genes are not only affected within a pathway but can generate alterations of other key signaling pathways and even involve epigenetic events that cause other epigenetic events. Although the mechanism causing a cascade of epigenetic silencing of genes remains unclear, whether epigenetic silencing of PPP1R1B and/or PPP1R3C directly affecting the cancer initiation and progress, or a consequence of a series of upstream epigenetic changes, the present study is important in terms of constructing a epigenome database tailor-made for specific types of cancer and also provides information for the signaling pathway affected by such pathological changes that may give rise to a cellular growth advantage to the malignant cells.

Another important aspect of the present study is that the reversal of transcription inhibition of PPP1R1B and PPP1R3C by promoter CpG island hypermethylation gives insight to the novel therapeutics approaches in both directly killing the cancer cells (through inducing cell death or activating the host immune response) and enhanced the chemosensitivity of the cancer towards the chemotherapeutics drugs. MLH1, a gene responsible for the DNA mismatch repair

was found to be frequently inactivated by promoter hypermethylation in different human cancers that has been associated with microsatellite instability (Esteller et *al.*, 1998; Herman et *al.*, 1998). MGMT is another example of a DNA repair gene that is frequently inactivated by promoter hypermethylation in human cancers (Esteller et *al.*, 1999). It has been found that restoration of MLH1 expression by combined treatment of 5-aza-CdR and histone deacetylase inhibitor would result in reconstitution of the mismatch repair function that sensitized the ovarian and colon tumor xenografts towards different chemotherapeutics drugs (Plumb et al., 2000). Similarly, restoring the defective expression of apoptosis effector proteins or re-establishing the expression of signal transduction or mediators of the apoptotic signal both pertaining to the mitochondrial and to the death receptor pathway by epigenetic drugs treatment can sensitize the neoplastic cells to apoptosis (Sigalotti et *al.*, 2007). It was suggested that re-expression of pro-apoptotic gene RASSF1A and prototypic apoptosis-associated IFN response gene XAF1 by 5-aza-CdR treatment and DNMT1 knockout could restore the sensitivity of the cancer cells to IFN-triggered apoptosis (Reu et *al.*, 2006a, 2006b). Restoring caspase-8 expression by 5-aza-CdR treatment was also proven to be effective in re-sensitive the cancer cells to TRAIL-, anti-FAS-, and drug-triggered apoptosis (Hopkins-Donaldson et *al.*, 2000; Fulda et *al.*, 2001). Now that epigenetic silencings of PPP1R1B and PPP1R3C by promoter hypermethylation have been confirmed in multiple carcinomas, it is important to know what signaling pathways are dysregulated upon the silencing of these genes and how those dysregulated pathways contribute to the growth advantage and/or aggressiveness of a particular tumor. There is a growing tendency in using the combination treatment of conventional chemotherapeutic drugs and epigenetic drugs to cure cancer, for example, the combined use of cisplatin and aza-CdR in patients

with solid tumors (Yoo et *al.*, 2006). Therefore, by knowing what sort of drug resistance will result from epigenetic silencing of PPP1R1B or PPP1R3C, we can make good use of epigenetic drugs as a way to alleviate the chemotherapeutics drug resistance and improve the response of the cancer treatment for the cancer patients. For instances, donwregulation of full-length DARPP-32 (PPP1R1B) would result in Herceptin resistance in breast cancer cells because of the upregulation of p-ser473 Akt (Gu et *al.*, 2009). Epigenetic drug to restore PPP1R1B expression will be useful to restore the sensitivity of breast cancer cells toward Herceptin if PPP1R1B is silenced in breast cancer cells through epigenetic mechanism. This is the reason why functional epigenetics is important in terms of elucidating the signaling pathways regulated by the PPP1R1B protein and also that of the PPP1R3C protein.

## 5.2 *Schematic model for the signal pathway involved by PPP1R1B*

Thr-34 phosphorylated DARPP-32 and Thr-75 phosphorylated DARPP-32 have often been the focus of research as they regulate the activity of PKA (by Thr-75 DARPP-32) and PP1 (by Thr-34 DARPP-32), which are important in the regulation of the phosphorylation status of many intracellular proteins and subsequently affects their downstream signaling pathway. At present, there are still many debates about the functions of PPP1R1B protein and DARPP-32, in terms of cancer. PKA is believed to be a mediator for promoting cell growth because it phosphorylates and activates the protein like Akt and CREB. Therefore, inhibition of PKA could be the key to suppress tumor growth. RIα is the regulatory subunit of PKA which confers the kinase activity of PKA. It has been suggested that depletion of RIα could potentiate the anti-proliferative effect of the anti-androgen bicalutamide in androgen-sensitive prostate cancer cells LNCaP (Desiniotis et *al.*, 2010). Thr-75

