The Hong Kong Polytechnic University

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A Study on the Hypermethylation of Tumor Suppressor Genes in Nasopharyngeal Carcinoma

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Certificate of Originality

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Abstract

Studies have shown that promoter hypermethylation of tumor suppressor genes may serve as a promising epigenetic biomarker for the diagnosis of nasopharyngeal carcinoma (NPC), which is of great significance in improving patient's survival rate. Resulting from DNA leakage due to tumor necrosis or apoptosis, cell-free circulating DNA in blood has been proven sharing a similar hypermethylation status as primary tumor and is considered desirable for promoter hypermethylation status screening. This study investigated the potential of promoter hypermethylation of five tumor suppressor genes in the detection of NPC in serum samples.

Cell-free circulating DNA was extracted from serum collected from 40 NPC patients before treatment and 41 age- and sex-matched healthy subjects. Promoter hypermethylation status of five tumor suppressor genes (*RASSF1A*, *CDKN2A*, *DLEC1*, *DAPK* and *UCHL1*) was assessed by methylation-specific polymerase chain reaction (MSP) after sodium bisulfite conversion. Methylation status of five tumor suppressor genes and clinicopathological parameters (age, gender and staging) were compared between NPC patients and healthy subjects.

The concentration of cell-free circulating DNA in the serum of NPC patients was significantly higher than that in normal controls. The five tumor suppressor genes *RASSF1A*, *CDKN2A*, *DLEC1*, *DAPK* and *UCHL1* were found to be methylated in 17.5%, 22.5%, 25%, 51.4% and 64.9% of the patients, respectively. The combination of four-gene markers - *CDKN2A*, *DLEC1*, *DAPK* and *UCHL1* - had the highest sensitivity and specificity in predicting NPC. Our results suggested

that screening of DNA hypermethylation of tumor suppressor genes in serum may be a promising approach for the diagnosis of nasopharyngeal carcinoma.

Promoter hypermethylation has been reported to be associated with the transcription inhibition of tumor suppressor genes and the initiation and progression of tumor cells. To explore the role of three candidate tumor suppressor genes *DLEC1*, *DAPK* and *UCHL1* in NPC tumorigenesis, changes in methylation status and transcription of these three genes were examined by methylation-specific PCR and RT-PCR in NPC cell line HNE1 treated with the demethylation reagent, 5-aza-2' deoxycytidine. Results showed that promoter hypermethylation could lead to transcriptional silencing of *DLEC1*, *DAPK* and *UCHL1* in NPC cell line HNE1.

Presentations and Publications Arising from the Thesis

1. Tian F, Yip SP, Kwong LW, Wu WC. Promoter hypermethylation of tumor suppressor genes in serum as potential biomarker for the early diagnosis of nasopharyngeal carcinoma. The 43rd European Human Genetics Conference, 23-26 June 2012, Nürnberg, Germany. (Abstract, poster presentation)
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List of Abbreviations

Abbreviations	Full name
3DCRT	Three dimensional conformal RT
5mC	5-methylcytosine
AJCC	American Joint Committee on Cancer
APS	Ammonium persulfate
Aza	5-aza-2-deoxycytidine
BCL2	B-cell CLL/Lymphoma 2
BSP	Bisulfate sequencing
CCND1	Cyclin D1
CDKN2A	Cyclin-dependent kinase inhibitor 2A
cf-DNA	Cell-free circulating DNA
CGH	Comparative genomic hybridization
CGI	CpG island
CHARM	Comprehensive high-throughput array-based relative methylation analysis
ChIP	Chromatin immunoprecipitation
CML	Chronic myelomonocytic leukemia
CT	Computed tomography
CTLs	Cytotoxic T lymphocytes
DAPK	Death associated protein kinase 1
DLEC1	Deleted in lung and esophageal cancer 1
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DNMTs	DNA methyltransferases
dNTPs	Deoxyribonucleoside triphosphates
EA	Early antigen
EB1/Zta	EBV transcription factor
EBNA1	Nuclear antigen1
EBV	Epstein-Barr virus
EGFR	Epidermal growth factor receptor

ER	Estrogen receptor
FBS	Fetal bovine serum
FDA	Food and Drug Administration
HELP assay	HpaII tiny fragments enrichment by ligation-mediated PCR
HLA	Leukocyte antigen
IGF-II	Insulin-like growth factor-II
IMRT	Intensity modulated radiotherapy
LOH	Loss of heterozygosity
MAGE	Melanoma-associated CT antigens
MBDs	Methyl-binding proteins
MDM2	Transformed 3T3 cell double minute2
MDS	Myelodysplastic syndromes
Me-DIP	Combination of ChIP with DNA microarray
MGMT	O-6-methylguanine-DNMT
MMPs	Matrix metalloproteinase
MRI	Magnetic resonance imaging
MSI	Microsatellite instability
MSP	Methylation-specific Polymerase chain reaction
MS-RAD	Methylation-sensitive representational difference analysis
MYC	v-myc myelocytomatosis viral oncogene homologue
MZ	Monozygotic
NPC	Nasopharyngeal carcinoma
PBS	Phosphate-buffered saline
PcG	Polycomb group proteins
PTLDs	Post-transplant lymphoproliferative disorders
QMSP/Methylight	Quantitative MSP
RASSF1A	RAS association domain family member 1
RB	Retinoblastoma
RLGS	Restriction landmark genomic scanning
RT	Radiotherapy
RT-PCR	Reverse transcription-Polymerase chain reaction

SAH	S-adenosylhomocysteine
SAM	S-adenosyl-methionine
Sat 2	Satellite 2
Sat α	Satellite α
SNCG	Synuclein-γ
TBE	Tris-Borate-EDTA
TNM	Tumor-node-metastasis
UCHL1	Ubiquitin C-terminal hydrolase L1
UICC	International Union against Cancer
VCA	Viral capsid antigen

Chapter 1: Literature review

1.1 Nasopharyngeal carcinoma

1.1.1 Epidemiology

Nasopharyngeal carcinoma (NPC) is a head and neck cancer that is rare among Caucasian but prevalent in southern China and South East Asia (Parkin et al., 2002). The incidence of NPC in the USA and Canada is under 1 per 100,000 population. By contrast, in endemic areas like southern China, the incidence is about 25 ~ 30 per 100,000. Even within China, the incidences of NPC are different with a decreasing tendency of 50-fold from south to north (Yu & Yuan, 2002). A comparative study in North America showed that Chinese immigrations continued to have a high NPC incidence when compared with local people, but the incidence reduced with prolonged duration (Buell, 1974; McCredie et al., 1999). The racial and geographic predisposition of NPC implies that both genetic susceptibility and environmental factors play important roles in NPC pathogenesis.

1.1.2 Histology and staging

NPC usually originates from the fossa of Rosenmuller and develops from the ostum of the Eustachian tube in nasopharynx (Sham et al., 1990). According to the degree of differentiation, the World Health Organization classifies NPC into three histological categories (Shanmugaratnam & Sobin, 1993): keratinizing squamous carcinoma (type I), differentiated nonkeratinizing squamous carcinoma (type II) and

undifferentiated nonkeratinizing squamous carcinoma (type III). In North America, the histological distribution of type I, II and III NPC tumor is 25%, 12% and 63%, respectively. However, these three categories account for 2%, 3% and 95% of NPC patients respectively in southern China (Nicholls, 1997; Pearson et al., 1983). It has been reported that Epstein-Barr virus (EBV) is generally absent in type I, but prevalent in type II and type III NPC, suggesting the existence of a different pathogenesis in type I NPC (Marks et al., 1998). Since 1950, approximately twenty staging systems for NPC have been reported (Myers & Iko, 1987). The major ones are proposed based on tumor-node-metastasis (TNM) staging. Currently, the most widely accepted system for NPC staging is the one established by the American Joint Committee on Cancer (AJCC) and International Union against Cancer (UICC) (**Appendix Ⅰ**)

1.1.3 Etiology

Epidemiological studies reveal that NPC is affected by three major factors: genetic susceptibility, environmental factors and EBV infection.

Genetic susceptibility

Human leukocyte antigen (*HLA*) genes have been reported to be involved in NPC pathogenesis. They are supposed to encode peptides and proteins that participate in recognizing foreign antigens, subsequent presentation to immune system and inducing NPC. In a genotyping study among Chinese, individuals with HLA A*0207 and B*4601 were found to be associated with increased risk of developing NPC (Hildesheim et al., 2002). Up to now, two NPC susceptibility loci on chromosome 4p15.1-q12 and 3p21 have been identified in a Chinese NPC pedigree via linkage analysis (Feng et al., 2002). However, NPC susceptibility genes have not been totally identified since there are a large number of candidate genes locating in those regions. Furthermore, polymorphisms of genes participating in carcinogen metabolism (*CYP2E1*), detoxification (*GSTM1*) and DNA repair (*XRCC1* and *hOGG1*) have been demonstrated to be linked to 2- to 5-fold increased risk of developing NPC (Hildesheim et al., 1997; Nazar-Stewart et al., 1999; Cho et al., 2003).

Environmental factors

Environmental factors that play a key role in inducing NPC include exposure to salted fish and preserved food which contains mutagenic chemical nitrosamines (Mirvish, 1995). Chemical carcinogen could result in genetic damage of nasopharyngeal epithelial cells and finally lead to NPC. In rat experiment, malignant nasal and nasopharyngeal tumors were found in animals after the animals were exposed to salted fish (Huang, 1978; Yu et al., 1989). Consumption of salted and preserved food in childhood has been reported to be connected with higher risk of developing NPC than adulthood (Yu et al., 1987). Intake of preserved food was found associated with 2-fold increase in the risk of developing NPC. Meanwhile, a 36% decrease was observed in individuals with high non-preserved vegetable intake (Gallicchio et al., 2006). Besides, toxic pollutants, tobacco, other smoke and alcohol have also been reported as potential environmental factors that may lead to NPC (Armstrong et al., 2000; Hildesheim et al., 2001; Yu & Yuan, 2002).

Epstein-Barr virus

A unique feature that distinguishes NPC from other head and neck cancer is its relationship with EBV, a human B-lymphotropic herpesvirus. Higher titer of IgA antibody against EBV was detected in a large percentage of NPC patients in previous studies (Chien et al., 2001). Lymphocytic origins and epithelial origins are the two ways through which EBV leads to disease. Diseases with lymphocytic origins include infectious mononucleosis, Hodgkin's disease and Burkitt's lymphoma whereas NPC, oral hairy leukoplakia and undifferentiated gastric carcinoma have an epithelial origin (Macsween & Crawford, 2003).

Since clonal viral genome of EBV was detected in DNA from NPC primary tumor, it is suggested that EBV plays an important role in NPC pathogenesis (Raab-Traub, 2002). At present, two sets of latent genes that attribute to the tumorigenic ability of EBV have been identified in NPC tumors. One group of genes encodes latent membrane proteins LMP1, LMP2A and LMP2B and another group encodes EBV-determined nuclear antigens EBNA1 and EBNA2 (Brooks et al., 1992). Of these genes, *LMP1* is widely considered as the principal oncogene of NPC as it is present in 80 ~ 90% of NPC tumors (Wang et al., 1985). In vitro, *LMP1* performs transforming activity in different cell types. When expressed in immortalized nasopharyngeal epithelial cells, *LMP1* induces the expression of genes controlling cell growth, survival and invasion (Ozyar et al., 2004; Liu et al., 2003). *LMP2A* could reduce the expression of *LMP1* by down-regulating transcription factor NF-κB (Stewart et al., 2004). *EBNA1* has been revealed playing an important role in immune evasion, and *EBNA2* is reported to be associated with the transactivation of *LMP1* (Lo et al., 2006; Wang et al., 1990). **Figure 1.1** summarizes the functions of EBV-related genes in NPC pathogenesis.

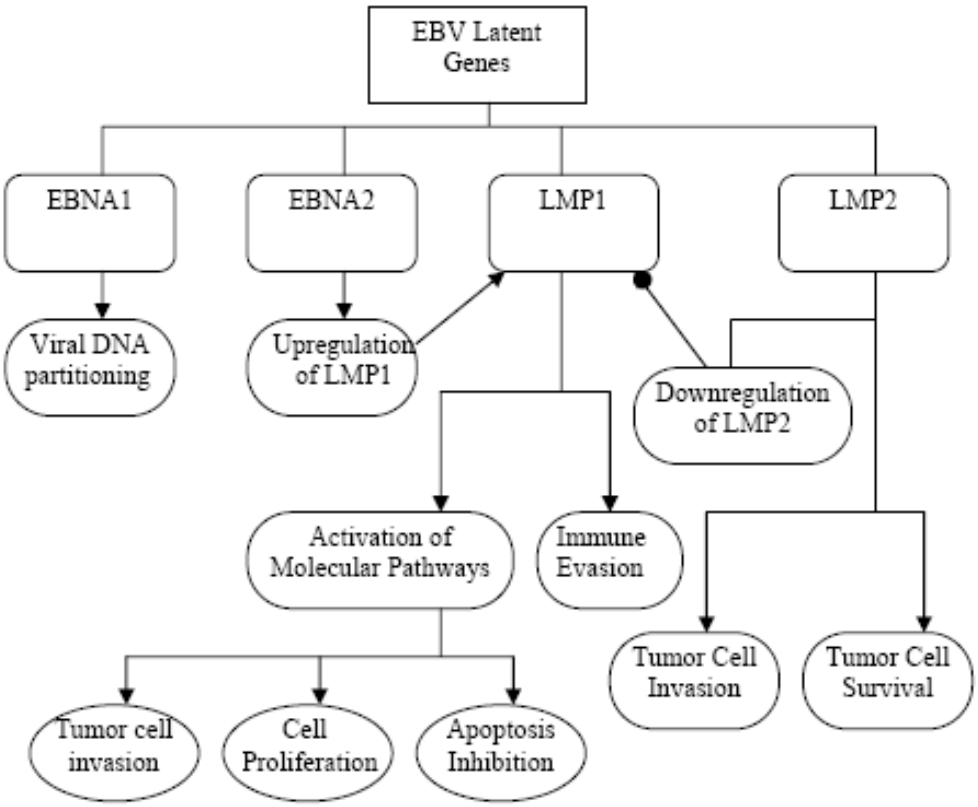

Figure 1.1 Roles of Epstein-Barr virus latent proteins in the development of nasopharyngeal carcinoma

LMP1 plays a major role in activating various molecular pathways and immune evasion. LMP1 is positively regulated by EBNA2 and negatively by LMP2. LMP2 could also mediate tumor cell invasion and survival. EBNA1 is responsible for viral DNA partitioning during replication. → Stimulatory effect; —● Inhibitor effect.

(Chou et al., 2008)

1.1.4 Diagnosis

Various examinations have been exploited to facilitate the diagnosis of NPC. Routine physical examinations involve examining the function of cranial nerve, and clinical evaluation of cervical lymph-node size and location. Other assessments include serum biochemistry, chest X-ray, blood cell count, nasopharyngoscopy, biopsy, computed tomography (CT) and magnetic resonance imaging (MRI) scans (Brennan, 2006).

In recent decades, molecular diagnosis taking advantage of the specific presence of EBV in NPC patients has been developed and is considered as a promising approach for the detection of NPC. Based on the specific existence of EBV in NPC, various detection systems have been established. High antibody titer against EBV viral proteins like early antigen (EA) IgA, viral capsid antigen (VCA) IgA, nuclear antigen1 (EBNA1) IgA, and EBV transcription factor (EB1/Zta) IgG have been identified in NPC patients. In one study, the sensitivity of VCA, EBNA1, Zta and EA detection were 93%, 84%, 74%, and 73%, respectively (Chan et al., 2003). Though the sensitivity of VCA IgA is the highest, its specificity is only 60%. High sensitivity and specificity (96% and 93%) were reported through quantitative analysis of cell-free EBV DNA in NPC patients in one study (Tao & Chan, 2007). However, there are also some studies reporting the low sensitivity and high false-positive rate of plasma EBV DNA detection, which makes it an unsatisfactory method for the detection of NPC (Leung et al., 2003; Chan et al., 2003; Hsiao et al., 2002).

Detection of methylated tumor suppressor genes in serum samples from NPC patients has been reported. It is suggested that the combination of tumor-specific DNA methylation detection in serum and serological EBV antibody test could greatly improve the diagnostic sensitivity and specificity (Wong et al., 2004). Detection of DNA hypermethylation hopefully compensates for the shortcomings of the EBV detection system or becomes an alternative choice for the diagnosis of NPC.

1.1.5 Treatment

Radiotherapy

Radiotherapy (RT) is the standard treatment method for NPC, with which initial stage I NPC patients demonstrated a 10-year survival rate of 98 % (Chua et al., 2003). Conventional two dimensional RT could control 75% ~ 90% of T1 and T2 tumors cases, and 50% ~ 75% of T3 and T4 tumors cases (Lee et al., 1992; Wang., 1991). For nodal stages, the survival rate was 90% for N0 and N1 cases. But for N2 and N3 cases, the survival rate dropped to 70% (Lee et al., 1992). The challenge of RT for the treatment of NPC is that the location of tumors is usually closely surrounded by radiation dose-limiting organs such as brain stem, spinal cord, pituitary-hypothalamic axis, temporal lobes, eyes, middle and inner ears, and parotid glands. Since NPC tumors tend to infiltrate and spread toward these organs, some of these organs might suffer great damage from RT (Wei & Sham, 2005). Therefore, radiation-induced complications, such as xerostomia, trismus and hearing deficiency are likely to occur in post-radiotherapy patients (Lee et al., 1992). Three-dimensional conformal RT (3DCRT) and intension-modulated radiotherapy (IMRT) that are able

to conform high dose to tumor tissues while reducing the dose to normal tissues are gradually becoming the new standard treatment for NPC (Waldron et al., 2003; Cheng et al., 2001). By using static or dynamic multi-leaf collimator in IMRT, the dose heterogeneity could be reduced by 36% compared with conventional RT, while the total tumor dose could be increased from 66 Gy to 70 Gy (Hunt et al., 2001).

Chemotherapy

Despite the effectiveness of radiotherapy for primary tumor, about 30% of patients with locoregionally advanced disease died due to distant metastases (Hui et al., 2004). The medium survival rate for these patients was 9 ~ 12 months (Tao & Chan, 2007). As conventional RT may not be adequate to treat patients with advanced disease, chemotherapy, either adjuvant or concurrent with radiotherapy has been introduced as new treatment approaches. Meta-analysis using original data from 1753 patients showed that patients could significantly benefit from concurrent chemoradiotherapy (Baujat et al., 2006). Nowadays, concomitant chemoradiotherapy with the use of standard chemotherapy agent cisplatin has been adopted as the standard treatment for locoregionally advanced NPC patients (Ma & Chan, 2006; Guigay et al., 2006).

Other novel therapies

With the advances of molecular biology, EBV-based immunotherapy, EBV-based gene therapy and epigenetic therapy have been proposed as possible methods for the treatment of NPC. Post-transplant lymphoproliferative disorders (PTLDs), EBV-positive disease, could be treated by EBV-specific cytotoxic T lymphocytes (CTLs) (Rooney et al., 1995; Papadopoulos et al., 1994; Khanna et al, 1999). But immunotherapy targeted at EBV may not be effective for the treatment of NPC as immunodominant EBV antigens (EBNA2, EBNA3 family) are not expressed in NPC. The exploration on EBV-based gene therapy is also at a preliminary stage due to the difficulty of targeting every individual tumor cell without affecting surrounding normal cells (Tao & Chan, 2007). Epigenetic therapy based on the methylation of tumor suppressor genes may provide new therapeutic opportunities. As DNA methylation could be reversed by demethylation reagent such as 5-aza-2' deoxycytidine (Aza), 5-azcitidine and zebularine, via demethylating tumor suppressor genes that are originally methylated in NPC tumors. Through recovering the normal expression of tumor suppressor genes in tumors, the malignant growth of cancer cells could be inhibited (Cheng et al., 2004).

1.2 NPC carcinogenesis

1.2.1 Genetic and epigenetic alterations

Genetic susceptibility, environmental factors and EBV infection, in combination or alone, contribute to NPC carcinogenesis through the accumulation of various genetic and epigenetic alterations including cytogenetic change, oncogene activation and tumor suppressor genes inactivation in normal cells.

Cytogenetic changes

With the use of cytogenetic analysis, spectral karyotyping, microsatellite and comparative genomic hybridization (CGH), multiple genetic changes have been detected in NPC tumors and NPC cell lines. By using 3 Mb array-based CGH, deletions of allelic genes in chromosomes 3p, 9q, 11q, 13q and 14q were observed in NPC tumors, implying that tumor suppressor genes located at these regions might participate in NPC tumorigenesis (Hui et al., 1999). Recurrent chromosome gains were also identified on chromosome 1q, 3q, 8q, 11q and 12p (Lo et al., 2000). The combined examination of high-resolution (1Mb) array-based CGH with whole-genome arrays revealed the deletion of 3p12-14, 8p22, 10p and 18q and amplification of 3q26 in chromosomes (Seng et al., 2007; Ying et al., 2006a; Ying et al., 2006b). **Table 1.1** lists the alterations on chromosomes detected by microsatellite marker analysis, CGH and array-CGH in NPC cell lines and primary tumors. The identification of chromosome alterations in NPC tumors and cell lines lays a foundation for the future investigation of novel tumor suppressor genes and oncogenes associated with the development of NPC.

Table 1.1 Genetic alterations detected by microsatellite marker analysis, CGH and array-CGH in NPC cell line and primary tumors

Chromosome	Deletions	Amplifications
1	1p36, 1p34	1q24.3-32.1, 1q42-44
2	2p12	2q24-31
3	3p25.3-26.3, 3p22-21.3, 3p14-12	3q26, 3q27.3-28
4	4q8	4q12-21
5	5q11-14, 5q31-33	5q21
6		6q14-22
7	7q31.3-36	7p15-14, 7q11.2-21
8		8q21.1-22, 8q23-24
9	9p21-23, 9q2.33-q31.2	
10	10p	
11	11q13-14, 11q14-23, 11q25	11p15, 11q13.1-13.3
12		12p12-13, 1213-15, 12q22-24.1
13	13q12-14.3, 13q21-32	
14	14q11-13, 14q24-31	
16	16q21, 16q22.3-23.1, 16q24.1	
17	17p13.3, 17q11	17q21, 17q25
18	18p11.21, 18q23	18q12-22

(Tao & Chan, 2007)

Oncogenes

By using cytogenetic or array-based CGH, several putative oncogenes of NPC have been identified. B-cell CLL/Lymphoma 2 (*BCL2*), an integral outer mitochondrial membrane protein, could interact with activated Bax and neutralizes its pro-apoptotic activity (Yu et al., 2003). Cyclin D1 (*CCND1*) could regulate cell cycle G1-S transition, interact with pRb and affect its function (Lai et al., 2002). Epidermal growth factor receptor (*EGFR*) could participate in a variety of cell regulation pathways such as signal transduction, cell communication, cell cycle regulation, proliferation, adhesion and migration (Leong et al., 2004). Other candidate oncogenes that have been identified in NPC are v-myc myelocytomatosis viral oncogene homologue (*MYC*) (Yu et al., 2003), transformed 3T3 cell double minute 2 (*MDM2*) (Wu et al., 2004) and *PIK3CA* (Or et al., 2005), which exhibit amplification, overexpression or gain of function mutations in NPC. **Table 1.2** is a summary of putative oncogenes in NPC pathogenesis.

Table 1.2 Candidate oncogenes in nasopharyngeal carcinoma pathogenesis

Name	Description	References
A20	Tumor necrosis factor, alpha induced protein 3	Codd et al., 1999
AKT3	v-akt murine thymoma viral cocogene homolog 3 (protein kinase B, gamma)	Hu et al., 2012
ASAP1	Development and differentiation enhancing factor1	Hu et al., 2012
ATF2	Activating transcription factor 2	Hu et al., 2012
BCL2	B-cell CLL/lymphoma 2	Sheu et al., 1997
BIRC3	Baculoviral IAP repeat-containing 3	Hu et al., 2012
CCND1	Cyclin D1	Lai et al., 2002
CIITA	Class II, major histocompatibility complex, transactivator	Hu et al., 2012
CLTC	Clathrin, heavy chain (Hc)	Hu et al., 2012
CTNNB1	Catenin (cadherin-associated protein), beta 1, 88 kDa	Hu et al., 2012
DEK	DEK oncogene (DNA binding)	Hu et al., 2012
ECT2	Epithelial cell transforming sequence 2 oncogene	Hu et al., 2012
EGFR	Epidermal growth factor receptor	Leong et al., 2004
EIF4A2	Eukaryotic translation initiation factor 4A, insoform 2	Hu et al., 2012
ETS1	v-ets erythroblastosis virus E26 oncogene homolog 1(avian)	Hu et al., 2012
EVI1	Ecotropic viral integration site 1	Guo et al., 2002
EZH2	Enhance of zeste homolog 2 (Drosophila)	Hu et al., 2012
FUS	Fusion (involved in t(12:16) in malignant liposarcoma)	Hu et al., 2012
GNA13	Guanine nucleotide binding protein (G protein), alpha 13	Hu et al., 2012
HER2	v-erb-b2 erythroblastic leukemia viral oncogene homologue 2	Ma et al., 2003
HRAS	v-Ha-ras Harvey rat sarcoma viral oncogene homologue	Yung et al., 1995
ID1	Inhibitor of DNA biding 1	Cheung et al., 2004
INT2	Fibroblast growth factor 3	Fan et al., 2000
ITGAV	Intergrin, alpha V (vitronectin receptor, alpha polypeptide, antigen CD51)	Hu et al., 2012
ITGB6	Integrin, beta 6	Hu et al., 2012
JAK2	Janus kinase 2(a protein tyrosine kinase)	Hu et al., 2012
JUN	Jun oncogene	Hu et al., 2012
KAT6A	K(lysine) acetyltransferase 6A	Hu et al., 2012
KDSR	3-ketodihydrosphingosine reductase	Hu et al., 2012
LCP1	Lymphocyte cytosolic protein 1 (L-plastin)	Hu et al., 2012

Table 1.2 Candidate oncogenes in nasopharyngeal carcinoma pathogenesis

(Continued)

Name	Description	References
MDM2	Mdm2, transformed 3T3 cell double minute 2	Wu et al., 2004
MET	Met proto-oncogene; hepatocyte growth factor receptor	Qian et al., 2002
MYC	v-myc myelocytomatosis viral oncogene homologue	Porter et al., 1994
NCOA3	Nuclear receptor coactivator 3	Hu et al., 2012
NRAS	Neuroblastoma RAS viral oncogene homologue	Hu et al., 2012
NRAS	Neuroblastoma RAS viral (v-ras) oncogene homolog	Hu et al., 2012
NTRK2	neurotrophic tyrosine kinase, receptor, type 2	Hu et al., 2012
PDE4DIP	Phosphodiesterase 4D interacting protein (myomegalin)	Hu et al., 2012
PICALM	Phosphatidylinositol binding clathrin assembly protein	Hu et al., 2012
PIK3CA	Phosphoinositide, catalytic, alpha polypeptide	Or et al., 2005
PSIP1	PC4 and SFRS1 interacting protein 1	Hu et al., 2012
RAB18	RAB18, member RAS cocogene family	Hu et al., 2012
RAB28	RAB28, member RAS cocogene family	Hu et al., 2012
RAN	RAN, member RAS oncogene family	Hu et al., 2012
RAP1A	RAP1A, member of RAS oncogene family	Hu et al., 2012
RAP1B	RAP1B, member of RAS oncogene family	Hu et al., 2012
RAP2C	RAP2C, member of RAS oncogene family	Hu et al., 2012
RBM15	RNA binding motif protein 15	Hu et al., 2012
RHEB	Ras homolog enriched in brain	Hu et al., 2012
ROBO1	Roundabout, exon guidance recepotor, homolog1	Hu et al., 2012
RPL22	Ribosomal protein L22	Hu et al., 2012
SKIL	SKI-like oncogene	Hu et al., 2012
SPARC	Secreted protein, acidic, cysteine-rich (osteonectin)	Hu et al., 2012
TFPC	Transferring receptor (p90, CD71)	Hu et al., 2012
TOP1	Topoisomerase (DNA)	Hu et al., 2012
TP73L	Tumor protein p73 like, p63 splicing variants lacking NH-terminal transactivating domain	Chu et al., 2006
TPM3	Tropomyosin	Hu et al., 2012
TPR	Translocated promoter region (to activated MET oncogene)	Hu et al., 2012
TRIM24	Tripartite motif-containing 24	Hu et al., 2012
WHSC1	Wolf-Hirschhorn syndrome candidate 1	Hu et al., 2012
XIAP	Baculoviral IAP repeat-containing 4	Hu et al., 2012

Tumor suppressor genes

A large number of tumor suppressor genes have been detected in different regions on the chromosomes of NPC tumor cells, in particular chromosome 9p21 (*CDKN2A*, *p15* and *p14 ARF*) (Huang et al., 1994; Lo et al., 1996; Kwong et al., 2002) and 3p21.3 (*RASSF1A*, *BLU/ZMYND10* and *CACNA2D2*) (Lo et al., 2001; Qiu et al., 2004), which account for 85-90% deletion rate in invasive tumors. The deficiency of the normal function of these tumor suppressor genes stems from promoter hypermethylation, deletion or mutation. Aberrant methylation, deletion and mutation of tumor suppressor genes contribute to NPC pathogenesis mainly through disrupting the normal regulation of crucial cellular regulation processes such as apoptosis, cell proliferation and cell adhesion. For example, *CDKN2A,* a cell-cycle-related tumor suppressor gene, could act as cyclin-dependent kinase inhibitors in G1 progression (Drexler, 1998). Hypermethylation of O-6-methylguanine-DNMT *(MGMT*) may result in the inactivation of DNA repair process (Esteller et al., 1999a; Weller et al., 2010). Methylated death associated protein kinase 1 (*DAPK*) and Ras association domain family 1 (*RASSF1A*) have been reported to be associated with apoptosis inhibition (Michie et al., 2010). The candidate tumor suppressor genes that may play a role in NPC pathogenesis are summarized in **Table 1.3**.

Table 1.3 Candidate tumor suppressor genes in nasopharyngeal carcinoma pathogenesis

Name	Full Name	Reference
ANXA1	Annexin	Hu et al., 2012
ARF	Alternate open reading frame	Baba et al., 2001
BRD7	Bromodomain containing 7	Zhou et al., 2004
CADM1	Cell adhesion molecule 1	Lung et al., 2004
CASP8	Caspase 8 apoptosis-related cysteine peptidase	Wong et al., 2003a
CBFA2T3	Core-binding factor, runt domain, alpha subunit 2; translocated to 3	Hu et al., 2012
CDH1	Cadherin 1, type 1, E-cadherin	Tsao et al., 2003
CDKN1B	Cyclin-dependent kinase inhibitor 1B	Baba et al., 2001
CDKN2A	Cyclin-dependent kinase inhibitor 2A	Lo et al., 1996
CDKN2B	Cyclin-dependent kinase inhibitor 2B	Kwong et al., 2002
CEACAM1	Carcinoembryonic antigen-related cell adhesion molecule 1 (biliary glycoprotein)	Hu et al., 2012
CEBPD	CCAAT/enhancer binding protein (C/EBP), delta	Hu et al., 2012
CHFR	Checkpoint with forkhead and ring finger domains	Cheung et al., 2005
CLCA2	Chloride channel, calcium activated, family member 2	Hu et al., 2012
CLU	Clusterin	Hu et al., 2012
CSK	c-src tyrosine kinase	Hu et al., 2012
CXCL14	Chemokine (C-X-C motif) ligand 14	Hu et al., 2012
DAB2	Disabled homolog 2, mitogen responsive phosphoprotein	Tong et al., 2010
DAPK	Death-associated protein kinase 1	Kwong et al., 2002
DLC1	Deleted in liver cancer 1	Seng et al., 2007
DLEC1	Deleted in lung and esophageal cancer	Kwong et al., 2007
DLG1	Discs, large homolog 1 (Drosophila)	Hu et al., 2012
DUOX1	Dual oxidase 1	Hu et al., 2012
DUOX2	Dual oxidase 2	Hu et al., 2012
EDNRB	Endothelin receptor type B	Lo et al., 2002
EHF	Ets homologous factor	Hu et al., 2012
EPAS1	Endothelial PAS domain protein 1	Hu et al., 2012
FHIT	Fragile histidine triad gene	Ko et al., 2002
GADD45G	Growth arrest and DNA-damage-inducible, gamma	Ying et al., 2005
GJB2	Gap junction protein, beta 2, 26 kDa	Hu et al., 2012

Table 1.3 Candidate tumor suppressor genes in nasopharyngeal carcinoma pathogenesis (Continued)

Name	Full Name	Reference
GLTSCR2	Glioma tumor suppressor candidate region gene 2	Hu et al., 2012
GPX3	Glutathione peroxidase 3 (plasma)	Hu et al., 2012
GSTP1	Glutathione S-transferase pi 1	Kwong et al., 2002
H19	Imprinted maternally expressed, untranslated mRNA	Ng et al., 2003
HRASLS	HRAS-like suppressor	Hu et al., 2012
IGFBP5	Insulin-like growth factor binding protein 5	Hu et al., 2012
ING2	Inhibitor of growth family, member 2	Hu et al., 2012
JUP	Junction plakoglobin	Hu et al., 2012
KLF5	Kruppel-like factor 5 (intestinal)	Hu et al., 2012
KLK11	Kallikrein-related peptidase	Hu et al., 2012
LCN2	Lipocalin 2 (oncogene 24p3)	Hu et al., 2012
LLGL2	Lethal giant larvae homolog 2 (Drosophila)	Hu et al., 2012
MGMT	O-6-methylguanine-DNA methyltransferase	Wong et al., 2003b
MIPOL1	Mirror-image polydactyly 1	Cheung et al., 2009
MLH1	Mut L homolog 1, colon cancer, nonpolyposis type 2	Wong et al., 2003b
MMP19	Matrix metallopeptidase 19	Chan et al., 2011
MSMB	Microseminoprotein, beta	Hu et al., 2012
MSRA	Methionine sulfoxide reductase	Hu et al., 2012
OPCML	Opioid binding protein/cell adhesion molecule-like	Cui et al., 2008
RBL2	Retinoblastoma-like 2	Claudio et al., 2000
PCDH10	Protocadherin 10	Ying et al., 2006b
PDCD4	Programmed cell death 4 (neoplastic transformation inhibitor)	Hu et al., 2012
PER2	Period homolog 2 (Drosophila)	Hu et al., 2012
PERP	PERP, TP53 apoptosis effector	Hu et al., 2012
PPP1R13B	Protein phosphatase 1, regulatory (inhibitor) subunit 13B	Hu et al., 2012
PRDM2	PR domain-containing 2, with ZNF domain	Chang et al., 2003a
PTPRG	Protein tyrosine phosphatase, receptor type, G	Cheung et al., 2008
PYCARD	PYD and CARD domain containing	Hu et al., 2012
RAB25	RAB25, member RAS oncogene family	Hu et al., 2012
RARB	Retinoic acid receptor, beta	Wong et al., 2003b
RASAL1	RAS protein activator like 1	Jin et al., 2007
RARRES1	Retinoic acid receptor responder 1	Kwong et al., 2005

Table 1.3 Candidate tumor suppressor genes in nasopharyngeal carcinoma pathogenesis (Continued)

Name	Full Name	Reference
RASSF1A	Ras association domain family 1	Lo et al., 2001
RASSF4	Ras association domain family 4	Chow et al., 2004b
RPS6KA2	Ribosomal protein S6 kinase, 90 kDa, polypeptide 2	Hu et al., 2012
S100A2	S100 calcium binding protein A2	Hu et al., 2012
SCGB3A1	Recretoglobin, family 3A, member 1	Wong et al., 2003a
SDHC	Succinate dehydrogenase complex, subunit C, integral membrane protein, 15 kDa	Hu et al., 2012
SEMA3F	Sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3F	Hu et al., 2012
SERPINB13	Serpin peptidase inhibitor, clade B (ovalbumin), member 13	Hu et al., 2012
SERPINB2	Serpin peptidase inhibitor, clade B (ovalbumin), member 2	Hu et al., 2012
SFN	Stratifin	Hu et al., 2012
SLC9A3R1	Solute carrier family 9 (sodium/hydrogen exchanger), member 3 regulator 1	Hu et al., 2012
SOX7	SRY (sex determining region Y)-box 7	Hu et al., 2012
TACC2	Transforming, acidic coiled-coil containing protein 2	Hu et al., 2012
THBS1	Thrombospondin1	Wong et al., 2003b
THY1	Thy-1 cell-surface antigen	Lung et al. 2005
TMSB10	Thymosin, beta 10	Hu et al., 2012
TP53	Tumor protein p53	Effert P et al., 1992
TP73	Tumor protein p73	Wong et al., 2003b
UCHL1	Ubiquitin carboxyl-terminal esterase L1	Li et al., 2010
VWA5A	von Willebrand factor A domain containing 5A	Hu et al., 2012
WIF1	WNT inhibitory factor 1	Chan et al., 2007
ZMYND10	Zinc finger, MYND-type containing 10	Qiu et al., 2004
ZNF185	Zinc finger protein 185 (LIM domain)	Hu et al., 2012

1.2.2 Cell regulation pathways

In NPC, activation of oncogenes and disruption of tumor suppressor genes could disturb the normal regulation of various cellular pathways and drive tumor progression. Upregulation of Wnt pathway leads to the accumulation of β-catenin, which plays an important role in the proliferation of NPC, by activating several downstream proliferation signals (Jou et al., 1995). High levels of NF-κB, which is critical to the regulation of cell growth and inflammation, is observed in almost all NPC tumors (Lo et al., 2006; Shi et al., 2006). Inhibition of apoptosis that is resulted from the up-regulation of anti-apoptotic factor oncoprotein bcl-2 (Hockenbery et al., 1990), overexpression of survivin (Ambrosini et al., 1997) and high activity of telomerase (McCaul et al., 2002) is regarded crucial to NPC tumorigenesis. Disruption of normal cell cycle by aberrant expression of cell cycle proteins including Cyclin D1 (Lee & Yang, 2003), Cyclin E (Sherr, 2000) and C-myc (Nesbit et al., 1999) could result in the development of NPC. Dysregulation of E-cadherin (Vleminckx et al., 1991) and matrix metalloproteinase (MMPs) (Nasr et al., 2007), could increase the invasive and metastasis potential of NPC tumors. Aberrations in PI3-K (Dackour, 2005), MAP kinases (Treisman, 1996), and EGFR (Mainou et al., 2005) signaling have also been reported playing key roles NPC tumorigenesis. Molecular mechanisms involved in NPC development are shown in **Figure 1. 2**.

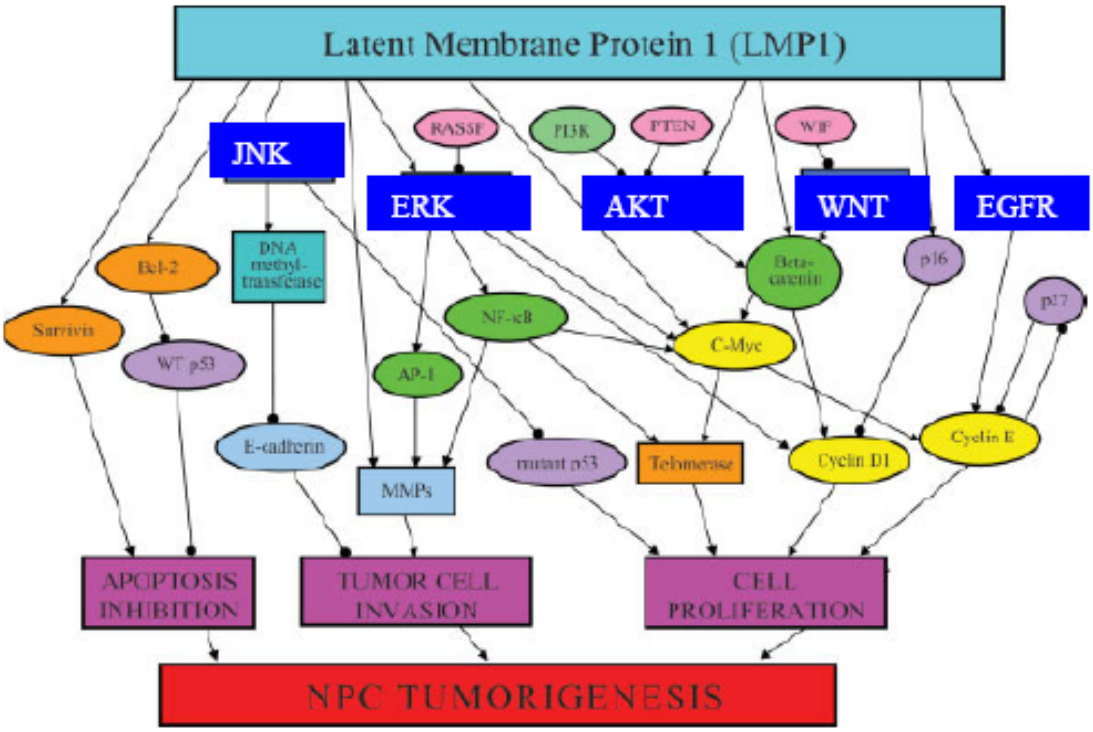

Figure 1.2 Molecular mechanisms involved in nasopharyngeal carcinoma development

Stimulatory effect ⟶, inhibitory effect —●; orange color indicates apoptosis regulators, light blue color indicates cell adhesion proteins, yellow color indicates cell cycle regulators, dark blue color indicates proliferative pathways, green color indicates transcription factors and purple color indicates tumor suppressors. (Chou et al., 2008)

1.2.3 Basic model of carcinogenesis

Based on the hypothesis that the accumulation of genetic and epigenetic changes in normal nasopharyngeal epithelium that stems from environmental exposure to carcinogens, genetic factors and EBV infection could transform the normal epithelium to pre-invasive lesions and invasive cancer, a multi-step model for NPC pathogenesis and development has been proposed (**Figure 1.3**). After exposure to carcinogens, normal nasopharyngeal epithelium with predisposed susceptibility undergoes genetic changes. Initiated cells with allelic loss on 3p and 9p first gain growth advantages and form multiple clonally cell populations throughout nasopharynx. Under the influence of additional genetic and environmental factors, genes such as *RASSF1A* and *CDKN2A* located on chromosome 14q are inactivated and result in the phenotypic and morphological changes in low-grade dysplastic lesions on nasopharynx. When the lesion cells are infected by virus, the cells would clonally expand throughout the epithelium, which is critical for the transformation of low-grade dysplastic lesions to high-grade dysplastic lesions. After that, genetic changes including deletion of 11q, 13q, 16q and inactivation of *TSLC1* and *ENDRB* contribute to the development of invasive carcinoma. Mutation of p53 and down regulation of *E*-cadherin further leads to the metastasis of carcinoma cells.