phosphorylated DARPP-32 is an endogenous PKA inhibitor. In our study, we found that p-ser473 Akt and p-ser552 β-catenin were downregulated upon the re-expression of PPP1R1B gene in which Akt and β-catenin are the substrate phosphorylated by PKA. We also found that the transcriptional activity of AP-1 was decreased when PPP1R1B was ectopically expressed in PPP1R1B silenced HCT116 and H1299 cells. Based on this finding and the result of colony formation assay, we were convinced that DARPP-32 itself played an important role in suppressing tumor growth through its ability of inhibiting PKA activity. In normal epithelial cells, kinase activity of PKA will be turned on in response to external stimulus such as the influence of growth factor. Then, it phosphorylates its targets like Akt, β-catenin and CREB of which the phosphorylated form of these proteins are the components to promote the proliferation and survival of the cells. Phosphorylation of DARPP-32 on Thr-75 by Cdk5 serves as a negative feedback on the kinase activity of PKA in which the presence of Thr-75 DARPP-32 is to inhibit the continuous phosphorylation on Akt, β-catenin and CREB by PKA. This can prevent excessive cell proliferation. In the cancerous tissue, epigenetic silencing of PPP1R1B by promoter CpG island hypermethylation leads to the reduction in the amount of DARPP-32 present inside the cells and hence the negative feedback on PKA. It can be expected that the accumulation of p-ser473 Akt, p-ser552 β-catenin and the upregulation of CREB and AP-1 transactivating activity in cancerous tissue is due to the lessening or abolishment of PKA activity inhibition which will subsequently enhance the survival of malignant cells and allow them to proliferate in an uncontrolled manner (Fig. 5.1).

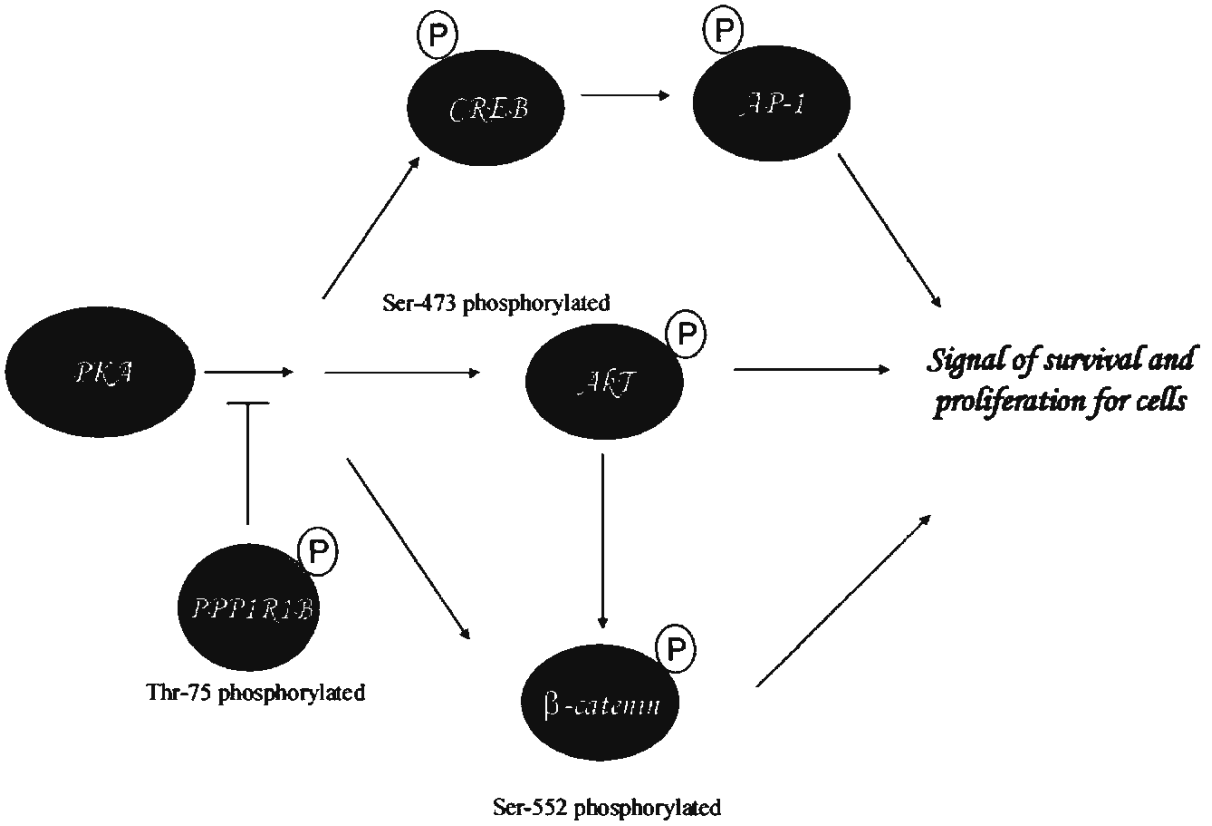



Fig. 5.1 Schematic model for the signaling pathway involves with PPP1R1B in suppressing tumor growth. Phosphorylated β-catenin, Akt, CREB and AP-1 are important activators of cell proliferation. Thr-75 phosphorylated PPP1R1B protein (DARPP-32) inhibits the kinase activity of PKA so that phosphorylation on β-catenin, Akt, CREB and AP-1 will decrease.