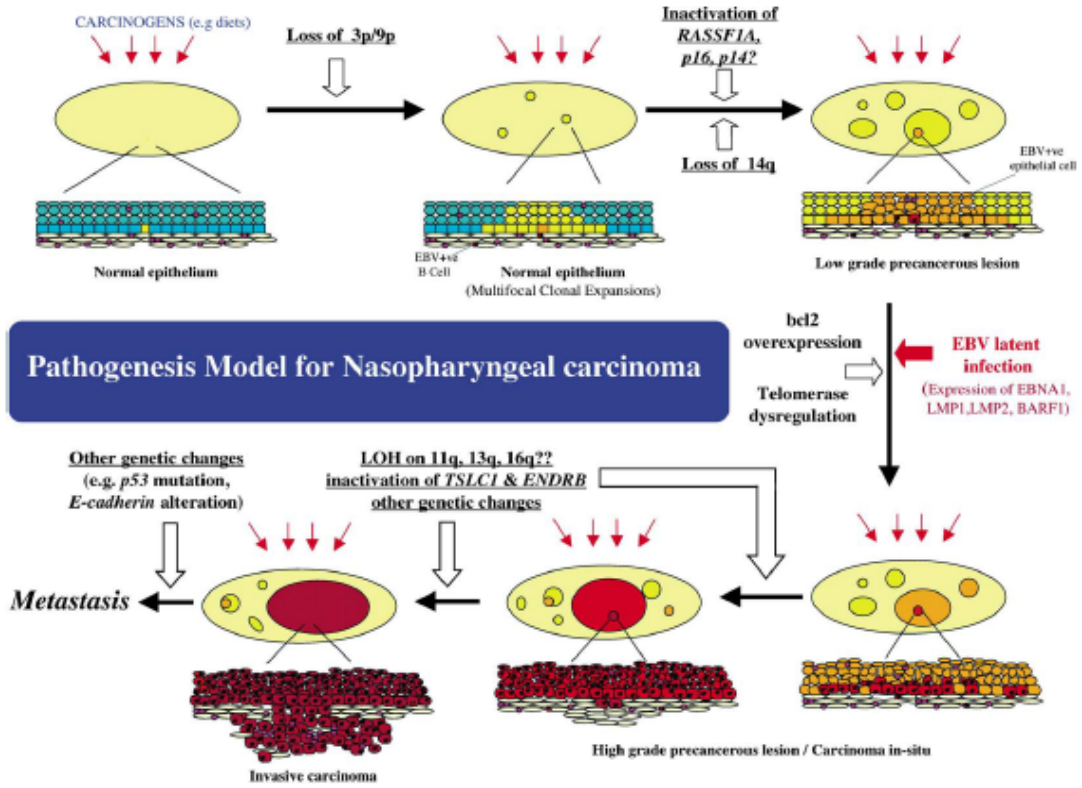

Figure 1.3 Pathogenesis model for nasopharyngeal carcinoma

See Section 1.2.3 for details.

(Lo & Huang, 2002)

1.3 DNA methylation

1.3.1 Introduction

The development of cancer is typically regarded as the result of genetic abnormalities including gene mutation, amplification, deletion and loss of heterozygosity (LOH) of DNA sequences that contain oncogenes or tumor suppressor genes (Clark, 2007). However, recent advances in cancer research reveal the importance of epigenetic modifications in carcinogenesis. Different from genetic events, in which the changes occur in primary DNA sequence, epigenetic changes are described as the inheritable changes at the expression level that occur independently of alterations in primary DNA sequences. Epigenetics can also be defined as the interaction between genetics and environment (Esteller, 2006). There are generally three types of epigenetic networks: DNA methylation (adding of a methyl group to the carbon-5 position of cytosine), histone modifications (posttranslational modifications occurring at the N-terminal of histones) and microRNAs (small RNAs located within the protein-coding genes or in intergenic regions that regulate the expressions of genes) (Bartel, 2004; Calin & Croce, 2006). Among them, DNA methylation is the most studied epigenetic phenomenon in mammals. In 1983, DNA methylation reduction of specific genes in human colon cancer cells was first observed (Feinberg & Vogelstein, 1983). In the same year, a global reduction of 5-methylcytosine DNA has been identified in tumor samples (Gama-Sosa et al., 1983). Recent advances in cancer research further confirm the significance of DNA methylation in carcinogenesis of various cancers.

1.3.2 Mechanism of DNA methylation

DNA methylation is characterized by the transfer of a methyl group to the carbon-5 of cytosine to create 5-methylcytosine (5mC) (Christman, 1982; Chiang et al., 1996) (**Figure 1.4**). 5mC is preferentially observed within CpG dinucleotides, although 5mC has also been found in the context of CpA and CpT (Ramsahoye et al., 2000). In the 3-billion-nucleotide human genome, there are 28 million CpGs in which 80% are methylated and located in repeat sequences that account for 20-30% of human genome. The remaining 20% CpG sites are unmethylated and located in CpG Island (CGI), short sequence stretches containing large clusters of CpG dinucleotides (Herman & Baylin, 2003; Colot & Rossigonol, 1999). According to a survey, about 60% of gene promoters in humans are correlated with CGI (Antequera & Bird, 1993).

The process of DNA methylation is mediated by a family of DNA methyltransferases (DNMTs) that catalyze the transfer of a methyl group from S-adenosyl-methionine (SAM) to cytosine (Schmitt et al., 1997). In mammals, there are five members in the DNA methyltransferase family: DNMT1, DNMT2, DNMT3a, DNMT3b, and DNMT3L. However, only DMNT1, DMNT3a, DMNT3b are considered to have cytosine methyltransferase function. DNMT1 is responsible for maintaining parental DNA methylation patterns through DNA synthesis. With the presence of DNMT1, methylation status can be ensured through each cell division. In mice, *DNMT1* mutation results in anomalous development and embryonic lethality (Li et al., 1992). DNMT3a and DNMT3b are associated with the establishment of *de novo* DNA methylation in early embryonic development. Studies

based on knock-out mouse models showed that the lack of any of these two enzymes would led to *de novo* DNA methylation blockage and lethality (Okano et al., 1999).

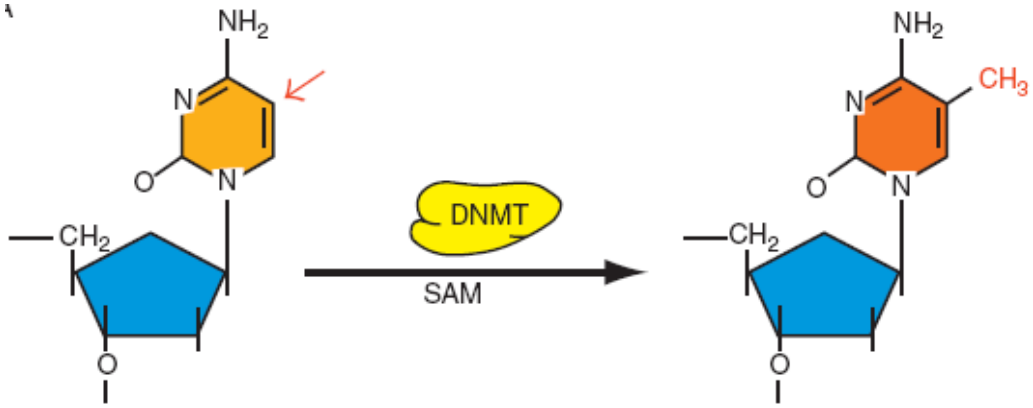

Figure 1.4 Mechanism of DNA methylation

Under the catalysis of DNA methyltransferases and its co-factor S-adenosyl-methionine, a methyl group is added to the 5 position of cytosine moiety. (Kulis & Esterller, 2010)

1.3.3 Mechanism of gene transcriptional silencing

Although the molecular mechanism of how the addition of the methyl group in CpG sites leads to gene silencing remains unclear, several models have been proposed to explain the mechanism. In one model, methyl groups are thought to act as a physical barrier to prevent transcription factors like AP-2, c-myc, CREB/ATF, E2F, MLTF/USF, and NF-κB from accessing the binding sites in the promoter region of gene. However, this model is only applicable to a subset of transcription factors since it cannot well explain the transcriptional silencing mediated by transcription factors such as CTF and Sp1 that are not sensitive to methyl CpG (Tate & Bird, 1993). Another model assumes the participation of methyl-binding domain proteins (MBDs), which contain methyl-CpG-binding domain and are able to bind methylated CpG sites specifically in suppressing the initiation of transcription (Boyes & Bird, 1991; Nan et al., 1993) (**Figure 1.5**). However, the Kaiso-like family of proteins (Filion et al., 2006; Prokhortchouk et al., 2001), proteins containing SET and RING finger-associated domain (Unoki et al., 2004), are also capable of acting as transcriptional repressors and lead to gene silencing. Recent studies demonstrate the involvement of a group of polycomb group proteins (PcG) in DNA methylation pattern establishment. The exact interaction between PcG and DNA methyltransferase needs further investigation.

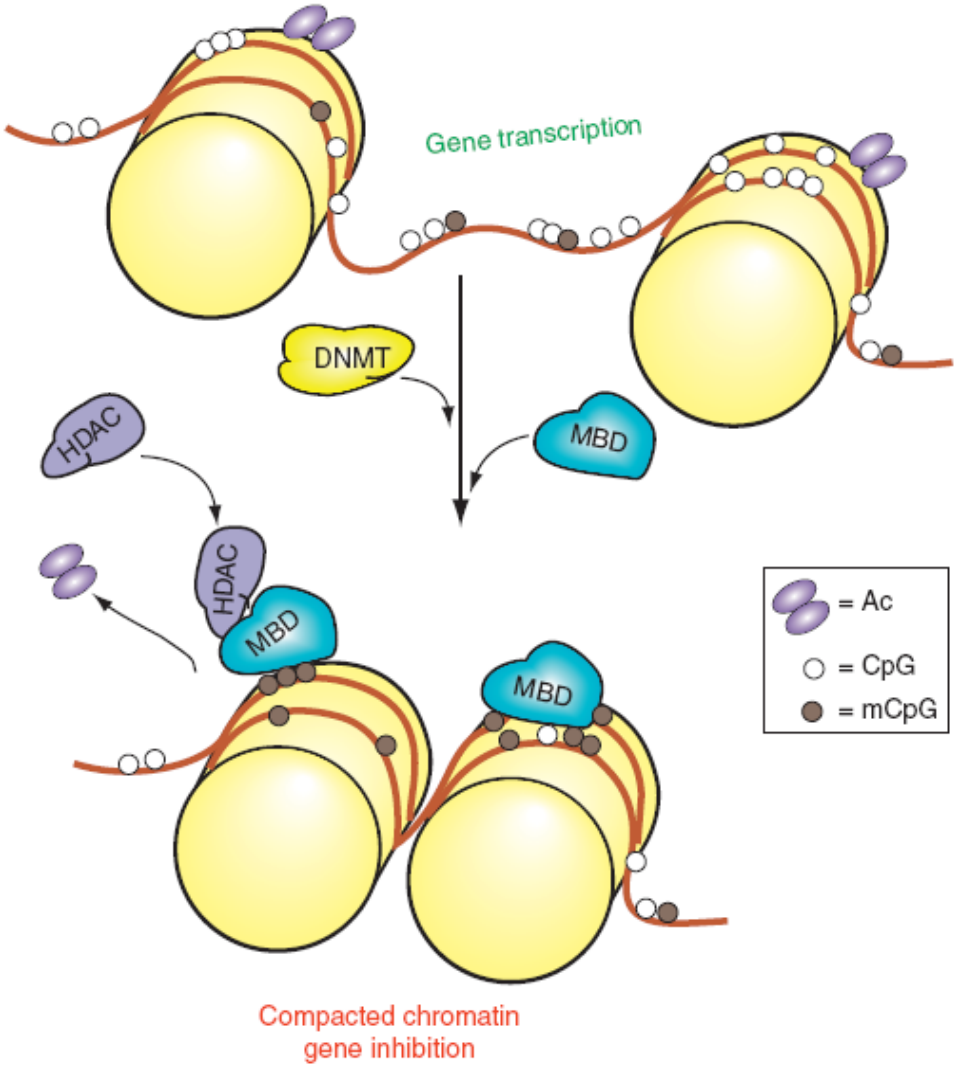

Figure 1.5 Gene silencing mediated by DNA methylation

Methyl-CpG-binding domain proteins (MBDs) first bind to DNA sites, which have been methylated by DNA methyltransderases (DNMTs). Histone deacetylases (HDACs) are then associated with MBDs, resulting in chromatin compaction. Finally, gene expression is inhibited.

(Kulis & Esterller, 2010)

1.3.4 DNA methylation in normal cells

DNA methylation has multiple functions in normal cell development, such as viral sequences inactivation (Yoder et al., 1997), embryonic development, chromosome integrity maintenance, X chromosome inactivation, imprinting, and gene transcriptional regulation (Esteller, 2005). Except viral sequence inactivation which occurs outside CGI, all the other events can be found within CGI.

In the development of mammal embryos, DNA methylation patterns are first established during gametogenesis and then continually undergo changes during development. Before the full differentiation of cells and establishment of tissue-specific-methylation, two global DNA demethylation events occur. The first global demethylation is called reprogramming which occurs soon after fertilization. In this period, the highly methylated gametes undergo non-synchronized demethylation in male and female genomes (Mayer at al., 2000; Oswald et al., 2000). Imprinted genes are protected from this global demethylation to ensure the proper mono-allelic expression of imprinted genes that are important for early embryogenesis. After implantation, DNA methylation patterns are restored and these patterns are sustained in the course of somatic cell divisions (Gaudet et al., 2004). The second global DNA demethylation takes place in primordial germ cells before gametogenesis (Santos et al., 2002). Methylation of imprinted genes is re-established before birth and maintained after birth.

Abnormal DNA methylation during development can cause a variety of congenital malignancies in humans. ICF syndrome (Immunodeficiency, Centromere instability and Facial anomalies syndrome) has be reported to be associated with the

de novo mutation of the DNA methyltransferase DNMT3B, which leads to the hypomethylation of juxtacentrometic regions in chromosomes 1, 9 and 16 (Okano et al., 1999). Imprinting disorders, including Beckwith-Wiedemann and Prader Willi/Angelman syndromes, are resulted from the deficiency in the normal mono-allelic expression of imprinted genes (Grandjean et al., 2000).

1.3.5 DNA methylation in cancer cells

In cancer cells, there are two types of DNA methylation: global hypomethylation and hypermethylation in CGI (**Figure 1.6** on p.35).

Hypomethylation

In DNA hypomethylation, DNA methylation of normally inactivated regions is damaged or loss. Compared to its normal counterpart, the malignant cell has 20 ~ 60% less genomic 5mC (Esteller, 2006). Oncogene activation, chromosomal instability, and imprinting loss have been put forward as the three mechanisms by which DNA hypomethylation contribute to carcinogenesis (Feinberg & Vogelstain, 1983; Feinberg et al., 2006).

To ensure the integrity and stability of the genome, pericentromeric heterochromatin, which contains repetitive DNA sequences such as LINE, SINE, IAP and Alu elements, is highly methylated in normal cells. However, in cancer cells, global hypomethylation disrupts normally inactivated region and induces undesired mitotic recombination. Reduced genomic stability may finally lead to the

development of tumor. For example, global hypomethylation of juxtacentromeric satellite 2 (*Sat 2*) and centromeric satellite α (*Sat α*) has been linked with the tumorigenesis of breast cancer and ovarian cancer (Costa et al., 2006; Widschwandter et al., 2004). Global hypomethylation is thought to occur at an early stage of tumorigenesis. After predisposing cells to genomic instability and genetic changes, subsequent gene-specific hypomethylation allows tumor cells to adapt to local environment and promote metastasis (Robertson, 2005).

Many oncogenes have been reported to be demethylated in cancer. C-myc, a transcription factor that acts as an oncogene, was found hypomethylated in cultured cell line (Cheah et al., 1984), hepatocellular carcinoma (Kaneko et al., 1985; Nambu et al., 1987), leukemia (Tsukamoto et al., 1992), gastric carcinoma (Fang et al., 1996), bladder cancer (Del Senno et al., 1989) and colorectal cancer (Sharrard et al., 1992). The promoter region of melanoma-associated cancer/testis antigens (*MAGE*) was hypomethylated in melanoma and colorectal cancer cell line and cancer tissues (Kim et al., 2006). Hypomethylation of the *CDH3* gene promoter was found in colorectal and breast carcinomas (Milicic et al., 2008; Paredes et al., 2005). Oncogene synuclein-γ (*SNCG*) has also been reported to be hypomethylated in breast, ovarian and gastric cancer (Gupta et al., 2003; Yanagawa et al., 2004). Other genes that have been reported to be hypomethylated in cancers include *WNT5A*, *CRIP1* and *S100P* (prostate cancer) (Wang et al., 2007), *L1CAM* (colorectal cancer) (Kato et al., 2009) and *XAGE-1* (gastric cancer) (Lim et al., 2005).

Hypermethylation

Hypermethylation is much more frequently reported than hypomethylation and is considered as the hallmark of many cancer types. DNA hypermethylation was first discovered in retinoblastoma (*RB*), in which the promoter region of *RB1* was methylated (Greger et al., 1989). Since then, a large number of tumor suppressor genes that are unmethylated in normal cells have been found to be methylated in primary tumors of various cancers. **Table 1.4** (p.36) summarizes the most frequently reported methylated tumor suppressor genes in various cancers. These tumor suppressor genes participate in many important cellular regulation pathways including cell cycle, DNA repair, apoptosis, metastasis, detoxification, hormone response, Ras signaling, and Wnt signaling. Tumor suppressor genes like *CDKN2A*, *RASSF1A* and *MGMT* involved in these extremely important cellular pathways can be observed to be methylated in many cancer types. However, there are also some tissue-specific genes that only exist in particular type of cancer. It is hypothesized that each cancer type may own a specific "methylome" that can be used for cancer classification, diagnosis and monitoring (Kulis & Esteller, 2010).

Despite the importance of promoter hypermethylation in tumorigenesis, more than 97% of DNA hypermethylation exist in intergenic regions or introns (Cheung et al., 2012). The reason for the differential methylation patterns in non-coding regions of cancer genome is unclear. It is proposed that the expression of microRNA, a group of small noncoding RNAs that play a role in matching the 3'-untraslated region of a gene at transcriptional level, was regulated by DNA hypermethylation. In one study, the expression of microRNA was found restored in cell line demethylated by 5-aza-2'-deoxycytidine, suggesting that microRNA could be epigenetically

adjusted as tumor suppressor genes (Lujambio et al., 2008). DNA hypermethylation of microRNA-127 was found associated with the aberrant expression of oncogenic factor BCL-6 in bladder cancer (Saito et al., 2006). It was also reported that microRNA-124 could act as a negative regulator of CDK6 (Lujambio et al., 2007), and microRNA-1 might play a role in hepatocellular carcinogenesis via methylation-mediated silencing of the respective microRNA genes (Datta et al., 2008). The growing number of studies on investigating the relation between DNA hypermethylation and microRNA reflects the importance of non-coding RNA in tumor formation and progression.

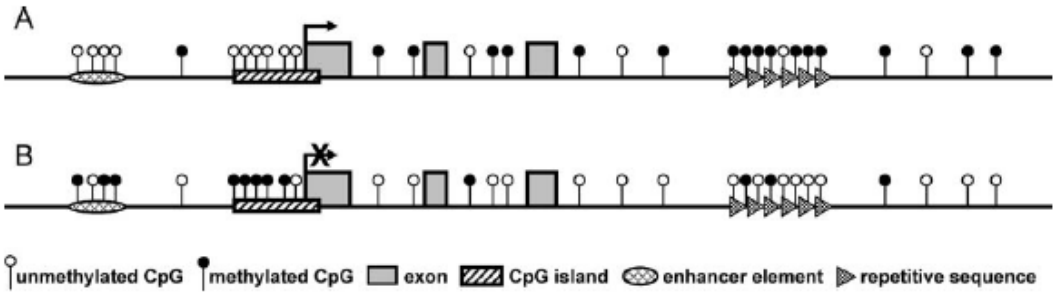

Figure 1.6 DNA methylation changes in cancer cells

A. In normal cells, most of the CpG islands and enhancer are methylation-free while interspersed CpG dinucleotides in repetitive sequences are heavily methylated. B. In cancer cells, there is a global DNA demethylation and regional hypermethylation of CpG islands and other regulatory regions.

(Brena et al, 2006)

Table 1.4 Most frequently reported methylated tumor suppressor gene

Gene	Function	Nasopharyngeal carcinoma	Breast Cancer	Lung Cancer	Prostate cancer	Leukemia/lymphomas	Colon cancer
APC	Antagonist of Wnt signaling pathway involved in cell migration and adhesion		√	√	√		√
BMAL1	Core component of the circadian clock					√	
BRCA1	DNA repair double-stranded breaks, transcription		√				
CDH1	E-cadherin, cell adhesion	√	√	√	√	√	√
CDH13	H-cadherin, cell adhesion		√	√		√	√
CDKN2A	CDK4 inhibitor, control of cell-cycle G1 progression	√	√	√	√	√	√
CDKN2B	CDK4 and CDK6 inhibitor, control of cell-cycle G1 progression	√				√	
P14 ARF	Control of cell-cycle G1 progression, stalinizer of the tumor-suppressor protein p53	√					√
COX2	Cyclooxygenase, prostaglandin biosynthesis						√
CRBP1	Transport of retinol necessary for growth or differentiation of epithelial tissues					√	√
DAPK	Positive mediator of γ-interferon induced programmed cell death	√		√		√	√
ESR1	Estrogen receptor, regulation of gene expression		√	√	√	√	
FHIT	Cleaves A-5'-PP-5'A to yield AMP and ADP; possible tumor suppressor	√					√
GATA4	GATA family of zinc-finger transcription factors						
GATA5	GATA family of zinc-finger transcription factors						√
GSTP1	Metabolism, detoxification, and elimination of genotoxic foreign compounds	√		√	√		
HIC1	Transcription factor		√	√		√	√
IGFBP3	Insulin-like growth factor-binding protein			√			
MGMT	DNA repair	√		√		√	√
MLH1	DNA mismatch repair, DNA damage signaling	√					√
NORE1A	Ras effector homolog			√			
PYCARD	TMS1/ASC, apoptotic signaling pathways	√	√	√			
RARB2	Retinoic acid receptor, limits growth of cells by regulating gene expression	√		√		√	√
RASSF1A	Inhibit the accumulation of cyclin D1, cell cycle arrest at G1/S phase transition, DNA repair	√	√	√	√	√	√
TLE1	Groucho homolog					√	
TP73	P53 family of transcription factors, apoptotic response to DNA damage	√				√	

(Kulis & Esteller, 2010)

1.3.6 DNA methylation with aging

DNA methylation patterns in cancer cells have been reported to be similar to those present in aging cells in normal tissues. Early studies showed that global DNA hypomethylation could be observed in various tissues during the process of aging in mice, hamsters and humans but not in immortal cells (Wilson & Jones, 1983). A global loss of DNA methylation during aging has also been reported by studies performed in different tissues from rat, mouse, cow (Romanov & Vanyushin, 1981), and lymphocytes (Drinkwater et al., 1989) and peripheral blood cells from humans (Bjornsson et al., 2008; Fuke et al., 2004). Progressive loss of DNMT1 efficacy on heterochromatic domains was proposed as a possible reason accounting for global DNA hypomethylation during aging (Casillas et al., 2003).