In the present study, full-length PPP1R1B gene and hence DARPP-32 protein were deliberately express in our *in vitro* experiments. However, DARPP-32 and the truncated isoform t-DARPP are produced through alternative splicing in normalphysiological condition. As suggested in many studies, t-DARPP is an oncoprotein in which its overexpression will promote the cancer survival and conferred resistance to the treatment of cancer through an Akt-dependent mechanism (Belkhiri et *al.*, 2008; Hamel et *al.*, 2009; Gu et *al.*, 2009). Thus, it is far more complicated to say whether the expression of PPP1R1B will promote or suppress tumorigenesis in physiological condition. The crucial factor may depend on other external factor deciding the transcription of full-length PPP1R1B or the truncated isoform of PPP1R1B.

## 5.3 *The difference between DARPP-32 and t-DARPP*

The tumor suppressor function of PPP1R1B has been revealed in our study. Such findings raised the question: what are the factors that will make DARPP-32 and t-DARPP behave so differently in terms of intracellular function. Our data has suggested that the tumor suppressor function of PPP1R1B depends on Thr-75 phosphorylated DARPP-32 but it is inadequate to explain the functional differences between DARPP-32 and t-DARPP as both proteins have Thr-75 phosphorylation site. Structurally, t-DARPP differs from full length DARPP-32 by not having the phosphoyrlation site Thr-34. Hansen et *al.* (Hansen et *al.*, 2006) suggested that inhibiting the motility of cells required the present of Thr-34 phosphorylated DARPP-32 by showing the abolishment of the ability of DARPP-32 to impair breast cancer cell migration after mutation of Thr-34 to an alanine residue. In addition, they observed an increase in Thr-34 phosphorylated DARPP-32 upon detachment of

breast cancer cells. Thus, it could be expected that expressing t-DARPP, which is lacking the Thr-34 instead of DARPP-32, may favour the cancer cell metastasis. They proposed that phosphorylated DARPP-32 is a potent PP1 inhibitor that favours phosphorylation and activation of signaling pathways possibly at the leading edge of a migrating cell. Alternatively, it exerts anti-migratory effect by increasing the phosphorylation status of motor or motor associated proteins (Hansen et *al.*, 2006). However, such claim could only provide the explanation for any association between PPP1R1B expression and the aggressiveness of the tumor. The difference between DARPP-32 and t-DARPP is possibly related to their influences on the activation of PI3K/Akt signaling pathway. As mentioned, it has been suggested that t-DARPP expression preserves the mitochondrial transmembrane potential through upregulation of Bcl-2 level in a Akt-dependent manner (Belkhiri et *al.*, 2008). In the study of the Herceptin resistance of breast cancer cells, Gu et *al.* (Gu et *al.*, 2009) suggested that Thr-34 DARPP-32 phosphorylated by PKA, positively regulated the level of active p-ser473 Akt as it inhibited the phosphatase activity of PP1. t-DARPP appears likely to be the activator of PKA or has a dominant negative effect on DARPP-32's inhibitory activity on PKA.

However, we should be very careful about the structural difference between t-DARPP and DARPP-32. We believe that the binding affinity and specificity of t-DARPP may be greatly different from that of DARPP-32 because t-DARPP is 36 amino acids shorter than full-length DARPP-32. It can be expected that the protein folding and hence the three dimensional structure of t-DARPP should be very different from that of DARPP-32. There is still the possibility that t-DARPP binds strongly to PP1 and then inhibits the activity of PP1 even without the presence of Thr-34 phosphorylated site. Such inhibition is expected to allow the accumulation of

ser-473 phosphorylated Akt and thus increases the level of Bcl-2 as Bcl-2 is phosphorylated by Akt. Nevertheless, it is necessary to resolve the three dimensional structure of t-DARPP and DARPP-32. It is also important to study the binding affinity of t-DARPP and DARPP32 towards PKA and PP1 and the details of how they interact with PKA and PP1, in order to understand how t-DARPP and DARPP-32 behave as oncoprotein and tumor suppressor protein respectively.

It is still unclear what mechanism controls the transcription of DARPP-32 and t-DARPP. It will be beneficial to understand what circumstances will induce the transcription of truncated variant t-DARPP or whether it is simply a mutant only found in cancer cells that gives the growth advantage for the cancer cells.

## 5.4 *Schematic model for PPP1R3C signaling*

PPP1R3C protein, more commonly called PTG, is an important regulator for the glycogen storage. The expression level of PTG is suggested to be directly proportional to the glycogen storage. It was found that overpexression of PTG markedly increased the glycogen accumulation while downregulation of PTG expression would lower the glycogen storage (Worby et *al.*, 2008). PTG and glycogen debranching enzymes bind to the glycogen particle through its carbohydrate binding module, which are both involved in glycogen synthesis. During normal glycogen metabolism, the carbohydrate binding motif of laforin allows the binding of laforin to the glycogen particles. In the meantime, it interacts with PTG and acts as a phosphatase to dephosphorylate the glycogen particles. Upon the intracellular stimulus, Malin will bind to the complex and ubiquitinate laforin, glycogen debranching enzyme and PTG to target them for proteasomal degradation while the glycogen particles will be released and metabolized for energy production

(Worby, et *al.*, 2008).

It was suggested that the phosphatase activity of laforin required the presence of PTG (Fernandez-Sanchez et *al.*, 2003). In a recent study, EPM2A (gene encoding Laforin) was suggested to be a tumor suppressor gene. EPM2A was found to be frequently silenced by promoter CpG island hypermethylation which was essential in carcinogenesis (Wang, et *al.*, 2006). In the same study, the authors found that the phosphatase activity of Laforin was necessary for maintaining the level of active dephosphorylated GSK-3β because active dephosphorylated GSK-3β could phosphorylate β-catenin and target it for ubiquitination dependent proteosomal degradation. The Wnt signaling was hence inhibited. It was once thought that PTG could also be an important regulator in Wnt signaling because of its necessity to the phosphatase activity of Laforin. In contrast to this hypothesis, our data revealed that the level of inactive ser-9 phosphorylated GSK-3β increased instead of decrease in the presence of PPP1R3C re-expression. Combining the result of NF-κB transcription activity analysis with western blot analysis on the level of p53, we proposed a model about the signaling networks involving in PPP1R3C, GSK-3β and NF-κB for suppressing the tumor growth (Fig.5.2). In our model, PPP1R3C protein is an inhibitor of PP1 rather than a PP1 activator. The binding of PPP1R3C seems to inhibit the phosphatase activity of PP1 and thus decrease the dephosphorylation of GSK-3β. The present hypothesis is an analogy to the action of the antifungal antibiotics tauomycetin and tautomycin, which are the potent chemical inhibitor of PP1. Adler et *al.* (Adler et *al.*, 2009) has studied the anti-proliferative effect of tauomycetin and tautomycin on medullary thyroid cancer. In that study, they were able to use these antibiotics to inhibit the growth of medullary thyroid cancer through inhibition of the kinase activity of GSK-3β. It was also shown that inhibition of

GSK-3β was through the inhibition of PP1 phosphatase activity (increased phosphorylated form of PP1 and decreased in the total amount of PP1) which subsequently led to the increase in ser9 phosphorylated GSK-3β. Thus, we believe that PPP1R3C protein has similar function as tautomycetin and tautomycin according to our findings and the data presented by Adler et *al.*'s (Adler et *al.*, 2009) study. In the study about the role of PTG in the regulation of PP1 activity, it was suggested that PTG increased the phosphatase activity of PP1 by lowering the ability of DARPP-32 to inhibit PP1 (Brady, et *al.*, 1997). Gu et *al.* (Gu et *al.*, 2009) supported a similar claim by suggesting PTG as a positive regulator of PP1 that there would be an increase in dephosphorylation of p-ser473 Akt by PP1 in the presence of PTG. To elaborate more, it means that expression of PTG will ultimately increase the level of dephosphorylated GSK-3β as GSK-3β is phosphorylated by active p-ser473 Akt. The data of our study and the proposed model here have ruled out the possibility that PPP1R3C exhibits anti-cancer activity by stimulating PP1 phosphatase activity. PPP1R3C as a positive regulator of PP1 may only link to the glycogen synthesis in adipocytes.

Rates of glucose usage and glycolysis are often higher in many poorly differentiated and rapidly growing malignant tumors in order to fulfill the higher energy demand when compared with the corresponding normal tissue counterpart (Pedersen, P. L., 1978; Warburg, O., 1956). It is interesting to know that Laforin is as an important tumor suppressor in terms of inhibiting Wnt signaling (Wang, et *al.*, 2006) may increase the sensitivity of cancer cell towards energy deprivation cancer therapy (Wang et *al.*, 2008). It has been suggested that laforin can be important in regulating the cell survival under energy deprivation. Wang et *al.* (Wang et *al.*, 2008) found that treatment of hexose kinase inhibitor 2-deoxyglucose could preferentially