Several specific regions of the human genome have been found hypermethylated in normal tissues during aging (Issa, 2003). About 1% of the loci examined in T-lymphocytes isolated from individuals of different ages were reported hypermethylated in one study (Tra et al., 2002). Currently, a number of genes have been shown to be methylated in non-tumorigenic tissues, which include estrogen receptor (*ER*) (Issa et al., 1994), insulin-like growth gactor-II (*IGF-II*) (Issa et al., 1996), p14ARF (Shen et al., 2003), *CDKN2A* (So et al., 2006), E-cadherin (Bornman et al., 2001), c-fos (Choi et al., 1996) and collagen-α1 (Takatsu et al., 1999). The concordance of methylation status in aging and cancer cells suggests that there might be a relationship between aging and tumorigenesis. It is hypothesized that the age-related methylation may be a precursor for the transformation of normal tissue to malignant tissue (Ahuja & Issa, 2000) (**Figure 1.7**). But the definitive relationship

between DNA methylation and aging needs more experimental evidence from further studies.

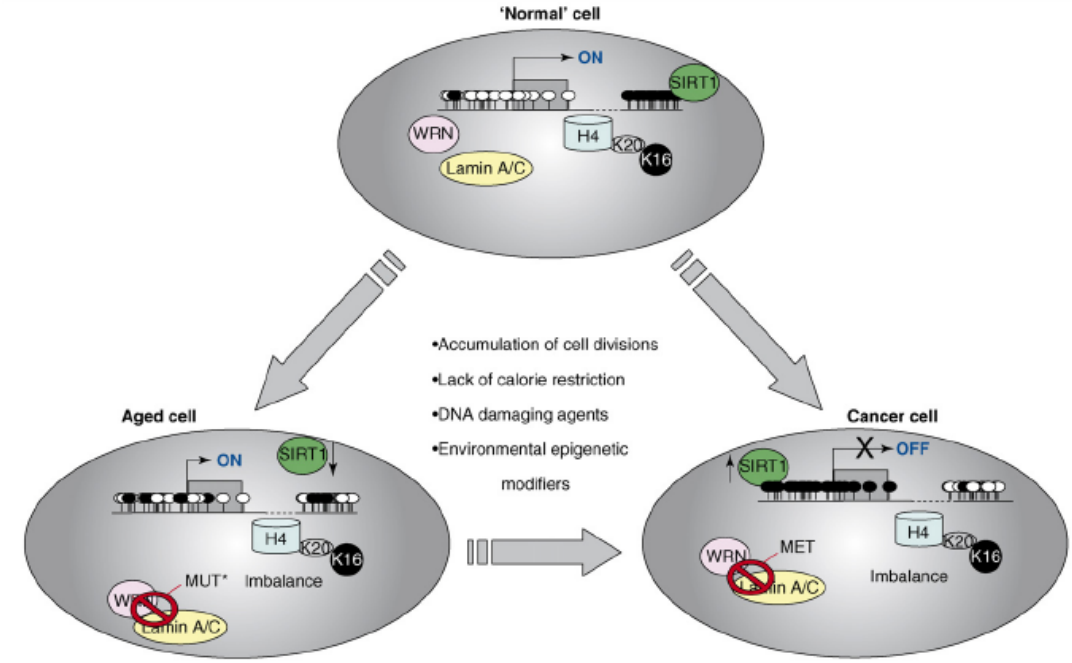

Figure 1.7 The epigenomes of normal, aged and cancer cells

In normal cells (top), there is a dense DNA methylation in repetitive sequences, unmethylated CpG islands. Cancer cells (botton right) undergo DNA hypometlation of repetitive DNA sequences, and CpG island hypermethylation of tumor suppressor genes associated with transcriptional silencing. In aged cells (botton left), there may be a progressive loss of methylated cytosines in the repetitive regions and the presence of patched sites of 5-methylcytosines in the promoter regions. Genes from aging network such as *WRN* and lamin A/C work correctly in normal cells, but become hypermethylated and silenced in cancer cells and have mutations in the aged cells. White lollipops indicate unmethylated CpG dinucleotides and black lollipops indicate methylated CpG dinucleotides.

(Fraga & Esteller, 2007)

1.3.7 Interaction with other epigenetic modifications

In addition to DNA methylation, histone modifications and RNA-mediated gene silencing also play important roles in the normal development of cells. The intimate communications between these three types of epigenetic modifications is important for the accurate transmission of chromatin states and gene expression across cell generations. Several studies have suggested that the interplay between DNA methylation and histone modifications might be involved in gene transcription and aberrant gene silencing in tumors (Jaenisch & Bird, 2003; Murr et al., 2007; Vaissiere et al., 2008). Hypermethylation of CpG could trigger deacetylation of histones while lower histone acetylation levels preferentially target DNA methylation (Herceg & Ushijima, 2010). Although it is known that the disruption of one of these three epigenetic events will inevitably affect the other, the hierarchical order of epigenetic events during unscheduled gene silencing in tumor cells remains unknown.

1.3.8 Induction of DNA methylation

Both epidemiological and laboratory-based studies have proven the importance of dietary and environmental factors in inducing cancers (Herceg, 2007). Studies of monozygotic (MZ) twins show that, at early age, MZ twins are epigenetically impossible to be differentiated from each other, while in older MZ twins, the content and distribution of 5mC are significantly different (Fraga et al., 2005). Moreover, the difference in 5mC content between twins who reported having spent less of their lifetime together was shown to be the highest (Fraga et al., 2005). Thus, it is believed

that environmental and dietary factors play crucial roles in the establishment of DNA methylation patterns. However, as the environmental factors are often cumulative and are likely to induce subtle changes, it is difficult to establish a clear-cut causal relationship between environmental exposure and DNA methylation. Even so, nutrients such as folate, choline, methionine, and vitamin B12 that are directly involved in the regeneration or supplying methyl group may influence the conversion of SAM to S-adenosylhomocysteine (SAH) when deficient, and have been found participating in the regulation of DNA methylation (Cantoni, 1985; Yi et al., 2000). Nutrients that take part in 1-carbon metabolism like riboflavin and vitamin B6 have also been proposed as factors that would affect the regulation of DNA methylation (Yi et al., 2000).

1.3.9 Applications of DNA methylation

Biomarker

In view of the important role of aberrant DNA hypermethylation in carcinogenesis, efforts have been made to apply DNA methylation screening to clinical practice to facilitate the early detection of cancer. Compared with other molecules like RNA, proteins and small metabolites, DNA as a marker has the advantages of easy extraction and can be preserved without denaturation for a long time. Besides, as DNA can be amplified by PCR, only small amounts of samples are needed in the detection process. In human epithelial cells, DNA hypermethylation is one of the earliest molecular alterations during malignant transformation and is often present in the precursor lesions of many cancers. Therefore, promoter methylation analysis of

tumor suppressor genes may be a promising method for the detection of tumors (Brooks et al., 1998; Esteller et al., 2000; Lamy et al., 2002).

An ideal biomarker usually has the characteristics of assessing non-invasively. Thus, using biomarkers present in blood or other body fluids as specimen sources for malignancy screening is highly desirable. For example, body fluids like sputum (Botezatu et al., 2000) and urine (Goessl et al., 2001) are potential specimens for DNA methylation detection. **Table 1.5** lists several examples of DNA methylation being used as biomarkers in biological materials of various cancers.

Table 1.5 Examples of potential DNA hypermethylation biomarkers in biological materials of various cancers

Gene	Cancer type	Detection material
BRCA1	Ovary	Serum
CDKN2A	Lung	Sputum
	Colon	Stool
CDKN2B	Leukemia	Blood
GSTP1	Prostate	Urine, ejaculate
MGMT	Colon	Stool
	Lung	Sputum
RASSF1A	Lung	Sputum
	Prostate	Urine
	Breast	Serum
SFRP2	Colon	Stool

(Mulero-Navarro & Esteller, 2008)

Treatment

As the process of DNA hypermethylation is reversible, agents with methylation inhibition abilities can be developed for cancer treatment. DNMT, the inactivation of which could result in DNA demethylation, is the main target of these agents. Two groups of DNMT inhibitors, nucleoside and non-nucleoside analogs, are currently under investigation. The fist group of analogs contributes to DNA demethylation through forming a covalent complex with DNMTs and inhibits its enzyme activity (Ghoshal et al., 2005). Two main types of DNA demethylation agents in this group are 5-aza-2-deoxycytidine (Aza) and 5-azacitidine, which have been approved by the Food and Drug Administration (FDA) for the treatment of myelodysplastic syndromes (MDS) and chronic myelomonocytic leukemia (CML) (Kaminskas et al., 2005; Steensma, 2009). These two agents can preferentially affect rapidly dividing cells, including cancerogenous cells, by incorporating into synthesized DNA (Liang et al., 2002). Although these drugs are used at low doses and are well tolerated in patients, side effects such as myelo-suppressions, nausea and infections also exist. Moreover, these two drugs are very unstable in solution and seem to be ineffective in solid tumors (Chabot et al, 1983; Ghoshal & Bai, 2007). Zebularine, another nucleoside analog with DNMT-inhibiting properties, is more stable in solution and is hopefully to become a better drug for cancer treatment (Zhou et al., 2002).

As the incorporation of nucleoside analogs into DNA may lead to toxicity and other side effects, non-nucleoside analogs that could bind to the catalytic site of DNMTs without incorporating into DNA have been investigated. Agents in this group are SGI-1027 (Datta et al., 2009), RG108 (Brueckner et al., 2005), hydralazine (Deng et al., 2003), procainamide (Lu et al., 2005a, b) and psammaplin (Pina et al.,

2003). MG98, which could inhibit DNMT at translational level, was investigated in phase I/II clinical trials and is a very promising agent for epigenetic therapy of tumors (Amato, 2007).

1.3.10 Detection methods

Nowadays, many techniques have been developed for the analysis of DNA methylation status. According to the methods of discrimination between 5-methyl cytosine and cytosine, these techniques can generally be divided into three categories: bisulfate-based methods, methylation-specific restriction enzyme-based methods and chromatin immunoprecipitation (**Table 1.6** on p.49).

Bisulfate-based methods

The principle of bisulfate treatment is to convert unmethylated cytosines to uracil while keeping methylated nucleotides unchanged in this process (Herman et al., 1996). The differences in methylation status could then be transformed to the differences in DNA sequences. Bisulfite-treated DNA could be analyzed by methylation-specific PCR (MSP) using specific primers designed to be complement to either methlylated or unmethylated DNA (Herman et al., 1996) (**Figure 1.8**). MSP that could detect 0.1% methylated DNA (≈ 50 pg) in unmethylated samples is the most commonly used method for assessing DNA methylation in laboratories (Herman et al., 1996). However, MSP has the limitations of being non-quantitative, restricting to CpG target sites, and being susceptible to positive detection error after many PCR cycles (An et al., 2002; Shaw et al., 2006).

Quantitative MSP (QMSP/Methylight) that can be performed in quantitative or semi-quantitative form is much more sensitive than MSP (Eads et al., 1999). To get the information of DNA methylation status of each CpG sites, bisulfate sequencing (BSP) of amplified DNA sequences could be performed. However, BSP has the shortcomings of requiring sequencing a large amount of clones, which is time-consuming and laborious. Pyrosequencing, based on the luminometric detection of pyrophosphate release following nucleotide incorporation, may be a more convenient method than BSP since it can be used for direct quantification of bisulfite-treated DNA and allows for the analysis of up to 10 CpG dinucleotides spanning a 75-nucleotide stretch in a single run (Uhlmann et al., 2002; Colella et al., 2003; Tost et al., 2003).

Methylation-specific restriction enzyme-based methods

Methylation-specific restriction enzyme could digest target sequence only if it is not methylated, and hence the methylated DNA sequence can be differentiated from its counterpart (Schumacher et al., 2008). The commonly used enzymes are HpaII, SacII, NotI, and BstUI. The limitation of this method is that it is only suitable for the analysis of CpGs located within the enzyme restriction site and is not applicable for clinical usage as it requires a large amount of genomic DNA.

For genome-wide methylation studies, methods like restriction landmark genomic scanning (RLGS) (Rush & Plass, 2002), methylation-sensitive representational difference analysis (MS-RAD) (Kaneda et al., 2003), HpaII tiny fragments enrichment by ligation-mediated PCR (HELP assay) (Khulan et al., 2006)

and comprehensive high-throughput array-based relative methylation analysis (CHARM) (Irizarry et al., 2008) based on methylation-specific restriction enzyme digestion have been developed. Among them, CHARM is highly quantitative. In this method, DNA samples are first digested with a frequent cutter and ligated to special linkers. The linked fragments are then cleaved by methylation-sensitive restriction enzymes. Linker-PCR is performed to amplify the methylated sequences that are not cleaved by the enzymes. After hybridization to microarray, the probes could be identified. Although costly, it is a powerful tool for identifying hundreds of specific genomic loci simultaneously (Schumacher et al., 2008; Zilberman & Henikoff, 2007).

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) makes use of specific antibodies against methyl-CpG-binding proteins, the proteins that could specifically recognize 5-methycytosine, to bind to methylated DNA sequences. Methylated and unmethylated cytosines can be distinguished through the use of DNA sequencing or microarray (Ballestar et al., 2003; Lopez-Serra et al., 2006). The combination of ChIP with DNA microarray (Me-DIP) could provide information about methylation status for large-scale studies.

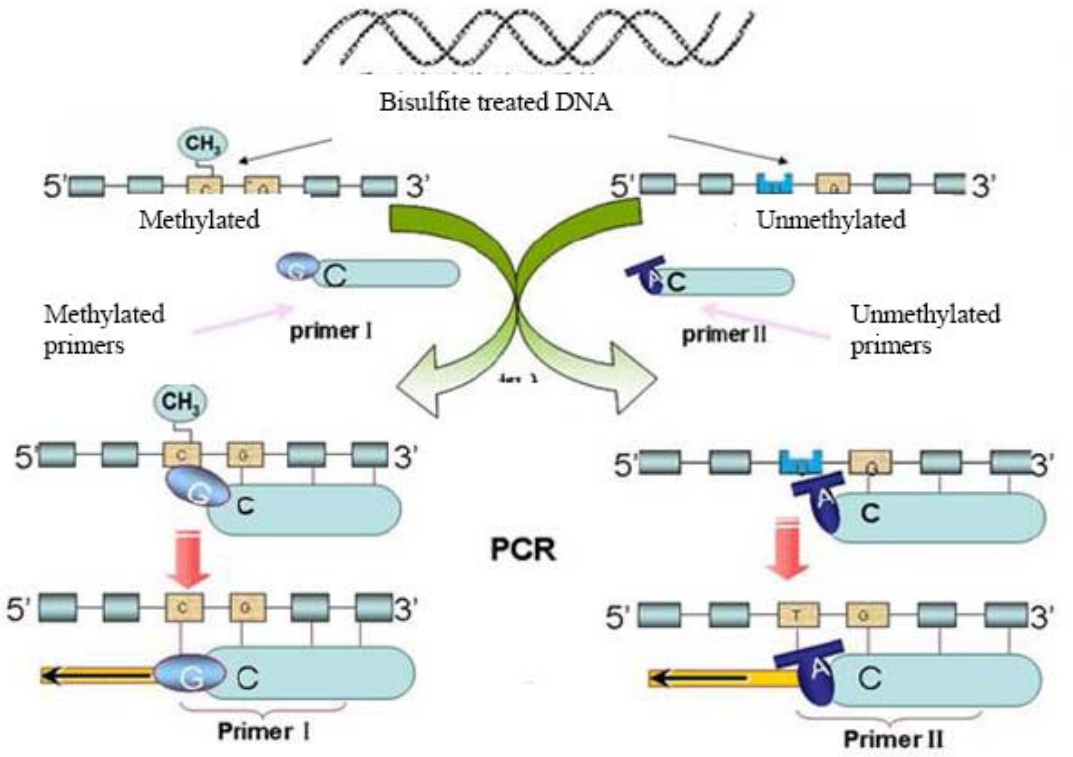

Figure 1.8 Principle of methylation-specific PCR

After sodium bisulfite conversion, all unmethylated cytosines are converted to uracils. But methylated cytosines remain unchanged. Then, the differences between methylated and unmethylated DNA have been transformed to the difference between DNA sequences. Primers can be designed either complement to methyalted DNA seuqnece or unmethylated DNA sequence.

(http://www.sangon.com/sangon_detail.aspx?newsID=416)

Table 1.6 Methods for methylation screening

Method	Description	Advantages	Disadvantages
Based on bisulfate conversion of DNA			
MSP	Methylation-specific PCR, two pairs of primers has to be designed to detect methylated and unmethylated CpGs	Easy to perform, quick analysis, low cost	Only few CpGs are detected, not quantitative
BSP	Bisulfite sequencing PCR, sequencing the amplified DNA sequence that contains CpGs	Several CpGs in a CGI are checked	Costly, laborious, time consuming
MethyLight	Quantitative methylation-specific PCR	Sensitive, quantitative, high throughput	Costly, not quantify methylation at individual nucleotide level
Based on methylation-sensitive restriction enzymes			
RLGS	Restriction landmark genomic scanning; digested DNA fragments separated on a 2D gel, detection based on radioactive labeling of unmethylated digestion sites	Genome-wide screening, quantitative, limited sensitivity	Detect only CpGs in restriction site, large amounts of DNA needed, lack of signal for methylated sites
MS-RAD	Methylation-sensitive representational difference analysis; digested DNA fragments ligated with universal adaptors. After amplification, libraries of products with unmethylated sites are obtained	Genome-wide screening, limited sensitivity	Detect only CpGs located within restriction site, lack of signal for methylated sites
CHARM	Comprehensive high-throughput array-based relative methylation analysis; digested DNA fragments analyzed on a specially design array, smoothing the data of neighboring genomic location increases sensitivity and specificity	Genome-wide screening, regions of lower CpGs density taken into the consideration, highly quantitative	Enzymatic digestion limitations
Based on affinity assays			
MsDIP	Methylated DNA immunoprecipitation; immunoprecipitation of methylated DNA fragments using antibody against 5-methylcytosine, following by hybridization to DNA microarray	High-resolution maps of methylome, global distribution of mCpGs studied, genome-wide screening	Large amounts of DNA needed, restricted to the antibody specificity, low resolution of detection

(Kulis & Esteller, 2010)

1.4 Cell-free circulating DNA

Cell-free circulating DNA (cf-DNA) refers to the extracellular DNA found in blood. It is comprised of genomic, epigenomic, mitochondrial and viral DNA. Although higher concentrations of cf-DNA in the serum of cancer patients than healthy individuals were first identified in 1977 (Leon et al., 1977), it is only recently that cf-DNA has been investigated for its value in the diagnosis and prognosis of cancer due to the poor method for cf-DNA quantification. With the improvement of assay techniques, the concentration of cf-DNA can now be determined by florescence-based methods (PicoGreen and ultraviolet spectrophotometer) and quantitative PCR (SYBR Green and TaqMan).

1.4.1 Origin and characterization

Most cf-DNA presents in serum or plasma is double-stranded with a size between 0.18 and 21 kilobases (Stroun et al., 1987； Jahr et al., 2001). cf-DNA in serum or plasma often exists in the form of nucleoproteins, the composition of which is not clear (Stroun et al., 1987). The concentration of cf-DNA in healthy individuals ranges from 0 to 100 ng/mL with an average of 30 ng/mL (Anker & Stroun, 2000). However, in cancer patients, a range of 0 ~ 1000 ng/mL with an average concentration of 180 ng/mL, has been reported (Shapiro et al., 1983). The mechanism by which cf-DNA is released to bloodstream is not yet fully understood. In healthy individuals, cf-DNA is suggested to be originated from apoptotic cells and is primarily of hematopoietic origin (Suzuki et al., 2008；Stroun et al., 2001b；Ziegler et al., 2002). As all living cells could actively release DNA fragments, these DNA

fragments may be responsible for the cf-DNA in the blood of healthy individuals (Stroun et al., 2001b； Stroun et al., 2001a). The high level of cf-DNA in cancer patients is thought to be related to the necrosis or apoptosis of tumor cells during tumorigenesis (Lecomte et al., 2002). The DNA fragments resulted from apoptosis are around 180 base pairs or corresponding multiples while the fragments of larger size are derived from necrosis. Macrophages have been proposed to be involved in this process through phagocytosing necrotic and apoptotic cells (Choi et al., 2005). In addition to necrosis or apoptosis, secretion has also been regarded as a potential way of releasing cf-DNA in blood (**Figure 1.9**).

The proportion of cf-DNA derived from tumor cells varies from patient to patient. The state and size of the tumor are two important factors that could affect the amount of cf-DNA in blood. For a patient with a tumor weighing 100 g (equal to 3×10^{10} cancer cells), up to 3.3% of tumor DNA was reported to enter into the blood every day (Diehl et al., 2005). The amount of cf-DNA may also be influenced by physiological filtering, clearance, degradation and lymphatic circulation (Schwarzenbach et al., 2011).