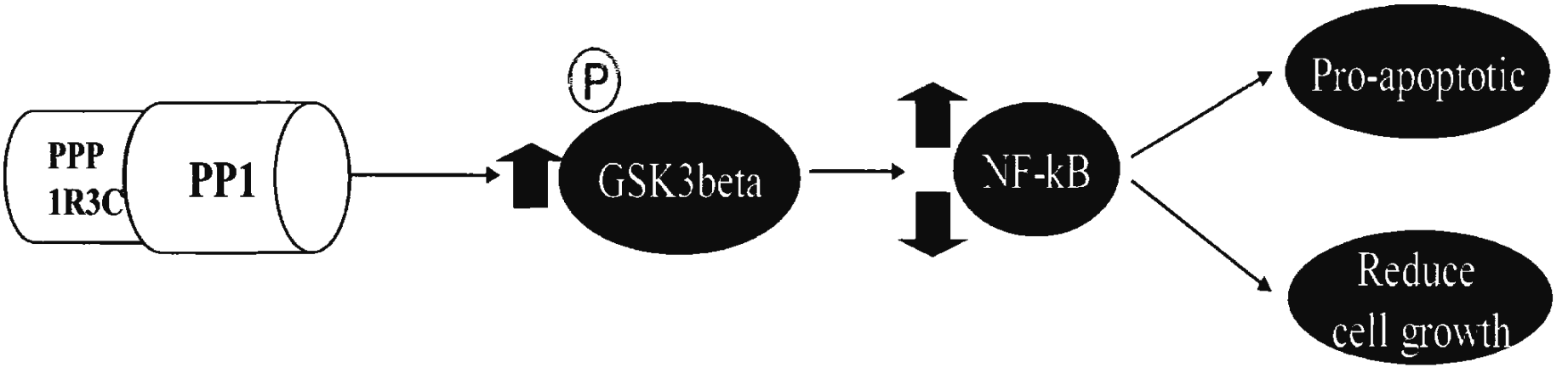



Fig. 5.2 Schematic model for the signaling pathway involved with PPP1R3C in suppressing tumor growth. Briefly, PPP1R3C protein (PTG) may inhibits the phosphatase activity of PP1 so that the dephosphorylation of p-ser9 GSK-3β will decrease. P-ser9 GSK-3β will affect the transcriptional activity of NF-κB which in turn suppresses the tumor growth or induce the apopotosis of the tumor cells.

kill the cancers with defective laforin expression and increase the survival of mice with aggressive lymphoma in which the laforin encoding EPM2A gene of the mice were genetically knockout. The accumulation of glycogen will increase in the presence of PTG expression while glycogen particles will be metabolized when PTG is degraded in the presence of laforin and malin in response to the energy need of the cell (Worby, C. A.). Epigenetic silencing of PPP1R3C in the cancer cells may allow glycogen particles to be continuously metabolized in order to fulfill the high energy demand of the cancer cells. Re-expressing the PPP1R3C by epigenetic treatment may be able to prevent the excessive glycogen metabolism where the cancer cells may suffer reduced energy production and deprived with energy. It will be intriguing to know whether combing the restoration of PPP1R3C expression with energy deprivation cancer therapy will bring synergistic cancer killing effect.

## 5.5 *Future perspectives*

*Subcellular localization of PPP1R1B and PPP1R3C*

In the present study, the subcellular localizations of PPP1R1B and PPP1R3C were determined by using the GFP-fusion protein. Although the reliability of using this method to demonstrate the subcellular localization of the target proteins has been well-documented, it should be pointed out that this method only gives us a preliminary idea of what further experiment should be done. It is because the size of GFP is around 26 kDa, which is comparable to PPP1R1B (~ 32 kDa) and PPP1R3C (~ 36 kDa), and the present of GFP may affect the folding and the trafficking of protein (because the masking of signal peptides by GFP). Immunohistochemical staining should be used in order to obtain a real picture of the subcelluar localization

of the target proteins. However, one should be careful that this method greatly relies on the specificity of the antibody in which cross reactivity of the other proteins will greatly affect the interpretation of the result, for example, fluorescence signal distributes all around the cells.

*Exploration of phosphatase regulatory subunits with tumor suppressor functions*

The importance for phosphatase in the regulation of cell cycle and apoptosis has been understood. The previous report about the tumor suppressor function of ASPP family protein and the present functional epigentics study of PPP1R1B and PPP1R3C reveals that protein phosphatase 1 regulatory subunits may be a class of proteins with important tumor suppressor functions. The successful use of gene expression profile study and epigenetic approaches to identify PPP1R1B and PPP1R3C as putative tumor suppressor gene indicated that epigenetic silencing of the regulatory subunits of protein phosphatase 1 might be one of a series of critical aberrant changes leading to carcinogenesis. The result of our study has provided a good foundation to prompt us for searching more regulatory subunits of protein phophatase 1 or even other phosphatases by using similar approaches as the present study in which their tumor suppressor functions may also be silenced by epigenetic mechanism and contribute to the carcinogenesis.

*Methylation status of PPP1R1B and PPP1R3C in primary tumors*

The current study has only showed the methylation status of PPP1R1B and PPP1R3C in a set of primary ESCC tumor samples. The methylation status for both putative tumor suppressor genes should be studied in other kinds of tumor such as NPC, hepatocellular carcinoma, etc, so as to evaluate the usefulness of

hypermethylation of the promoters of PPP1R1B and PPP1R3C as a marker for diagnosing cancer in clinical settings.

*Functional analysis of PPP1R1B and PPP1R3C in controlling the execution of cell cycle arrest and apoptosis*

Although we have successfully proved that expression of PPP1R1B and PPP1R3C both decrease the activity or the level of the protein related to cell proliferation, cell cycle progression, or upregulating the protein important in inducing apoptosis, we have yet to prove the present of cell cycle arrest and apoptosis upon the ectopic expression of PPP1R1B and PPP1R3C. Propidium iodide (PI) staining is a commonly used method for studying the cell cycle distribution because PI has a high affinity with double-stranded DNA which allows us to quantify the amount of DNA copy present in the cells in a particular time interval and hence gives us the information about the phase of the cell cycle that have been entered. Flipping of the membrane phosphatidyl-serine (PS) is one of the hallmark features of apoptosis. Annexin(V)-PI staining is the commonly used method for studying the induction of apoptosis in cells because Annexin(V) has a high affinity with the PS and only gives green fluorescence upon binding with PS. As PS is normally situated under the cell membrane and the cell membrane is impermeable to Annexin(V) and PI, the presence of green fluorescence is a good indicator for apoptosis. Thus, flow cytometric analysis using PI staining and Annexin(V)-PI staining should be performed in order to see whether cell cycle arrest or apoptosis will be induced in the carcinoma cells upon the re-expression of epigenetic silenced PPP1R1B and PPP1R3C genes.

*Analysis of for the activation of tumor suppressor genes or inactivation of oncogenes after ectopic expression of PPP1R1B and PPP1R3C*

Transcription factors AP-1 and NF-κB activate the transcription of their target genes in order to induce cell proliferation or apoptosis. The current study showed that there was downregulation of transcription factor AP-1 after reexpressing PPP1R1B while activity of the transcription factor NF-κB was altered upon the ectopic expression of PPP1R3C. It is also worthwhile to investigate the activity changes of the transcription of their target genes such as c-Fos by means of quantitative real-time PCR. It can help better understand the signaling pathways affected by PPP1R1B and PPP1R3C, which are important in controlling the cell cycle progression or apoptosis and hence the mechanism that leads to tumorigenesis.

*Determination of the phosphatase which its activity regulated by PPP1R3C in terms of controlling tumor growth*

In the present study, we are only able to show that the dephosphorylating activity of phosphatase of GSK-3β is inhibited by PPP1R3C and hence increases the amount of p-ser9 GSK-3β. There are many phosphtases present inside the cell besides PP1. Thus, experiment to determine the phosphatase inhibited by PPP1R3C in this particular circumstance has to be done. As PPP1R3C is a regulatory subunit of PP1, PP1 should be the most likely target. To prove PP1 is the responsible phosphatase inhibited by PPP1R3C in terms of controlling tumor growth, combination of ectopic expression of PPP1R3C with the knockdown of PP1 gene by small interference RNA or specific inhibition of PP1 by tautomycetin should be performed to see if there is any difference in the amount of p-ser9 GSK-3β compared

with that of the ectopic expression of PPP1R3C in carcinoma cells alone. Downregulation of p-ser9 GSK-3β will indicate that PP1 is the phosphatase dephosphorylating GSK-3β in this particular situation.

# Chapter 6

# Conclusions

Carcinogenesis is a complex and multi-steps process that involves a series of genetic or epigenetic alterations including the activation of pro-oncogenes and inactivation of TSGs. Phosphatases have been known for its importance in tumor suppression because they regulate the phosphorylation status of their target protein. PP1 as a member of the phosphatases family is regulated by protein phosphatase 1 regulatory subunits. In the present study, two protein phosphatase 1 regulatory subunits were identified with potential tumor suppressor functions. They are PPP1R1B and PPP1R3C. In the gene expression profile study by means of RT-PCR, both PPP1R1B and PPP1R3C were found to be frequently silenced in multiple carcinoma cells.