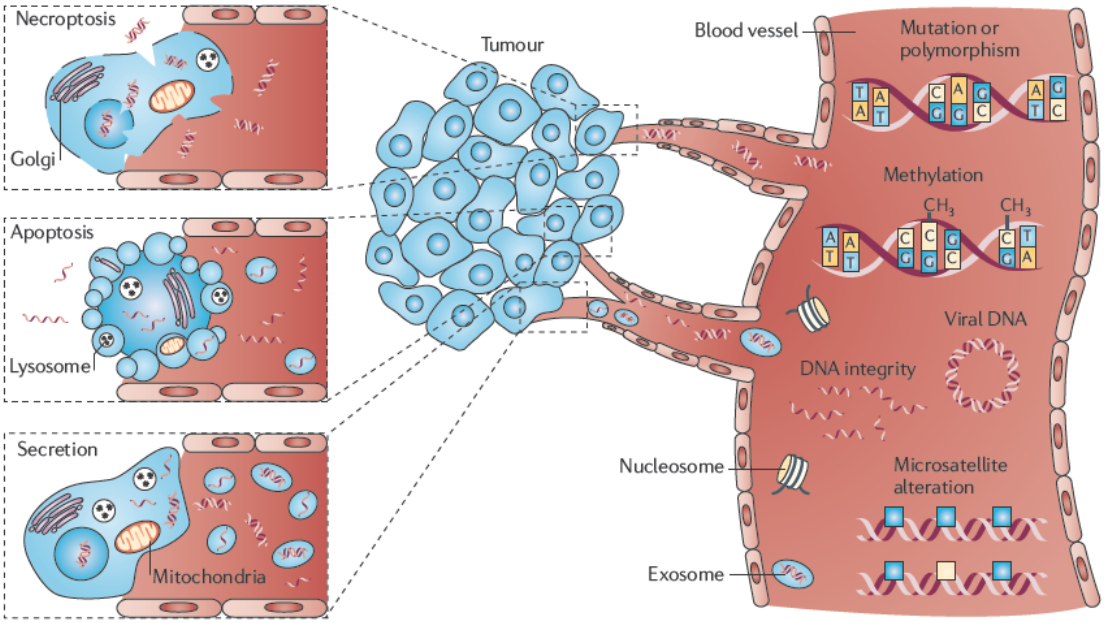

Figure 1.9 Cell-free circulating DNA in blood

Cell-free circulating DNA can exist in the form of single-stranded and double-stranded DNA. Apoptosis, necrosis and secretion are proposed as the three mechanisms by which DNA is released from tumor cells to blood.

(Schwarzenbach et al., 2011)

1.4.2 Tumor specific changes

Based on the tumor origin of cf-DNA in cancer patients, similar genetic and epigenetic alteration patterns should be detected in both tumor tissue and cf-DNA. Mutations of oncogenes and tumor suppressor genes, microsatellite alterations and promoter hypermethylation are the three most studied genetic and epigenetic changes of cf-DNA (**Table 1.7** on p.56).

Mutation

Mutations of oncogenes and tumor suppressor genes that participate in cell-cycle regulation may induce carcinogenesis. Several studies have been conducted to discover mutations in both the cancer tissues and the corresponding cf-DNA. In one study, 73.2% of the primary breast carcinoma patients were found to have tumor suppressor gene *TP53* mutations. Of these patients, 42.9% exhibited *TP53* mutation in the corresponding plasma DNA (Garcia et al., 2006). The consistency of *TP53* mutation in tumor tissue and plasma has also been reported in other cancers such as colorectal and ovarian cancers (Swisher et al., 2005； Ito et al., 2003). In pancreatic carcinoma, *KRAS* was reported to be mutated in 80 ~ 90% of patients. The same mutation existed in 27% of plasma DNA in pancreatic ductal adenocarcinoma patients (Castells et al., 1999). Detection of mutations of other oncogenes and tumor suppressor genes such as *BRAF*, *EGFR* and *APC* in serum or plasma cf-DNA are underway. The study of mutations in these genes in cf-DNA may lay a good foundation to the discovery of maker to monitor the treatment of therapeutic agents target at *KRAS*, *BRAS*, *EGFR* and *p53* pathways (Downward, 2003； Levine & Oren, 2009).

Microsatellite

Microsatellite is defined as a region in chromosome that contains DNA sequences with 1 ~ 6 nucleotides and 1 ~ 60 times of tandem repeats (Jung et al., 2010). Two common microsatellite alterations, microsatellite instability (MSI) and loss of heterozygosity (LOH), which occurs when the normal function of one allele is lost, has been detected in cf-DNA. In one study, MSI (D21S1245) and LOH (FHIT) were detected in 56% of non-small cell lung cancer tumors and 40% of plasma DNA (Sozzi et al., 2003). However, discrepancy in LOH between primary tumors and cf-DNA has also been reported (Fleischhacker & Schmidt, 2007). These contradictory data might be explained by technical problems and the low proportion of cf-DNA derived from tumor cells in some samples (Diehl et al., 2005; Coulet et al., 2000; Hibi et al., 1998; Kopreski et al., 1997). Furthermore, remarkable differences were found when comparing the data of microsatellite alterations in cf-DNA from different studies even though the same cf-DNA microsatellite markers were used for patients with both the same and different tumors (Jung et al., 2010). The low repeatability and analytical sensitivity reduce the application value of microsatellite alterations for cancer screening.

DNA methylation

Besides genetic changes, epigenetic alterations such as DNA methylation can also be detected in cf-DNA. The detection of promoter hypermethylation is more sensitive and specific than mutation and microsatellite since aberrant DNA methylation always occurs at specific CpG dinucleotides (Klose & Bird, 2006). Currently, concordant methylation patterns in tumor tissues and corresponding plasma or serum

samples have been reported in a large number of genes in various cancers. A summary of 31 studies examining methylation alterations in cf-DNA from patients with different cancers demonstrated a range of 5% to 80% methylation rate using single hypermethylation markers (Board et al., 2008). To reduce the basis from using single-gene marker and increase diagnostic sensitivity, the selection of a panel of appropriate genes from a list of candidate genes that are methylated in specific type of tumor is indispensable. The first commercial real-time PCR plasma test for *SEPT9* gene in colorectal cancer was developed by Epigenomics AG and Abbott Molecular. Although this biomarker is still under validation, the application value of promoter hypermethylation detection of tumor suppressor genes in cf-DNA for cancer diagnosis is obvious.

Table 1.7 Detection of cell-free circulating DNA in patients with different cancers

Cancer	Mutation	Microsatellite	Methylation
Bladder		√	√
Breast	√	√	√
Cervical			√
Colorectal	√		√
Hepatocelular carcinoma	√		√
Lung		√	√
Non-Hodgkin's lymphoma			√
Melanoma	√		
Ovarian			√
Pancreatic	√		√
Prostate		√	√
Nasopharyngel carcinoma			√

(Schwarzenbach et al., 2011)

1.4.3 Technical and practical aspects

In spite of the great application potential in cancer diagnosis of cf-DNA, there are still some problems in manipulating and measuring cf-DNA from different samples.

The first problem concerns with the sample type and various pre-analytical factors in blood sampling and processing. Currently, there is no consensus as to whether plasma or serum is preferable for circulating nucleic acid analysis. Serum reportedly contains 3 ~ 24 folds higher amount of cf-DNA than plasma, but the underlying reason is not fully understood (Thijssen et al., 2002). The blood collection, processing, storage methods and conditions can also influence the quality and quantity of cf-DNA (Sturgeon et al., 2008). It has been suggested that the rapid processing of plasma or serum before storage at – 20℃ is critical. But the optimal cryopreservation conditions of cf-DNA have not been established (Gormally et al., 2007). The above diversities in manipulating cf-DNA make it difficult to compare data from different laboratories.

Second, the variations in extraction protocols may affect the yield and quality of cf-DNA (Jahr et al., 2001). It has been reported that the differences of DNA extracted using different kits can be as high as 50%, especially for those based on silica-gel columns (de Kok et al., 1998). Since cf-DNA contains a lot of short DNA fragments, the ability of the method that can extract DNA fragments of different sizes is important for ensuring the reliability and comparability of extraction and downstream operation (Liu et al., 2010).

Third, the approaches for cf-DNA concentration measurement could influence the sensitivity and specificity of the test. For the measurement of cf-DNA concentration, fluorometric approach using different dyes and PCR assays is more sensitive than spectrophotometer method. Results from the comparison of these two methods for quantification of plasma DNA in lung cancer patients showed that PicoGreen was a more rapid, accurate and inexpensive method when compared with real-time PCR (Szpechcinski et al., 2008). Other problems with cf-DNA assessment include the variations in sensitivity and specificity of different assay techniques for promoter hypermethylation detection. New regulatory guidelines such as the one established by the National Academy of Clinical Biochemistry for tumor biomarker discovery for clinical practice are useful in minimizing the method-related errors in handling cf-DNA and improving its clinical utility (Sturgeon et al., 2008).

Chapter 2: Evaluating the potential of promoter hypermethylation of five methylated tumor suppressor genes in the detection of nasopharyngeal carcinoma in serum samples

2.1 Background

2.1.1 Introduction

The high incidence of NPC in endemic regions suggests that NPC is still a serious healthcare problem today. Based on the correlation between EBV and NPC in endemic regions, various EBV detection systems have been established for the diagnosis of NPC in recent decades. High antibody titers against EBV viral proteins such as EA IgA, VCA IgA, EBNA1 IgA, and EB1/Zta IgG have been identified in NPC patients. However, none of these markers is sensitive and specific enough to predict NPC (Tsang et al., 2004; Leung et al., 2003; Fachiroh et al., 2006). It has been suggested that the effectiveness of the combined detection of methylated DNA in plasma and serological EBV antibody is better than either one alone (Wong et al., 2004).

DNA hypermethylation, one of the most studied epigenetic events in mammals, has been described in a variety of human cancers and is considered the hallmark of many cancers (Reik et al., 2001; Jones & Wolffe, 1999; Baylin et al., 2001). DNA hypermethylation involves the binding of a methyl group to CpG dinucleotides in the promoter region of a gene by DNMTs. Aberrant promoter hypermethylation may result in expression silencing of tumor suppressor genes (Herman & Baylin, 2003).

Detection of DNA hypermethylation has the potential to compensate for the shortcomings of the EBV detection system or becomes an alternative choice for the diagnosis of NPC.

cf-DNA identified in serum and plasma is an emerging target for cancer screening in recent years (Sidransky, 1997). The concentration of cf-DNA in the blood of cancer patients was much higher than that from healthy individuals (Leon et al., 1975). The high level of cf-DNA in cancer patients was thought to be related to the necrosis or apoptosis of tumor cells during tumorigenesis (Leon et al., 1975). Currently, tumor-specific genetic and epigenetic alterations can be detected in serum DNA from patients with various cancers including head and neck, lung, gastric and colorectal cancer (Sanchez-Cespedes et al., 2000; Esteller et al., 1999b; Ichikawa et al., 2004; Hibi et al., 1998). Testing cf-DNA has the advantages of high sensitivity, low cost, non-invasiveness, and suitability for routine clinical application.

2.1.2 Hypothesis and objective

As DNA hypermethylation is one of the earliest molecular alterations during malignant transformation in human epithelial cells and is often present in the precursor lesions of cancers (Brooks et al., 1998; Esteller et al., 2000; Lamy et al., 2002), analysis of promoter methylation in tumor suppressor genes may serve as a promising method for the detection of NPC.

A number of tumor suppressor genes have been found to be frequently methylated in NPC. Among these genes are *RAS* association domain family member

1 (*RASSF1A*) (Chow et al., 2004a), cyclin-dependent kinase inhibitor 2A (*CDKN2A*) (Lo et al., 1996), deleted in lung and esophageal cancer 1 (*DLEC1*) (Kwong et al., 2007), death-associated protein kinase1 (*DAPK*) (Kwong et al., 2002) and ubiquitin C-terminal hydrolase L1 (*UCHL1*) (Li et al., 2010), all of which contribute to NPC pathogenesis by disrupting the normal regulation of apoptosis, DNA repair, cell proliferation, and signal transduction (Tao & Chan, 2007 ; Li et al., 2010). *RASSF1A* and *CDKN2A* have been reported giving good discrimination between NPC and non-NPC in NPC paraffin and brushing (Hutajulu et al., 2011). The combination of *DLEC1*, *UCHL1* and *KIF1A* was found to have a sensitivity of 84% and a specificity of 92% in primary tumor NCP samples (Loyo et al., 2011). *DAPK*, *CDKN2A* and *RASSF1A* could be found with high specificity in plasma of NPC patients but rarely in both EBV IgA serological-positive and serological-negative normal controls (Wong et al., 2004). Although these five genes have been studied for methylation status in NPC in separate studies, ours is the first study unifying them as a panel of biomarkers for the detection of NPC in serum samples.

The objective of this study is to investigate the potential of promoter hypermethylation of five tumor suppressor genes (*RASSF1A*, *CDKN2A*, *DLEC1*, *DAPK* and *UCHL1*) as markers in the detection of nasopharyngeal carcinoma in serum samples.

2.2 Materials and methods

To evaluate the potential of promoter hypermethylation of tumor suppressor genes in serum as a diagnostic tool for NPC, a case-control study was conducted (**Figure 2.1**). cf-DNA was extracted from serum collected from 40 NPC patients before treatment and 41 age- and sex-matched healthy subjects. Promoter hypermethylation status of five tumor suppressor genes was assessed by MSP after sodium bisulfite conversion. Differences in methylation status of the five tumor suppressor genes and clinicopathological parameters (age, gender and staging) between NPC patients and healthy subjects were compared.

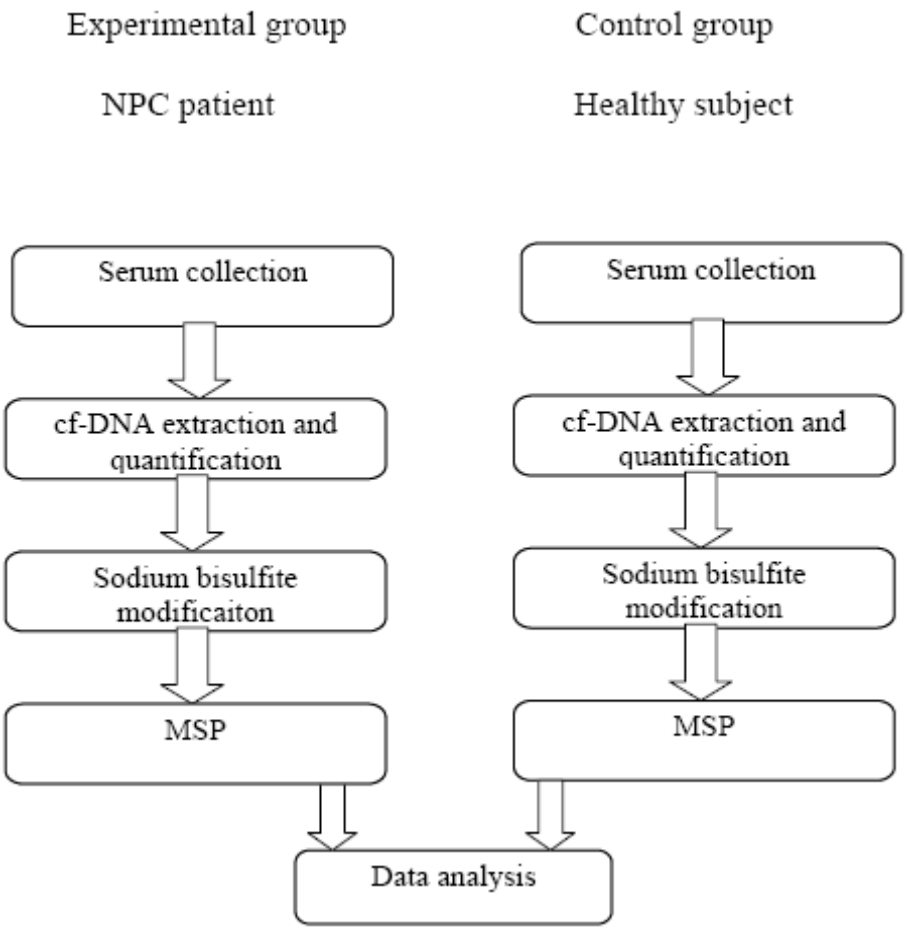

Figure 2.1 Workflow for the evaluation of the potential of five methylated tumor suppressor genes in the detection of nasopharyngeal carcinoma in serum samples

2.2.1 Sample size calculation

Sample size was estimated by the statistical software G*Power 3.1.2. Alpha Level = 0.05, anticipated effect size = 0.5875 (Wong, et al., 2004), desired statistical power = 0.8. According to the estimation, 39 NPC patients should be recruited in experiment group and 39 age- and sex-matched healthy subjects should be recruited as normal controls.

2.2.2 Subject recruitment and blood collection

Forty newly diagnosed NPC patients were recruited before treatment from Cancer Hospital, Shantou University Medical College between July 2011 and January 2012. The inclusion and exclusion criteria for experimental group were newly diagnosed NPC patients, which were confirmed by nasopharyngeal fiberoscope, before radiation treatment. All patients had no evidence of other cancers based on clinical and radiological evaluations by Shantou hospital. Blood samples (3 mL) were collected from the patients along with their clinical information. Blood samples (12 mL) were also obtained from 41 healthy individuals. The inclusion criteria for normal controls included age- and sex-matched, and did not have NPC. The objectives of the study and the relevant procedures were explained to the subjects, who then signed a consent form before joining the study. Consent form and information sheet are shown in **Appendix Ⅱ**. Ethics approval was obtained from both the Hong Kong Polytechnic University and the Shantou University Medical College.

2.2.3 Serum separation

Whole blood was placed at 37°C in water bath (Grant Instruments, Cambridge, England) for 20 minutes. After clotting, whole blood was placed in Allxegra®X-15R centrifuge (Beckman Coulter, CA, USA) and centrifuged for 10 minutes at 1900 × *g* at 4°C. Serum supernatant was carefully aspirated from blood samples without disturbing blood cells. An aliquot of about 4 ~ 5 mL serum was obtained from one 10 mL primary blood tube. Aspirated serum was transferred into fresh 1.5 mL microtubes (Axygen, CA, USA). Then, serum samples were centrifuged for 10 minutes at 16,000 × *g* in Microfuge®22R centrifuge (Beckman Coulter, CA, USA) at 4°C to remove residual cell debris, to which cellular nucleic acids might attach. Supernatant was carefully removed to a new tube with a pipette without disturbing the pellet. If serum was used for nucleic acid extraction on the same day, the sample was stored at 2 ~ 8°C until further processing. For longer storage, serum was kept frozen at − 80°C. Before using the serum for circulating nucleic acid extraction, serum tubes were thawed at room temperature.

2.2.4 Cell-free circulating DNA extraction

cf-DNA was extracted from serum samples with QIAamp Circulating Nucleic Acid Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

2.2.4.1 Reagents

(1) Phosphate-buffered saline (PBS) (Life Technologies, Carlsbad, CA).

(2) Buffer ACB (500 mL) was prepared by adding 200 mL isopropanol (100%) (SK Chemicals, Ulsan, Korea) to 300 mL buffer ACB concentrate (provided).

(3) Buffer ACW1 (44 mL) was prepared by adding 25 mL ethanol (100%) (Sigma, St. Louis, USA) to 19 mL buffer ACW1 concentrate (provided).

(4) Buffer ACW2 (43 mL) was prepared by adding 30 mL ethanol (100%) to 13 mL buffer ACW2 concentrate (provided).

(5) Buffer ACL was prepared by adding carrier RNA solution to buffer ACL concentrate (provided) that was made by adding 1550 µL Buffer AVE (provided) to the tube containing 310 µg lyophilized carrier RNA (provided) to obtain a 0.2 µg/µL solution.

(6) Proteinase K solution (provided).

2.2.4.2 Equipment

(1) Vac Elut-20 16 × 100mM Vacuum manifold (Varian Inc, CA, USA).

(2) DOA-V 130-BN Vacuum Pump (Waters, MA, USA).

2.2.4.3 cf-DNA extraction

Preparation

Before DNA extraction, serum samples and Buffer AVE were equilibrated to room temperature. If serum samples were < 2 mL, or < 5 mL, the volumes were brought up to 2 mL, or 5 mL with PBS, respectively. QIAvac 24 Plus was set up, water bath

was heated to 60°C and heating block was heated to 56°C before the experiments

Procedure

An aliquot of 200 µL, or 500 µL QIAGEN Proteinase K was pipetted into a 50-mL centrifuge tube (SPL Life Sciences, Pocheon, Korea). Then, 2 mL or 5 mL of serum and 1.6 mL or 4.0 mL Buffer ACL (containing 1.0 µg carrier RNA) were added to the 50-mL tube. The tubed was capped and the content was mixed by pulse-vortexing for 30 seconds. After incubation at 60°C for 30 minutes, Buffer ACB (3.6 mL or 9 mL) was added to the lysate in the tube. The mixture was mixed thoroughly by pulse-overtaxing for 15 ~ 30 seconds. Lysate-Buffer ACB mixture was incubated in the tube for 5 minutes on ice.

QIAamp Mini column was inserted into the VacConnector on the QIAvac 24 Plus. A 20-mL tube extender was inserted into the open QIAamp Mini column. Then, Lysate-Buffer ACB mixture was carefully applied into the tube extender of the QIAamp Mini column. Vacuum pump was switched on. When all lysates had been drawn through the columns completely, the vacuum pump was switched off and the pressure was released to 0 mbar. The tube extender was carefully removed and discarded. Next, 600-µL Buffer ACW1 was applied to the QIAamp Mini column. The lid of the column was left open, and the vacuum pump was switched on. After all of Buffer ACW1 had been drawn through the QIAamp Mini column, the vacuum pump was switched off and the pressure was released to 0 mbar. An aliquot of 750 µL Buffer ACW2 was applied to the QIAamp Mini column. The lid of the column was left open, and the vacuum pump was switched on. After all of Buffer ACW2 had

been drawn through the QIAamp Mini column, the vacuum pump was switched off and the pressure was released to 0 mbar.

An aliquot of 750 μL ethanol (100%) was applied to the QIAamp Mini column. The lid of the column was left open, and the vacuum pump was switched on. After all of ethanol had been drawn through the spin column, the vacuum pump was switched off and the pressure was released to 0 mbar. The column was removed from the vacuum manifold, and the VacConnector was discarded. The QIAamp Mini column was placed in a clean 2-mL collection tube, and centrifuged at full speed (20,000 × *g*; 14,000 rpm) for 3 minutes. QIAamp Mini Column was placed into a new 2-mL collection tube. With the lid left open, the assembly was incubated at 56°C for 10 minutes to dry the membrane completely. QIAamp Mini column was placed in a clean 1.5-mL elution tube and the 2-mL collection tube was discarded. An aliquot of 50 μL Buffer AVE was carefully applied to the center of the QIAamp Mini membrane. The lid was closed and the tube was incubated at room temperature for 3 minutes, and then centrifuged in a microcentrifuge at full speed (20,000 × *g*; 14,000 rpm) for 1 minute to elute the nucleic acids. The extracted DNA was stored at − 20°C.

2.2.5 Cell-free circulating DNA quantification

Extracted DNA samples were quantified by Quant-iT PicoGreen® dsDNA Reagent and Kits (Invitrogen, Carlsbad, CA) according to the manufacturer's directions.

2.2.5.1 Reagents

(1) TE working solution (1×) was prepared by diluting the concentrated 20× TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5) (provided) 20-fold in Ultrapure™ distilled water (Life Technologies, Carlsbad, CA).

(2) Aqueous working solution of the Quant-iT™ PicoGreen® reagent was prepared by making a 200-fold dilution of the concentrated DMSO solution (provided) in 1× TE buffer.

(3) A 500 ng/mL working solution of dsDNA was prepared by diluting 100 µg/mL lambda DNA standard (provided) 200-fold in 1× TE buffer.

2.2.5.2 Equipment

(1) 1420 Multilabel Counter, Victor™^{3}V Fluorometry (Perkinelmer, MA, USA).

(2) Fluotrac 200 Greiner 96-well microplate (Greiner bio-one, Neuberg, Germany).

2.2.5.3 cf-DNA quantification

Standard curve construction

The lambda DNA standard, provided at 100 µg/mL with the Kit, was diluted 200-fold in TE to make the 500 ng/mL working solution. To produce a standard curve with concentration ranging from 2.5 ng/ml to 250 ng/ml, 500 ng/mL DNA working solution (prepared in Step 1) was added into wells based on the volumes shown in **Table 2.1**. An aliquot of 100 µL aqueous working solution of Quant-iT™ PicoGreen® reagent was added to each well. This step was conducted in dark. The fluorescence intensity of each sample was measured using a spectrofluorometer with

standard fluorescent wavelengths (excitation 485 nm, emission 535 nm, 0.1 second).

Each sample was added into two identical wells and each well was measured twice to decrease the experimental error. Fluorescence value of the reagent blank was subtracted from that of each of the samples. A standard curve of fluorescence versus DNA concentration was generated using corrected data.

Table 2.1 Protocol for preparing standard curve

Volume (µL) of TE	Volume (µL) of lambda DNA 500 ng/mL	Volume (µL) of diluted Quant-iT™ PicoGreen® dsDNA Reagent	Final DNA concentration in Quant-iT™ PicoGreen® dsDNA Reagent
0	100	100	250 ng/mL
50	50	100	125 ng/mL
80	20	100	50 ng/mL
90	10	100	25 ng/mL
95	5	100	12.5 ng/mL
98	2	100	5 ng/mL
99	1	100	2.5 ng/mL
100	0	100	0

Sample Analysis

A volume of 2 μL experimental DNA solution was added to 98 μL TE to give a final volume of 100 μL. The aqueous working solution (100 μL) of the Quant-iT™ PicoGreen® reagent was added to each sample. The fluorescence of the sample was measured using instrument parameters corresponding to those used for generating the standard curve. Each sample was added into two identical wells and each well was measured twice to decrease the experimental error. The fluorescence value of the reagent blank was subtracted from that of each of the samples. DNA concentration of sample was determined from the standard curve generated in DNA Standard Curve.

2.2.6 Sodium bisulfite modification

Bisulfite treatment of DNA (up to 2 μg) was performed using EZ DNA Methylation-Direct Kit (Zymo Research, Orange, CA) following the manufacturer's protocol (**Figure 2.2**).

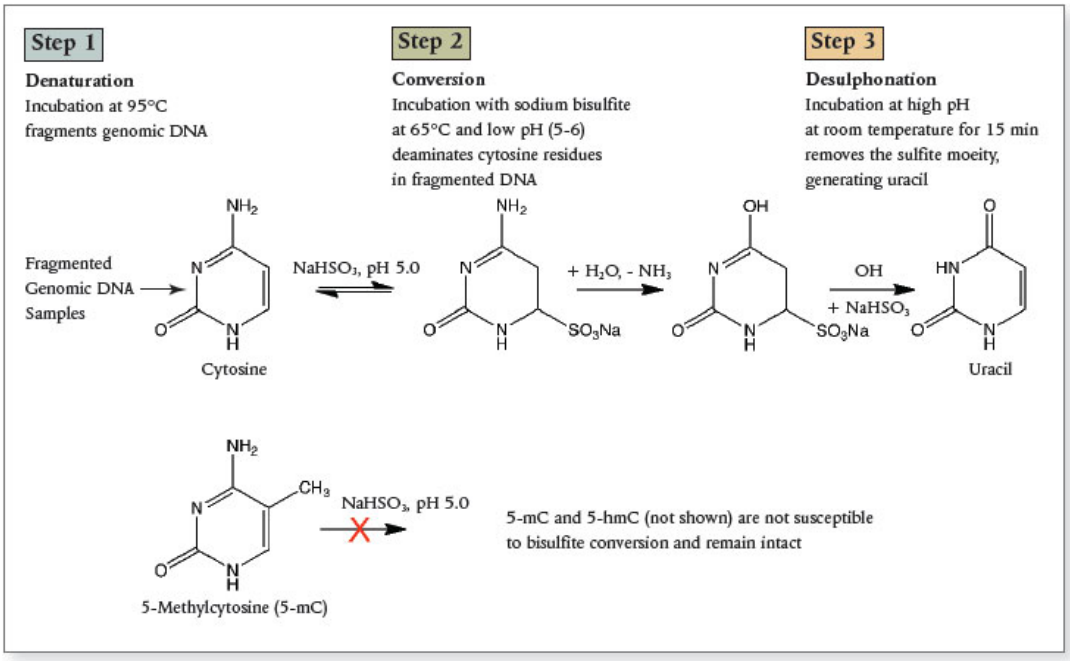

Figure 2.2 Mechanism of sodium bisulfite modification (http://www.neb.com/nebecomm/tech_reference/epigenetics/Bisulfite_Conversion.asp)

2.2.6.1 Reagents

(1) CT Conversion Reagent Preparation: 790 µL of M-Solubilization Buffer (provided) and 300 µL of M-Dilution Buffer (provided) were added to a tube of CT Conversion Reagent (provided) and mixed at room temperature with frequent vortexing or shaking for 10 minutes. M-Reaction Buffer (160 µL; provided) was added and mixed for an additional 1 minute.

(2) M-Wash Buffer was prepared by adding 96 mL of 100% ethanol to the 24 mL M-Wash Buffer concentrate (provided).

2.2.6.2 Equipment

96-well GeneAmp PCR system 9700 (Life Technologies, Carlsbad, CA)

2.2.6.3 Procedure

A volume of 20 µL DNA sample was added to 130 µL of CT Conversion Reagent solution in a PCR tube. The sample was mixed and then centrifuged briefly to ensure no droplets were in the cap or sides of the tube. PCR tube was placed in a thermal cycler and the following steps were performed: 1. 98 °C for 8 minutes, 2. 64 °C for 3.5 hours, 3. 4 °C storage for up to 20 hours.

An aliquot of 600 µL M-Binding Buffer was added into a Zymo-Spin™ IC Column placed into a provided Collection Tube. Sample from last step was loaded into Zymo-Spin™ IC Column containing M-Binding Buffer. The column was capped, and the contents were mixed by inverting the column several times and then

centrifuged at full speed (> 10,000 × *g*) for 30 seconds. The flow-through was discarded. A volume of 100 µL M-Wash Buffer was added to the column. Then, the column was centrifuged at full speed for 30 seconds. M-Desulphonation Buffer (200 µL) was added to the column with subsequent incubaton at room temperature (20°C ~ 30°C) for 15 ~ 20 minutes.

After incubation, the column was centrifuged at full speed for 30 seconds. A volume of 200 µL M-Wash Buffer was added to the column. Then, the column was centrifuged at full speed for 30 seconds. Another 200-µL aliquot of M-Wash Buffer was added and the column was centrifuged for an additional 30 seconds. The column was placed into a 1.5 mL microtube and an aliquot of 10 µL M-Elution Buffer was added directly to the column matrix. Then, the column was centrifuged for 30 seconds at full speed to elute the DNA. The concentration of all bisulfite-modified DNA was adjusted to 1 ng/µL by re-suspending in elution buffer. The DNA was stored at or below − 20°C for later use.

2.2.7 MSP for bisulfite treated DNA

2.2.7.1 Reagents

(1) AmpliTaq Gold DNA polymerase (5 U/µL), GeneAmp 10× PCR Buffer II (150 mM Tris-HCl at pH 8, 500 mM KCl, without $MgCl_2$) and 25 mM $MgCl_2$ (Life Technologies, Carlsbad, CA).

(2) Deoxyribonucleoside triphosphates (dATP, dCTP, dGTP, dTTP each at 20 mM) (GE Healthcare, Buckinghamshire, UK).

(3) Primers for PCR were ordered from Invitrogen (Life Technologies, Carlsbad,

CA).

(4) *SssI* methyltransferase (New England Biolabs, Beverly, MA).

(1) Stock Tris-Borate-EDTA (TBE; 10×) buffer was made from 890 mM Tris (hydroymethyl)-aminoethane (Tris) (United States Biochemical Crop., Cleveland, USA), 890 mM boric acid (United States Biochemical Crop., Cleveland, USA) and 250 mM disodium ethylenediamine tetraacetic acid, Na_2EDTA (Malinckrodt, St. Louis, USA).

(2) Working TBE buffer (0.5×) was diluted from 10× TBE buffer.

(3) Loading buffer (6×) was made from 30% glycerol (United States Biochemical Crop., Cleveland, USA), 10 mM Tris-HCl (pH 8.0) (United States Biochemical Crop., Cleveland, USA), 1 mM disodium ethylenediamine tetraacetic acid, Na_2EDTA (pH 8.0) (Riedel-de Haën, Seelze, Germany) and 0.05% bromophenol blue (Sigma, St. Louis, USA).

(4) 1 kb plus DNA Ladder (Life Technologies, Carlsbad, CA).

(5) Polyacrylamide gel was prepared with 40% acrylamide-bis (19:1) solution including acrylamide (United States Biochemical Crop., Cleveland, USA) and bis-acrylamide (United States Biochemical Crop., Cleveland, USA), TEMED (Bio-Rad laboratories, CA, USA) and 25% ammonium persulphate (APS) (BDH laboratory supplies, Poole, England).

(6) A solution for binding polyacrylamide gel to the glass plate contained 10% ethanol (Riedel-de Haën, Seelze, Germany), 0.5% acetic acid (BDH Laboratory Supplies, Poole, England) and silane (γ-methacryloxyptopyltrimethoxylsilane) (Sigma-Aldrich, St. Louis, USA).

(7) 100× SYBR Green I (Life Technologies, Carlsbad, CA).

2.2.7.2 Equipment

(1) 96-well GeneAmp PCR system 9700 (Life Technologies, Carlsbad, CA).

(2) Apparatus GNA-200 (Amersham Pharmacia Biotech, Uppsala, Sweden).

(3) PowerPac™ Basic Power Supply (BioRad, CA, USA).

(4) Gel documentation system-Chemi Genius^2 (Syngene, Cambridge, UK).

2.2.7.3 Procedure

Design of primer was based on the DNA sequence of the selected genes by MethPrimer (http://www.urogene.org/methprimer/) and OLIGO Primer Analysis Software (version 6.65, http://www.oligo.net/).

PCR amplification for five genes was carried out in a 15-μL reaction mixture consisting of 0.3 μM of each primer, 0.3 unit of AmpliTaq Gold polymerase, 0.2 mM of each dNTP, $MgCl_2$ (2.5 mM for *RASSF1A*, *DLEC1*, *DAPK* and *UCHL1*; 1.5 mM for *CDKN2A*) and GeneAmp 1× PCR buffer II. The primer sequences and PCR conditions for MSP are shown in **Table 2.2**. One μL of bisufite-modified DNA was added into each PCR reaction. PCR amplification was carried out in a 96-well 9700 thermal cycler. PCR conditions were as follows: 95 °C for 10 minutes; 40 cycles of 95°C for 30 seconds, specific annealing temperature (60°C for *RASSF1A*, *CDKN2A*, *DAPK* and *UCHL1*; 55°C for *DLEC1*) for 30 seconds, and 72°C for 30 seconds; and a final extension for 5 minutes at 72°C.

DNA extracted from leukocytes that were obtained from healthy individual and methylated in vitro by *SssI* methyltransferase was used as positive control for

methylation-specific primers. Specifically, 14 µL nuclease-free water, 2 µL 10× NEBuffer 2, 2 µL diluted SAM (SAM 32 mM stock was diluted by adding 1 µL SAM 32 mM stock into 19 µL Nuclease free water), 1 µL genomic DNA (1 µg), 1 µL *SssI* methylase (4 U/µL) were mixed, and pipetted up and down at least six times. The mixture was incubated for one hour at 37°C. Reaction was stopped by heating at 65°C for 20 minutes. Leukocytes from healthy individual and molecular grade water were used as positive control for unmethylation-specific primers and negative control, respectively. The PCR products were visualized under UV transillumination in 6% polyacrylamide gel stained with SYBR Green.

Table 2.2 Summary of primer sequences, annealing temperatures, cycles and product sizes for MSP in cf-DNA

Gene	Forward primers(5'-3')	Reverse primer(5'-3')	Annealing temp (°C)	Cycles	Product size(bp)
RASSF1A-M [a]	TCG TAT TCG GTT GGA GCG	ACC CCG CGA ACT AAA AAC G	60	40	110
RASSF1A-U	GGG TTG TAT TTG GTT GGA GTG	TAA CAA ACC CCA CAA ACT AAA AAC A	60	40	119
CDKN2A-M	TTA TTA GAG GGT GGG GCG GAT CGC	GAC CCC GAA CCG CGA CCG TAA	60	40	150
CDKN2A-U	TTA TTA GAG GGT GGG GTG GAT TGT	CAA CCC CAA ACC ACA ACC ATA A	60	40	151
DLEC1-M	GAT TAT AGC GAT GAC GGG ATT C	ACC CGA CTA ATA ACG AAA TTA ACG	55	40	193
DLEC1-U	TGA TTA TAG TGA TGA TGG GAT TTG A	CCC AAC TAA TAA CAA AAT TAA CAC C	55	40	193
DAPK-M	GAT AGT CGG ATC GAG TTA ACG TC	CAA ATC CCT CCC AAA CGC CGA	60	40	102
DAPK-U	GGA GGA TAG TTG GAT TGA GTT AAT GTT	CAC AAA TCC CTC CCA AAC ACC AA	60	40	108
UCHL1-M	TTT ATT TGG TCG CGA TCG TTC	CAA ACT ACA ACT ATA AAA CGC CG	60	40	105
UCHL1-U	GTT TGT ATT TAT TTG GTT GTG ATT GTT T	CCA AAC TAC AAC TAT AAA ACA CCA	60	40	113

[a] M, primers for methylated DNA; U, primers for unmethylated DNA

2.2.8 Interpretation of MSP results

Only when both the positive controls for methylated and unmethylated DNA template showed positive results and the blank control showed negative results in MSP would the results for PCR be regarded as valid. Interpretation for possible MSP results is shown in **Table 2.3**. Cases with unmethylated band but no methylated band were considered as unmethylated in the target region. Cases with methylation band but without unmethylated band were interpreted as methylated in the target region. Incomplete methylation applied to cases in which both methylated and unmethylated band were observed. Absence of both methylated and unmethylated band was interpreted as unsuccessful and the case was repeated.

Table 2.3 Interpretation of MSP results

Unmethylated/U band	Methylated/M band	Interpretation
Positive	Negative	Unmethylated
Negative	Positive	Complete methylation
Positive	Positive	Incomplete methylation
Negative	Negative	Invalid test

2.2.9 Statistical analysis

Differences of age and sex between NPC patients and normal controls were analyzed by independent sample *t*-test and Fisher's exact test. Difference of cell-free DNA concentration between NPC patients and normal controls was compared using Mann-Whitney test. Correlation of DNA concentration with sex and staging was analyzed using Mann-Whitney test and Krusakal-Wallis test.

Differences of methylation status for the five genes between NPC patients and normal controls were analyzed with Fisher's exact test. Sensitivity and specificity for each gene as well as different combinations of the five genes in the detection of NPC were calculated. Sensitivity is defined as the number of true positives divided by the number of true positives plus the number of false negatives. Specificity is defined as the number of true negatives divided by the number of true negatives plus the number of false positives.

Association between promoter methylation and sex, age and staging were analyzed through the use of the Fisher's exact test. Correlation between the methylation status of any two gene markers were compared with Spearman's Rho test. Logistic regression was used to establish multigene predictive model. All statistical analyses were performed using SPSS version 16.0, and $P < 0.05$ was considered as statistically significant difference.

2.3 Results

2.3.1 Demographic characteristics of NPC patients and normal controls

A total of 40 NPC patients and 41 normal controls were recruited in this study. Their demographic characteristics are summarized in **Table 2.4**. The experimental group was composed of 30 (75%) male and 10 (25%) female patients with age ranging from 15 to 74 years, and a mean of 50.23 years. In the control group, there were 31 (78%) male and 9 (22%) female subjects with age ranging between 16 and 77 years, and a mean of 48.2 years. There were no significant difference between the cases and controls in terms of gender (Fisher's exact test, $P = 0.798$) and age (Independent Sample *t*-test, $P = 0.701$).

Table 2.4 Demographic and clinical characteristics of nasopharyngeal carcinoma patients and normal controls

Characteristic		NPC[a] patients (n = 40)	Normal controls (n = 41)	*P* value
Gender	Male	30 (75%)	32 (78%)	0.798[b]
	Female	10 (25%)	9 (22%)	
Age	Mean (range)	50.2 (15-74)	48.2 (16-77)	0.701[c]
Stage	II	4 (10%)		
	III	16 (40%)		
	IVA	15 (37.5%)		
	IVB	5 (12.5%)		
Type	I	2 (5%)		
	II and III	38 (95%)		

[a] NPC, nasopharyngeal carcinoma

[b] Fisher's exact test

[c] Independent sample *t*-test

2.3.2 Cell-free circulating DNA concentration

Based on PicoGreen assay, the mean cf-DNA concentration in serum was 126.41 ng/mL (range: 22.41 ~ 890.91 ng/mL) in NPC patients and 28.60 ng/mL (range: 7.05 ~ 69.83 ng/mL) in normal controls. The concentration of cf-DNA in the serum was significantly higher in NPC patients than in normal controls (Mann-Whitney test, $P < 0.000$; **Figure 2.3**). However, the cf-DNA concentration was not significantly correlated with gender (Mann-Whitney test, $P = 0.611$) or stage (Krusakal-Wallis test, $P = 0.953$).

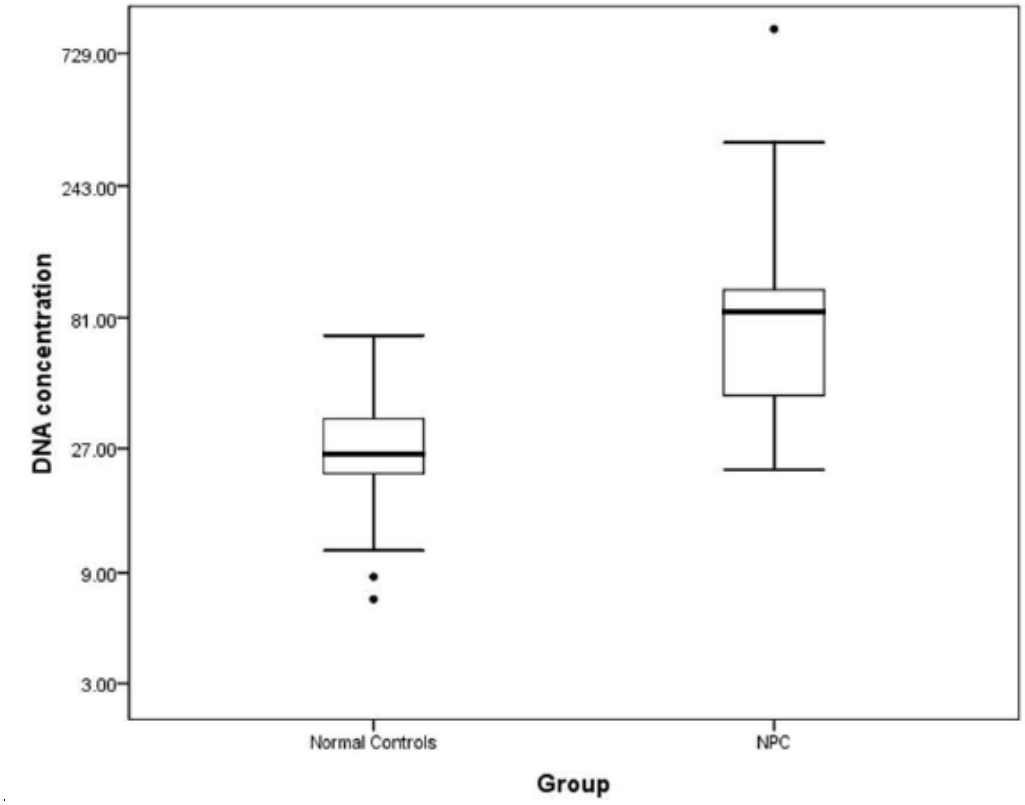

Figure 2.3 Distribution of serum DNA concentrations in normal controls and nasopharyngeal carcinoma patients

The *y* axis has a log scale.