Promoter CpG island hypermethylation, histone deacetylation are the examples of the epigenetic mechanisms leading to gene silencing. In this study, PPP1R1B and PPP1R3C were both found to be silenced by promoter CpG island hypermethylation according to the result of MSP and BGS analysis. Restoration of PPP1R1B and PPP1R3C expression by the use of DNA methyltransferase inhibitor 5' aza-cytidine and histone deacetylase inhibitor TSA or genetic knockout of DNMT1 and DNMT3b further proved the significance of promoter CpG island hypermethylation in expression silencing of PPP1R1B and PPP1R3C. Result of MSP and BGS analysis revealed that their promoter CpG islands were demethylated after the treatment. The methylation analysis of PPP1R1B and PPP1R3C in primary ESCC tumor showed that a significant amount of tumor samples were hypermethylated but remained unmethylated in their paired normal epithelial tissue

samples which was well-correlated with the result of using the carcinoma cell lines as the sample.

In the *in vitro* functional study, ectopic expression of PPP1R1B and PPP1R3C in the carcinoma cells lacking the expression could significantly inhibit anchorage-dependent growth of corresponding carcinoma cells. This strongly supports the notion that PPP1R1B and PPP1R3C possess important tumor suppressor function.

In the functional analysis, it was found that full-length PPP1R1B suppressed the cancer cell growth by inhibiting the kinase activity of PKA as indicated by the decrease in the level of p-ser473 Akt, p-ser552 β-catenin without the change in total Akt and total β-catenin upon ectopic expression of PPP1R1B. Also, a reduction in the transcriptional activity of AP-1 was observed in the same situation. Subcellular localization study revealed that PPP1R1B is likely to distribute throughout the cytoplasm. For PPP1R3C, its ectopic expression in carcinoma cells could significantly increase the level of p-ser9 GSK-3b and p53 level in wild-type p53 expressing HCT116 cells. Furthermore, a significant increase in the transcriptional activity of NF-κB was detected in HCT116 cells while a sharp decreased in the transcriptional activity of NF-κB was found in KYSE150 cells after re-expressing PPP1R3C. Fluorescence microscopic study showed that PPP1R3C is likely to localize around the nuclear membrane which may indicate the importance of PPP1R3C in conveying intracellular signal to the nucleus.

To sum up, PPP1R1B and PPP1R3C were identified as two putative tumor suppressor genes in which epigenetic silencing of both of them were believed to be essential in carcinogenesis. The findings of the present study provide important information for understanding the molecular pathogenesis of cancer. In addition,

using epigenetic silencing of PPP1R1B and PPP1R3C as biomarkers can be promising for the development of non-invasive molecular diagnostic method and evaluation of the prognosis for the cancer patients. Furthermore, the analysis for the functional consequences of epigenetic silencing of PPP1R1B and PPP1R3C provides insight for the development of novel therapeutics approaches for cancer treatment.