2.3.3 Methylation analysis

2.3.3.1 Methylation profile in NPC and normal controls

The frequency of promoter hypermethylation of five genes was determined by MSP. **Figure 2.4** (p.87) shows representative examples of MSP results. Promoter hypermethylation rate for five genes in the serum of NPC patients and normal controls are summarized in **Table 2.5** (p.88). The methylation frequencies were as follows: 17.5% for *RASSF1A*, 22.5% for *CDKN2A*, 25% for *DLEC1*, 51.4% for *DAPK* and 64.9% for *UCHL1*. DNA methylation rates of *CDKN2A*, *DLEC1*, *DAPK* and *UCHL1* were significantly higher in NPC patients than normal controls (Fisher's exact test). The sensitivity of individual genes ranged from 17.5% to 64.9% and the specificity ranged from 80.5% to 97.6%.

Concurrent methylation for different numbers of genes in NPC patients and normal controls are summarized in **Table 2.6** (p.89). In 40 NPC patients, only six cases were unmethylated for any of the five genes. DNA methylation for at least one gene was observed in 85.0% (34/40) of all the samples. In normal controls, no methylation for any of the five genes was detected in 26 subjects (63.4%). The concurrent methylation for two genes was detected in 7.3% (3/41) of all samples. No sample was found with concurrent methylation for three or more genes.

Correlation between the methylation status of any two gene markers was analyzed. Significant correlation of methylation status was found between *RASSF1A* and *DLEC1* ($r > 0.273$), *CDKN2A* and *UCHL1* ($r > 0.234$) and *DAPK* and *UCHL1* ($r > 0.301$) (Spearman's Rho test). Results for correlation analysis are shown in **Appendix Ⅲ**.

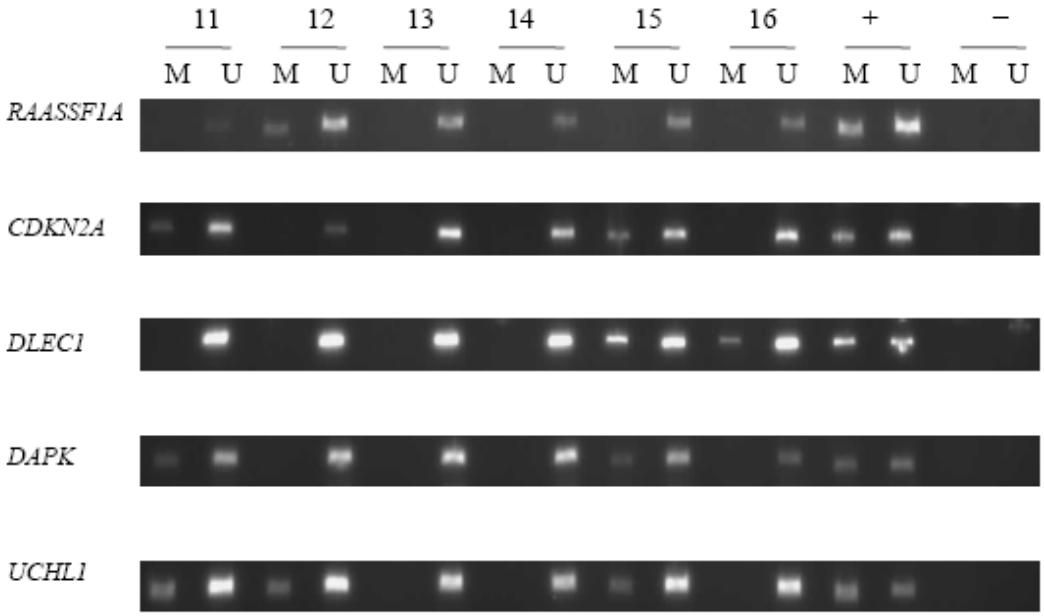

Figure 2.4 MSP results of representative samples from nasopharyngeal carcinoma patients

Values on the top are sample numbers. M, methylation band; U, unmethylation band; + M, positive control for methylation-specific primers; + U, positive control for unmethylaiton-specific primers; −, negative control.

Table 2.5 Promoter hypermethylation frequency for the five selected genes

	NPC [a] patients	Normal Controls	*P* [b] value	Sensitivity %	Specificity %
RASSF1A	7/40	2/41	$P = 0.088$	17.5	95.1
CDKN2A	9/40	1/41	$P = 0.007$	22.5	97.6
DLEC1	10/40	3/41	$P = 0.037$	25.0	92.7
DAPK	18/35	4/41	$P < 0.000$	51.4	90.2
UCHL1	24/37	8/41	$P < 0.000$	64.9	80.5

[a] NPC, nasopharyngeal carcinoma

[b] Fisher's exact test

Table 2.6 Number of concurrent genes with methylation versus sample number

Number of concurrent genes	Number of methylated samples in NPC [a] patients (Percentage)	Number of methylated samples in normal controls (Percentage)
0	6/40 (15.0%)	26/41 (63.4%)
1	11/40 (27.5%)	12/41 (29.3%)
2	14/40 (35.0%)	3/41 (7.3%)
3	7/40 (17.5%)	0/41 (0%)
4	2/40 (5.0%)	0/41 (0%)
5	0/40 (0%)	0/41 (0%)

[a] NPC, nasopharyngeal carcinoma

2.3.3.2 Sensitivity and specificity of different gene combinations

For the five genes, there are totally 26 different combinations. **Table 2.7** shows the sensitivity and specificity of all the combinations of methylation markers. The percentage of at least one gene that was methylated in NPC patients (Sensitivity 1) ranged from 35.0% to 85.0%, and the percentage of no one gene that was methylated in normal controls (Specificity 1) ranged from 63.4% to 92.7%. While in normal controls, the percentage of at least two genes that were methylated in NPC patients (Sensitivity 2) ranged from 2.5% to 57.5%. And the percentage of less than two genes that were methylated in normal controls (Specificity 2) ranged from 92.7% to 100.0%.

Table 2.7 Sensitivity and specificity of all combinations of methylation markers

Gene combinations	At least one gene is methylated [a]		At least two genes are methylated [b]	
	Sensitivity 1 (%)[c]	Specificity 1 (%)[d]	Sensitivity 2 (%)[c]	Specificity 2 (%)[d]
RASSF1A,CDKN2A	37.5	92.7	2.5	100.0
RASSF1A,DLEC1	35.0	90.2	7.5	97.6
RASSF1A,DAPK	55.0	85.4	7.5	100.0
RASSF1A,UCHL1	65.0	75.6	12.5	100.0
CDKN2A,DLEC1	45.0	90.2	2.5	100.0
CDKN2A,DAPK	57.5	87.8	10.0	100.0
CDKN2A,UCHL1	65.0	78.0	17.5	100.0
DLEC1,DAPK	60.0	82.9	10.0	100.0
DLEC1,UCHL1	70.0	75.6	15.0	97.6
DAPK1,UCHL1	72.5	73.2	32.5	97.6
RASSF1A,CDKN2A,DLEC1	52.5	87.8	12.5	97.6
RASSF1A,CDKN2A,DAPK	67.5	82.9	15.0	100.0
RASSF1A,CDKN2A,UCHL1	70.0	73.2	27.5	100.0
RASSF1A,DLEC1,DAPK	62.5	80.5	25.0	97.6
RASSF1A,DLEC1,UCHL1	72.5	73.2	25.0	95.1
RASSF1A,DAPK,UCHL1	75.0	68.3	42.5	97.6
CDKN2A,DLEC1,DAPK	72.5	80.5	17.5	100.0
CDKN2A,DLEC1,UCHL1	75.0	73.2	30.0	97.6
CDKN2A,DAPK,UCHL1	77.5	70.7	40.0	97.6
DLEC1,DAPK,UCHL1	80.0	68.3	42.5	95.1
RASSF1A,CDKN2A,DLEC1,DAPK	75.0	78.0	30.0	97.6
RASSF1A,CDKN2A,DLEC1,UCHL1	77.5	70.7	37.5	95.1
RASSF1A,CDKN2A,DAPK,UCHL1	80.0	65.9	50.0	97.6
RASSF1A,DLEC1,DAPK,UCHL1	80.0	65.9	50.0	92.7
CDKN2A,DLEC1,DAPK,UCHL1	85.0	65.9	50.0	95.1
RASSF1A,CDKN2A,DLEC1,DAPK, UCHL1	85.0	63.4	57.5	92.7

[a] As long as there is one gene methylated in the sample, this sample is considered as positive.

[b] Only if there are two genes or more than two genes methylated in the sample, this sample is considered as positive.

[c] Sensitivity = number of positive cases in NPC patients / total number of NPC patients

[d] Specificity = (total number of normal controls − number of positive cases in normal controls) / total number of normal controls

2.3.4 Promoter hypermethylation with clinical parameters

Of the 40 NPC patients, 30 were male and 10 female. Methylation rates of *RASSF1A* and *DLEC1* were higher in male than female patients while the methylation rates of *CDKN2A* and *DAPK* were lower in male than female patients. For *UCHL1*, male and female patients had the same methylation rate. Methylation rate of five genes in NPC patients was not correlated with gender.

Based on AJCC/UICC staging system, 4 samples belong to stage II, 16 samples were in stage III, 15 cases were in stage IVA and 5 cases were in stage IVB. Due to the limited number of samples, stages II and III were combined to form one group, and IVA and IVB another group, and the newly formed groups were compared by Fisher's exact test. No significant association was found between methylation and stage.

The age range of the patients was 15 ~ 74 years, with a mean age of 50.23 years and a median at 49 years. In order to have a balanced number of patients, 40 NPC patients were divided into two groups: ≤ 49 years old and > 49 year old. For *RASSF1A*, *DLEC1* and *DAPK*, the methylation rate was higher in patients older than 49 years old. But for *CDKN2A* and *UCHL1*, the methylation rate was higher in patients 49 years old or younger. With Fisher's exact test, no significant difference of methylation rate for any one of five genes was identified between patients in different age group (**Table 2.8**).

Table 2.8 Association between promoter hypermethylation and clinical pathological parameters

			Hypermethylation (%)				
		No.	*RASSF1A*	*CDKN2A*	*DLEC1*	*DAPK*	*UCHL1*
Sex	Male	30	6 (20.0%)	6 (20.0%)	8 (26.7%)	11 (36.7%)	18 (60.0%)
	Female	10	1 (10.0%)	3 (30.0%)	2 (20.0%)	7 (70.0%)	6 (60.0%)
P value [a]			*P* = 0.656	*P* = 0.665	*P* = 1.000	*P* = 0.140	*P* = 1.000
Staging	II	4	1 (25.0%)	2 (50.0%)	0 (0.0%)	2 (50.0%)	2 (50.0%)
	III	16	3 (18.8%)	4 (25.0%)	4 (25.0%)	6 (37.5%)	12 (75.0%)
	IVA	15	1 (6.7%)	1 (6.7%)	5 (33.3%)	7 (46.7%)	7 (46.7%)
	IVB	5	2 (40.0%)	2 (40.0%)	1 (20.0%)	3 (60.0%)	3 (60.0%)
P value [a]			*P* = 1.000	*P* = 0.451	*P* = 0.716	*P* = 0.751	*P* = 0.333
Age	≤ 49	21	2(9.5%)	5(23.8%)	4(19.0%)	9(42.9%)	13(61.9%)
	> 49	19	5(26.3%)	4(21.1%)	6(31.6%)	9(47.4%)	11(57.9%)
P value[a]			*P* = 0.226	*P* = 1.000	*P* = 0.719	*P* = 1.000	*P* = 0.756

[a] Fisher's exact test

2.4 Discussion

Previous studies have demonstrated the potential of DNA hypermethylation of tumor suppressor genes in cf-DNA as biomarker for the diagnosis of various cancers (Sanchez-Cespedes et al., 2000; Esteller et al., 1999b; Ichikawa et al., 2004; Hibi et al., 1998). However, studies on discovering biomarkers based on DNA hymethylation in NPC are very limited. Since the effective diagnosis of NPC plays an important role in improving the treatment effect and survival rate of NPC patients, this study on investigating the potential of DNA hypermethylation of five tumor suppressor genes as markers for the diagnosis of NPC may provide useful information for the management of NPC.

2.4.1 Methylation rate of tumor suppressor genes in cell-free circulating DNA

Based on previous studies, the methylation rates of these five genes in NPC primary tumor were 67% for *RASSF1A*, 33% for *CDKN2A*, 60% for *DLEC1*, 77% for *DAPK* and 66% for *UCHL1* in primary NPC (Loyo et al., 2011; Chang et al., 2003b). In our study, *RASSF1A*, *CDKN2A*, *DLEC1*, *DAPK* and *UCHL1* were found to be methylated in 17.5%, 22.5%, 25%, 51.4% and 64.9% of NPC patients respectively. The comparably lower methylation rate of each gene in serum samples might be resulted from the special source of cf-DNA. Different from tumors, which are mostly composed of tumor cells, the proportion of tumor DNA is much lower in serum as both tumor-derived and normal-cell-derived DNA can be released into blood (Schwarzenbach et al., 2011). This was concordant with our MSP findings, in which unmethylated DNA templates were amplified in all samples.

In this study, DNA methylation of four genes (*CDKN2A*, *DLEC1*, *DAPK* and *UCHL1*) was found to be significantly higher in NPC patients than that in normal controls. The low sensitivity and specificity of a single-gene biomarker limited the use of one gene for cancer diagnosis. To increase the diagnostic power, DNA methylation of 26 different combinations of the five genes was analyzed (**Table 2.7**). Results showed that a gain in sensitivity was always accompanied by a loss in specificity. When the methylation of at least one gene was taken into account, the sensitivity (Sensitivity 1) could reach to 85.0%. However, the corresponding specificity (Specificity 1) decreased to 65.9%. Given the high false positive rate of the EBV detection system, specificity may be a more important concern for the early diagnosis of NPC. When the methylation of at least two genes was considered, the specificity (Specificity 2) could increase to a value between 92.7% and 100%.

In clinical practice, to effectively distinguish NPC patients from healthy individuals, Sensitivity 1 could be used to identify potential NPC patients, while Specificity 2 could be utilized to exclude healthy individuals. According to this principle, the combinations with high sensitivity and specificity were *CDKN2A, DLEC1* and *DAPK* (72.5% versus 100%), *RASSF1A*, *CDKN2A*, *DAPK* and *UCHL1* (80% versus 97.6%), and *CDKN2A*, *DLEC1*, *DAPK* and *UCHL1* (85.0% versus 95.1%). When sensitivity and specificity were considered together, four-gene marker - *CDKN2A*, *DLEC1*, *DAPK* and *UCHL1* - could provide the highest sensitivity of 85.0% and specificity of 95.1%. Specifically, if two or more than two of the four genes are methylated in a subject, the ability of the test to correctly identify NPC patients is above 85.0%. If no one gene is methylated in a sample, the ability of the test to correctly identify normal individuals is above 95.1%. As to the case in which

there is only one gene methylated, the individual will be recommended to have serological EBV antibody screening or nasoendoscopy as supplement test.

Correlation analysis showed that the methylation status of these five genes did not show strong concordance with each other (**Table 2.6**), implying that these five genes might participate in different regulation pathways of NPC pathogenesis.

2.4.2 Association of DNA hypermethylation with clinical parameters

From the Fisher's exact test, no significant association was found between methylation status of the five genes with age (**Table 2.8**). This showed that the methylation status of these five genes was not age-related. However, DNA methylation of some tumor suppressor genes was reported to be correlated with aging (Issa, 2003). Since the age of subjects may have an influence on the methylation status of genes, age-matching should be one requirement for subject recruitment to avoid confounding due to age. In this study, no significant association was found between DNA methylation and gender and stage (**Table 2.8**). Promoter hypermethylation is an important epigenetic event in tumor initiation and progression, it was hypothesized that different DNA methylation patterns may be the reflections of different stages in tumorigenesis (Kulis & Esteller, 2010). The identification of NPC "methylome" corresponding to different stages of NPC development would be of great significance to NPC diagnosis, and monitoring of disease and prognosis. The value of DNA hypermethylation for cancer monitoring and prognosis remains to be elucidated in future studies.

2.4.3 Problems with serum DNA methylation biomarker discovery

An obstacle for serum DNA methylation biomarker discovery is the variation of experiment techniques used by different laboratories, which may cause discrepancy in results and make it hard to make comparison for data obtained by different researchers. These variations may include the methodology of manipulating cf-DNA and the analytical method for assessing methylation status of cf-DNA.

There is now no consensus as to whether plasma or serum is preferable for circulating nucleic acid analysis, and the impact of sample collection and processing on yield is unclear. Serum reportedly contains 3-24 folds higher amount of free circulating DNA than plasma does (Thijssen et al., 2002). In our study, the average concentration of cf-DNA in serum of NPC patients was 126.41 ng/mL, which was significantly higher than 28.60 ng/mL in normal controls (Section 2.3.2 and **Figure 2.3**). This result was concordant with previous findings (Leon et al., 1975). Some researchers advocated serum as a better specimen source for circulating cancer-related DNA analysis because the interferences from the presence of anticoagulant in plasma samples were eliminated (Umetani et al., 2006).

The variation in extraction protocols for cell-free circulating DNA, which contained small fragments of 70 ~ 200 base pairs, might also affect the yield and quality of DNA (Jahr et al., 2001). It has been reported that the differences of DNA extracted using different kits could be as high as 50%, especially for those based on silica-gel columns (de Kok et al., 1998). In our study, we used QIAamp Circulating Nucleic Acid Kit, which was optimized for recovery of short DNA fragments present

at low concentrations in the starting material for cell-free circulating DNA extraction and was superior at extracting fragmented DNA (Horlitz et al., 2011).

The approaches for DNA methylation status detection could also influence the sensitivity and specificity of the test. At present, the widely used technique for DNA methylation status detection is MSP. It is reported that MSP could detect 0.1% methylated DNA (≈ 50 pg) in unmethylated samples (Herman et al., 1996). However, MSP has the limitation of being non-quantitative and was restricted to targeted CpG sites, (An et al., 2002; Shaw et al., 2006). Despite the limitations of being non-quantitative, the results obtained from our study demonstrate the usefulness of MSP in preliminary investigation of methylation biomarkers. The low sensitivity results from the low amounts of cf-DNA in plasma or serum samples could also be solved by establishing MethylPlex libraries, through which the methylated CpG islands could be greatly enriched to increase the specificity and sensitivity of the analysis (Kim et al., 2011).

2.4.4 Limitations of this study

This study is limited by the following factors. First, only five genes were chosen for methylation status screening from dozens of genes that are frequently methylated in NPC. The recruitment of more candidate genes could facilitate the discovery of the genes with higher diagnostic sensitivity and specificity, and increase the validity of this study. Second, this study was limited by the small number of subjects recruited. The sensitivity, specificity, and prediction efficiency of the best gene combinations could be validated with a larger sample size in future studies. Due to the limited

number of early stage NPC patients (stage I-II = 4/40) recruited in this study, the results of statistical analysis on the association between DNA methylation and stage of the disease, were not conclusive. If certain relationships could be detected and established between DNA methylation and stage, aberrant methylation patterns may be used for the early diagnose of NPC, which might be potentially useful in improving the survival rate of NPC patients. Third, as the subjects in the control group did not undergo any clinical evaluations before blood taking, the possibility that certain individuals had NPC or other diseases could not be excluded (although the chance was small). Finally, the small amount of DNA (1 ng) added in each PCR might limit the repeatability of PCR. To evaluate the reproducibility and increase the accuracy of the assay, quantitative analytical techniques such as Real-time PCR and pyrosequencing are recommended in further experiments. Additional work is also recommended for detecting the existence of EBV antibody in serum simultaneously to see if EBV antibody testing and DNA methylation screen could effectively collaborate with each other.

2.4.5 Future research

This study only evaluated the potential of DNA methylation for use as biomarker for the diagnosis of NPC in training set, the sensitivity, specificity and prediction efficiency of the gene combinations could be validated in validation set in future studies. To achieve a higher diagnostic sensitivity and specificity for the best gene combinations, more tumor suppressor genes that are frequently methylated in NPC could be tested. By recruiting more NPC patients in different stages, the association between DNA methylation and stage could be further studied.

DNA methylation screening of cf-DNA in serum samples for disease diagnosis may also be extended to other cancers. This kind of analysis of blood samples could be beneficial to cancer patients if put into clinical practice. The probability of DNA methylation used for detecting or monitoring the metastasis and recurrence of certain cancers could also be investigated in further studies.

Chapter 3: Investigating the association between promoter hypermethylation and transcriptional silencing

3.1 Background

3.1.1 Introduction

A large number of tumor suppressor genes have been found to be methylated in NPC (Huang et al., 1994; Lo et al., 1996; Kwong et al., 2002; Lo et al., 2001; Qiu et al., 2004). Promoter hypermethylation of tumor suppressor genes contributes to NPC carcinogenesis through disrupting cellular regulation pathways including cell cycle, DNA repair, apoptosis, Ras signaling, and Wnt signaling (Drexler, 1998; Esteller et al., 1999a; Weller et al., 2010; Michie et al., 2010; Chou et al., 2008). *DLEC1*, *DAPK* and *UCHL1* have been reported frequently methylated in the primary tumor of NPC (Kwong et al., 2007; Kwong et al., 2002; Li et al., 2010), this is concordant with our findings in Chapter two (25% for *DLEC1*, 51.4% for *DAPK* and 64.9% for *UCHL1*). But the roles of these three genes in NPC carcinogenesis are not fully understood.