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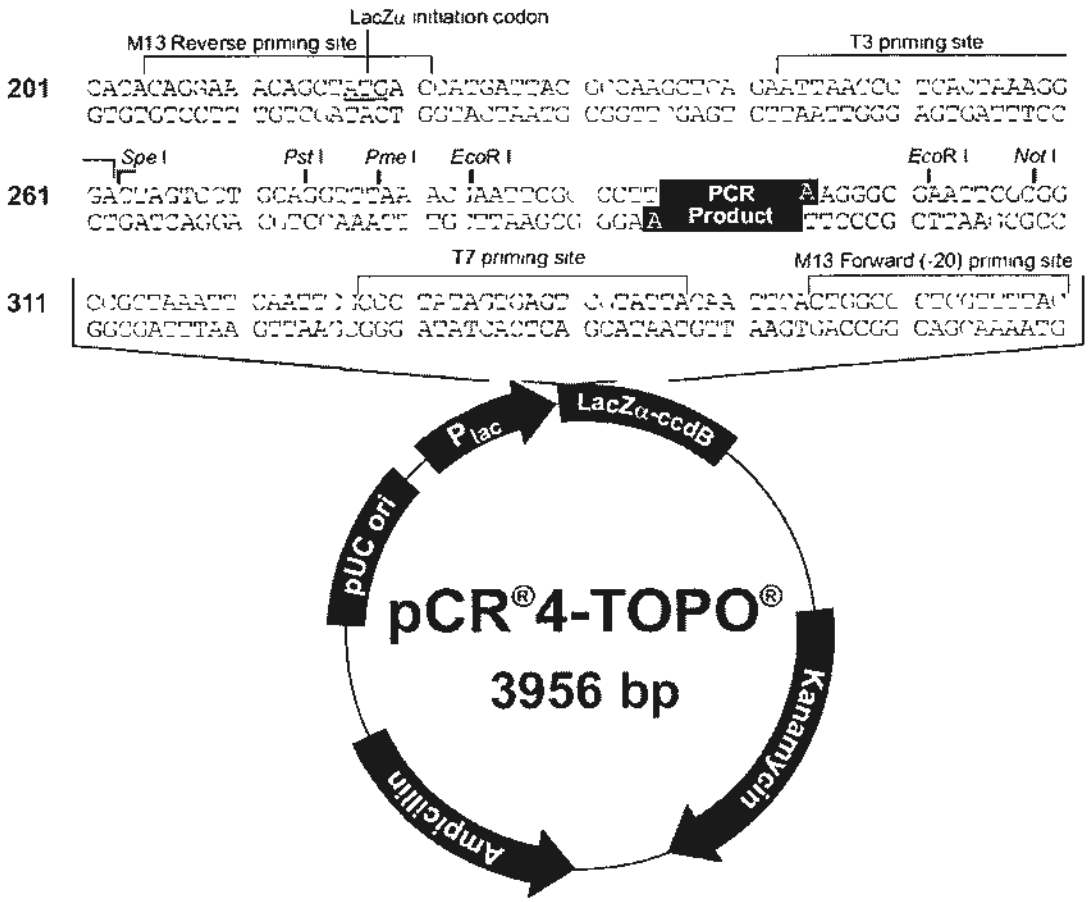
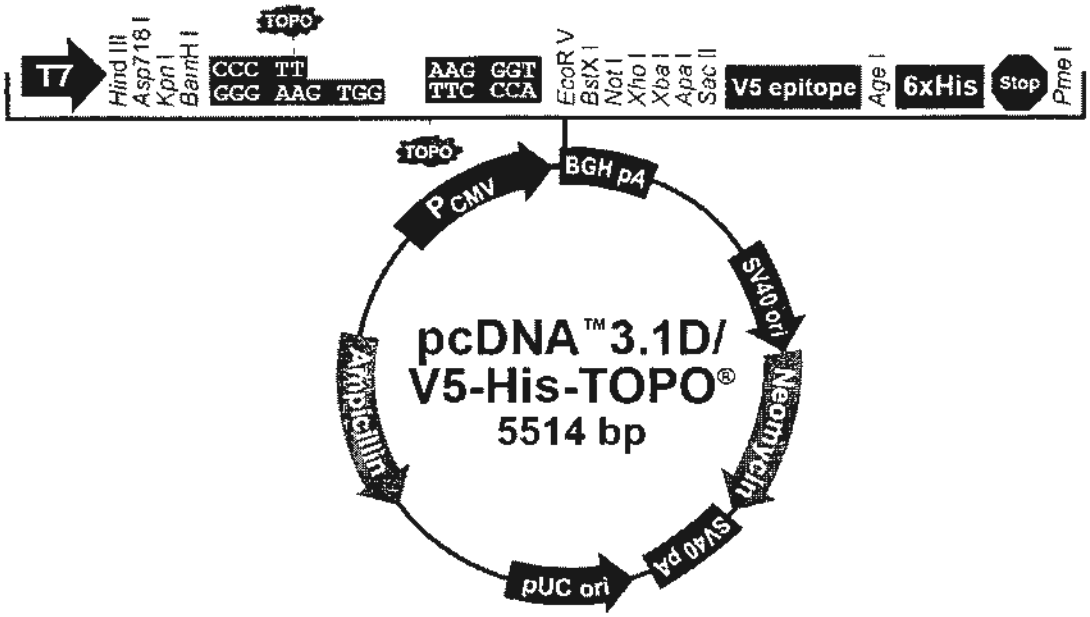
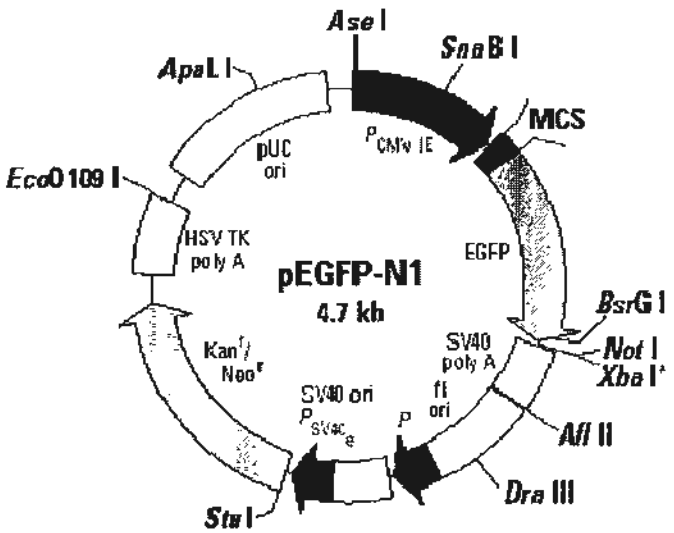
# Appendix




## Appendix A: pCR®4-TOPO vector map




**Appendix B: pcDNA™3.1D/V5-His-TOPO® vector map**




**Appendix C: pEGFP-N1 vector map**