DLEC1 is a candidate tumor suppressor gene that was first identified from chromosome region 3p21.2. It contains 37 exons and encodes a 1755 amino-acid protein without any significant homology to known proteins or conserved domains. The down regulation of *DLEC1* from hypermethylation has been proven in a variety of cancer cell lines, xenograft and tumors (Peng et al., 2002; Daigo et al., 1999). In NPC cell line HK-1 and HONE1, the expression of *DLCE1* can be restored by Aza and the overexpression of *DLEC1* could suppress the growth and invasiveness of

NPC cells, suggesting that the silencing of *DCLE1* by promoter hypermethylation may be important in NPC carcinogenesis (Kwong et al., 2007).

DAPK is a calcium/calmodulin-dependent serine threonine kinase protein that may play a role in regulating apoptosis pathway (Cohen et al., 1999). Aberrant expression of *DAPK* may result in the inhibition of apoptosis of normal cells. Promoter hypermethylation of *DAPK* has also been reported as an important epigenetic alteration in various cancers (Kissil et al., 1997). In NPC cell line CNE, mRNA expression of *DAPK* could be restored after Aza treatment (Kong et al., 2006), which shows that the loss of *DAPK* expression may be associated with promoter methylation in NPC.

UCHL1 is a member of ubiquitin C-terminal hydrolase family of proteins. As the ubiquitin modification of proteins plays a very important role in cell cycle regulation, DNA repair and many other cellular regulation pathways, the abnormal deubiquitination process may disrupt the normal cell control and lead to cancer (Ciechanover et al., 2000; Wilkinson, 1997; D'Andrea & Pellman, 1998). In NPC, *UCHL1* leads to NPC pathogenesis through deubiquitinating p53 and p14ARF and ubiquting MDM2 for p53 to promote p53 signaling (Li et al., 2010).

3.1.2 Hypothesis and objective

Promoter hypermethylation has been reported to be associated with the transcriptional inhibition of tumor suppressor genes and the initiation and progression of tumor cells. In this part of study, three genes (*DLEC1*, *DAPK* and *UCHL1*) with higher methylation rate in NPC patients than normal controls were selected from the five genes. The association between promoter methylation of *DLEC1*, *DAPK* and *UCHL1* and the transcriptional inhibition of these three genes was investigated in NPC cell line HNE1.

3.2 Materials and methods

NPC line HNE1 was treated with demethylation reagent, 5-aza-2' deoxycytidine of different concentrations (0 μM, 1 μM and 5 μM) for 4 days. Changes in methylation status and transcription for *DLEC1*, *DAPK* and *UCHL1* were examined by methylation-specific PCR and reverse transcription-PCR (RT-PCR) (**Figure 3.1**).

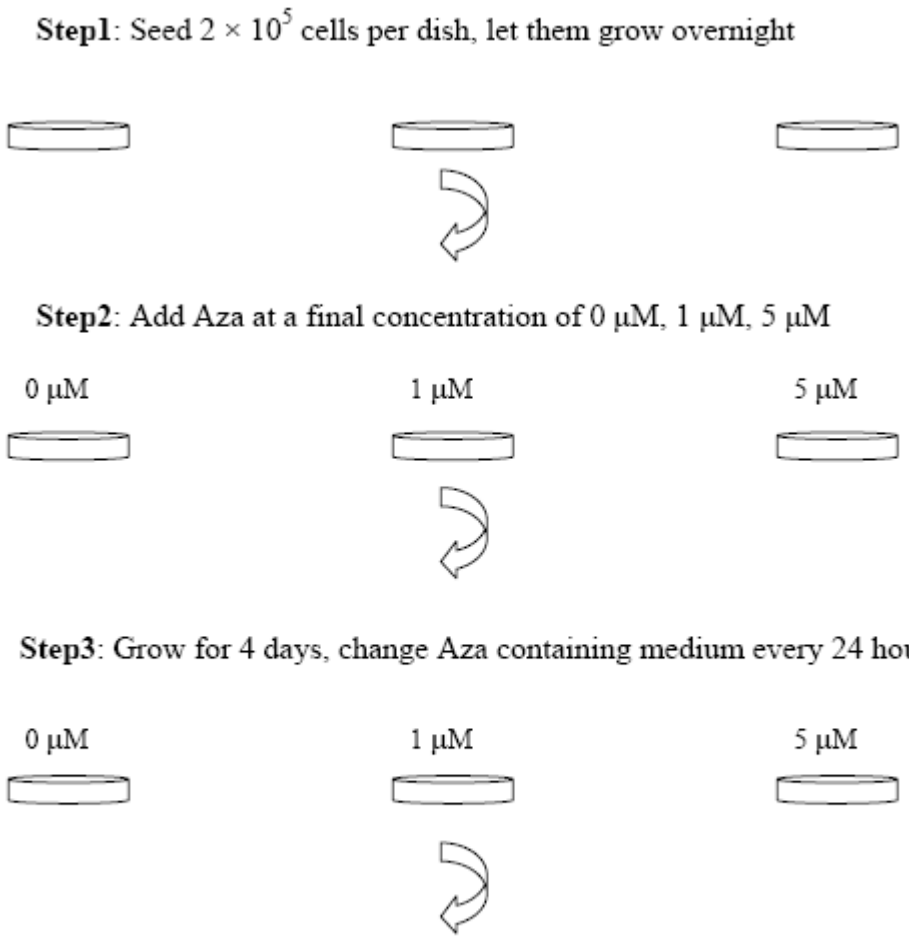

Figure 3.1 Workflow for analyzing the association between DNA methylation and transcriptional silencing of tumor suppressor gene in NPC cell line HNE1

3.2.1 Cell culture

Undifferentiated NPC cell line HNE1 (Glaser et al., 1989) was provided by AoE Hong Kong NPC Research Tissue Bank and underwent cell culture.

3.2.1.1 Reagents

(1) Dulbecco's Modified Eagle's Medium (DMEM) (Life Technologies, Carlsbad, CA).
(2) 10% fetal bovine serum (FBS) (Life Technologies, Carlsbad, CA).
(3) 0.25% Trypsin-EDTA (Life Technologies, Carlsbad, CA).
(4) 1× PBS (Life Technologies, Carlsbad, CA).
(5) 0.4% Trypan Blue (Life Technologies, Carlsbad, CA).
(6) Dimethyl sulfoxide (DMSO) (Sigma, St. Louis, USA).

3.2.1.2 Equipment

(1) Esco CYT-4A1 clean bench (Esco, Oregon, USA).
(2) Olympus CK2 Microscope (Olympus, Japan).
(3) Volac 9' pasteur pipette (Poulten & Graf, Wertheim, Germany).
(4) 10 mL and 25 mL stripette (Corning, NY, USA).
(5) 75 cm cell culture flask (SPL Life Sciences, Pocheon, Korea).
(6) Hemocytometer chamber (Boeco, Germany).
(7) CO_2 incubator (Shel lab, USA).
(8) Cryotube (Nunc™, Roskilde, Demark).
(9) NalgeneCryo 1 C Freezing container (Nalgene™, NY, USA).

(10) Filter (Nalgene™, NY, USA).

3.2.1.3 Protocol for cell culture

Count cells

Culture medium, trypsin and PBS were warmed in 37°C water bath. The chamber was cleaned using 75% alcohol, followed by distilled water. Culture medium was removed and cells were washed by 1× PBS. The cells were rinsed with 1.5 mL 0.25% trypsin, incubated in 37°C incubator for 4 minutes until cells detached. The status of cells was checked under phase microscope. A volume of 5 mL DMEM was added to inactivate trypsin. Cells were aspirated and dispensed using pipette to a 50-mL centrifuge tube, and centrifuged 5 minutes at 2500 rpm. Cell supernatant was discarded and the cell pellet was resuspended in 5 mL PBS. An aliquot of 20 µL cell suspension was pipette to 1.5-mL tube containing 80 µL trypan blue solution (Dilution factor **[**D**]** = (20 + 80) / 20 = 5). The contents were mixed well using mixer and pipetted up and down. Cell suspension was pipetted into a clean counting chamber (**Figure 3.2**). The total number of cells [N] in 8 squares (1 mm × 1 mm) of two chambers was counted. At least 100 cells within each central counting area of each chamber should be counted. Calculation: Cell density (cells/mL) = (N / 8) × D× 10^4.

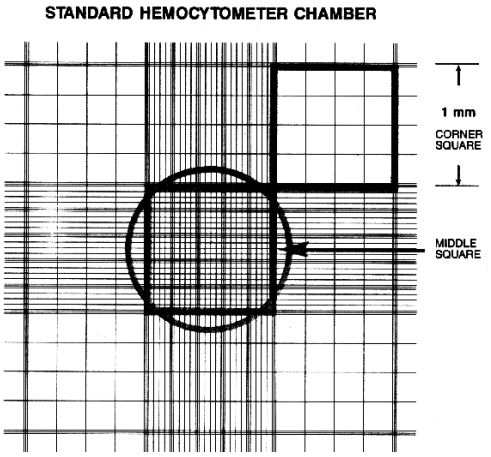

Figure 3.2 Hemocytometer grids in one chamber

Corner square = 1 mm^2, 100 nL at a depth of 0.1 mm.

Split cells

Culture medium, trypsin and PBS were warmed in 37°C water bath. The chamber was cleaned using 75% alcohol, followed by distilled water. Culture medium was removed and cells were washed by 1× PBS. The cells were rinsed with 1.5 mL 0.25% trypsin, incubated in 37°C incubator for 4 minutes until cells detached. The status of cells was checked under phase microscope. A volume of 5 mL DMEM was added to inactivate trypsin. Cells were pipetted to a new flask.

Collect cells

Culture medium, trypsin and PBS were warmed in 37°C water bath. The chamber was cleaned using 75% alcohol, followed by distilled water. Culture medium was removed and cells were washed by 1× PBS. The cells were rinsed with 1.5 mL 0.25% trypsin, incubated in 37°C incubator for 4 minutes until cells detached. The status of cells was checked under phase microscope. A volume of 5 mL DMEM was

added to inactivate trypsin. Cells were aspirated and dispensed using pipette to a 15-mL centrifuge tube, and centrifuged 5 minutes at 2500 rpm. Cell supernatant was discarded and cell pellet was resuspended in suitable medium immediately.

Freeze cells

Culture medium, trypsin and PBS were warmed in 37°C water bath. The chamber was cleaned using 75% alcohol, followed by distilled water. Culture medium was removed and cells were washed by 1× PBS. The cells were rinsed with 1.5 mL 0.25% trypsin, incubated in 37°C incubator for 4 minutes until cells detached. The status of cells was checked under phase microscope. A volume of 5 mL DMEM was added to inactivate trypsin. Cells were aspirated and dispensed using pipette to a 15-mL centrifuge tube, and centrifuged 5 minutes at 2500 rpm. Cell supernatant was discarded. The cell pellet was resuspended in 5 mL PBS and centrifuged 5 minutes at 2500 rpm. Cell supernatant was discarded and cell pellet was resuspended in 9 mL culture medium. A volume of 1 mL DMSO was pipetted into cell suspension. Cell suspension (10 mL) was pipetted up and down and aliquoted into 6 vials. Vials were wrapped by parafilm and stored at − 80°C freezer overnight. The vials were transferred to liquid nitrogen the next day.

Thaw cells

Culture medium, trypsin and PBS were warmed in 37°C water bath. A volume of 20 mL DMEM was pipepetted into flask. The vial tubes were warmed in 37°C water bath immediately until a little ice remained. An aliquote of 1 mL cells was pepitted

from vial to each flask. The cells were cultured in 37°C incubator.

3.2.2 Aza treatment

3.2.2.1 Reagent

5-aza-2' deoxycytidine (Sigma, St. Louis, USA).

3.2.2.2 Procedure

Preparation

Stock solution (1 mM) was prepared by dissolving 5 mg Aza into 21.9 mL PBS. Stock solution (21 mL) was aliquoted into forteen 1.5-mL microtubes and stored at − 80°C. The remaining 900 µL stock solution was aliquoted into eighteen 200-µL PCR tubes and stored at − 80°C. Working solution was prepared according to **Table 3.1**.

Table 3.1 Protocol for preparing Aza working stock

Concentration of working solution	0 µM	1 µM	5 µM
Volume of 1 mM Aza stock	0 µL	20 µL	100 µL
Volume of PBS	100 µL	80 µL	0 µL
Volume of DMEM	1900 µL	1900 µL	1900 µL

Aza treatment for HNE1

HNE1 was freshly seeded at 2×10^5 cells in three identical culture dishes and was allowed to grow overnight. Cell culture medium was replaced with fresh medium containing Aza at a final concentration of 0, 1, 5 μM. Cells were allowed to grow continuously for 4 days, with change of Aza containing medium every 24 hours. On the sixth day, cells were counted for cell viability and harvested for sodium bisulfite modification and RNA extraction. Each step for Aza treatment was repeated twice.

3.2.3 Sodium bisulfite modification

Bisulfite treatment of DNA (up to 2 μg) was performed using EZ DNA Methylation-Direct Kit (Zymo Research, Orange, CA) following the manufacturer's protocol.

3.2.3.1 Reagents

(1) Proteinase K was prepared by adding 1040 μL Proteinase K Storage Buffer (provided) to the tube containing Proteinase K (provided).
(2) CT Conversion Reagent was prepared using the same protocol as Chapter 2.
(3) M-Wash Buffer was prepared using the same protocol as Chapter 2.

3.2.3.2 Equipment

96-well GeneAmp PCR system 9700 (Life Technologies, Carlsbad, CA).

3.2.3.3 Procedure

A total of 5×10^4 cells were collected for each culture dish and subjected to bisulfite conversion. A volume of 26 μL digestion solution was set up by adding 13 μL M-Digestion Buffer (2×) and 1 μL Proteinase K to 12 μL Sample (10^5 cells). The reaction mixture was incubated for 20 minutes at 50°C and then centrifuged for 5 minutes at 10,000 × *g*.

A volume of 20 μL DNA sample was added to 130 μL of CT Conversion Reagent solution in a PCR tube. The sample was mixed and then centrifuged briefly to ensure no droplets were in the cap or sides of the tube. PCR tube was placed in thermal cycler and the following steps were performed: 1. 98 °C for 8 minutes, 2. 64 °C for 3.5 hours, 3. 4 °C storage for up to 20 hours.

An aliquot of 600 μL M-Binding Buffer was added into a Zymo-Spin™ IC Column placed into a provided Collection Tube. The sample from the last step was loaded into the Zymo-Spin™ IC Column containing M-Binding Buffer. The column was capped, and the contents were mixed by inverting the column several times and then centrifuged at full speed (> 10,000 × *g*) for 30 seconds. The flow-through was discarded. A volume of 100 μL M-Wash Buffer was added to the column. Then, the column was centrifuged at full speed for 30 seconds. M-Desulphonation Buffer (200 μL) was added to the column with subsequent incubaton at room temperature (20°C ~ 30°C) for 15 ~ 20 minutes.

After incubation, the column was centrifuged at full speed for 30 seconds. A volume of 200 μL M-Wash Buffer was added to the column. Then, the column was

centrifuged at full speed for 30 seconds. Another 200-µL aliquot of M-Wash Buffer was added and the column was centrifuged for an additional 30 seconds. The column was placed into a 1.5 mL microtube and an aliquot of 10 µL M-Elution Buffer was added directly to the column matrix. Then, the column was centrifuged for 30 seconds at full speed to elute the DNA. The DNA was stored at or below − 20°C for later use.

3.2.4 DNA quantification

The concentration of bisulfite-modified DNA was determined by Nanodrop 2000 Spectrophotometer (Thermo Scientific, DE, USA). "ssDNA-33" was chosen to measure the concentration of single-stranded DNA. The purity of eluted DNA was determined by A260/A280. Ratio around 1.8 was considered as pure for DNA.

3.2.5 MSP

3.2.5.1 Reagents

(1) HotStarTaq Plus DNA polymerase (5 U/µL), 10× PCR buffer (Tris-HCl, KCl, $(NH4)_2SO_4$, 15 mM $MgCl_2$: pH 8.7) and 25 mM $MgCl_2$ (Qiagen, Hilden, Germany).

(2) Deoxyribonucleoside triphosphates (dATP, dCTP, dGTP, dTTP each at 20 mM) (GE Healthcare, Buckinghamshire, UK).

(3) Primers for PCR ordered from Invitrogen (Life Technologies, Carlsbad, CA).

(4) Stock Tris-Borate-EDTA (TBE; 10×) buffer was prepared using the same protocol as Chapter 2.

(5) Working TBE buffer (0.5×) was diluted from 10× TBE buffer.

(6) Loading buffer (6×) was prepared using the same protocol as Chapter 2.

(7) 1 kb plus DNA Ladder (Life Technologies, Carlsbad, CA).

(8) SeaKem® LE Agarose (Lonza Group, Switzerland).

(9) Ethidium bromide, 20 μg/mL (Sigma, St. Louis, USA).

3.2.5.2 Equipment

(1) 96-well GeneAmp PCR system 9700 (Life Technologies, Carlsbad, CA).

(2) Electrophoresis tank (Jordan scientific Co., Bloominutesgton, USA).

(3) PowerPac™ Basic Power Supply (BioRad, Hercules, CA).

(4) Gel documentation system-Chemi Genious^2 (Syngene, Cambridge, UK).

3.2.5.3 Procedure

Design of primer was based on the DNA sequence of the candidate genes by MethPrimer (http://www.urogene.org/methprimer/) and OLIGO Primer Analysis Software (version 6.65, http://www.oligo.net/).

PCR amplification for five genes was carried out in a 15-μL reaction mixture consisting of 0.3 μM of each primer, 0.3 unit of HotStarTaq Plus DNA Polymerase, 0.2 mM of each dNTP (deoxyribonucleoside triphosphate), $MgCl_2$ (1.0 mM for *UCHL1*) and 1× PCR buffer. The primer sequences and PCR conditions for MSP were shown in **Table 3.2**. One μL (20 ng) of bisufite-modified DNA was added into each PCR reaction. PCR amplification was carried out in a 96-well 9700 thermal

cycler. PCR conditions were as follows: 95°C for 5 minutes; 35 cycles of 95°C for 30 seconds, specific annealing temperature (60°C for *UCHL1*; 55 °C for *DLEC1* and *DAPK*) for 30 seconds, and 72°C for 30 seconds; and a final 5 minutes extension at 72°C.

DNA extracted from leukocytes that were obtained from healthy individual and methylated in vitro by *SssI* methyltransferase was used as positive control for methylation-specific primers. Specifically, 14 μL nuclease-free water, 2 μL 10× NEBuffer 2, 2 μL diluted SAM (SAM 32 mM stock was diluted by adding 1 μL SAM 32 mM stock into 19 μL Nuclease free water), 1 μL genomic DNA (1 μg), 1 μL *SssI* methylase (4 U/μL) were mixed, and pipetted up and down at least six times. The mixture was incubated for one hour at 37°C. Reaction was stopped by heating at 65°C for 20 minutes. Leukocytes from healthy individual and molecular grade water were used as positive control for unmethylation-specific primers and negative control, respectively. The PCR products were visualized under UV transillumination in 2% agarose gel stained with ethidium bromide.

Table 3.2 Summary of primer sequences, annealing temperatures, cycles and product sizes for MSP in cell line

Gene	Forward primers(5'-3')	Reverse primer(5'-3')	Annealing temp (°C)	Cycles	Product size(bp)
DLEC1-M [a]	GAT TAT AGC GAT GAC GGG ATT C	ACC CGA CTA ATA ACG AAA TTA ACG	55	35	193
DLEC1-U	TGA TTA TAG TGA TGA TGG GAT TTG A	CCC AAC TAA TAA CAA AAT TAA CAC C	55	35	193
DAPK-M	GAT AGT CGG ATC GAG TTA ACG TC	CAA ATC CCT CCC AAA CGC CGA	55	35	102
DAPK-U	GGA GGA TAG TTG GAT TGA GTT AAT GTT	CAC AAA TCC CTC CCA AAC ACC AA	55	35	108
UCHL1-M	TTT ATT TGG TCG CGA TCG TTC	AAA CTA CAT CTT CGC GAA ACG	60	35	175
UCHL1-U	TTG TAT TTA TTT GGT TGT GAT TGT TT	AAC TTA AAC TAC ATC TTC ACA AAA CA	60	35	185

[a] M, primers for methylated DNA; U, primers for unmethylated DNA

3.2.6 RNA extraction

Total RNA from cell line was extracted with RNeasy Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. After determine the number of cells, culture medium was removed and cells were washed by 1× PBS. Then, cells were rinsed with 1.5 mL 0.25% trypsin and incubated in 37°C incubator for 4 minutes until cells detached. The status of cells was checked under phase microscope. A volume of 5 mL DMEM was added to inactivate trypsin. The cells were transferred to RNase-free polypropylene centrifuge tube, and centrifuged at 300 × *g* for 5 minutes. After completely aspirated the supernatant, the cell pellet was loosen thoroughly by flicking the tube. A volume of 350 µL Buffer RLT was added. The reaction mixture was vortexed or pipetted to mix. A volumn of 350 µL 70% ethanol was added to the homogenized lysate. The reaction mixture was vortexed or pipetted to mix. Up to 700 µL of the sample including any precipitate that may have formed was transferred to an RNeasy spin column placed in a 2-mL collection tube. The column was capped and centrifuged for 15 seconds at 8000 × *g*.

A volume of 700 µL Buffer RW1 was added to the RNeasy spin column. The column was capped and centrifuged for 15 seconds at 8000 × *g*. Next, a volume of 500 µL Buffer RPE was added to the RNeasy spin column. The column was capped and centrifuged for 15 seconds at 8000 × *g*. After that, another volume of 500 µL Buffer RPE was added to the RNeasy spin column. The column was capped and centrifuged for 2 minutes at 8000 × *g*.

The RNeasy spin column was placed in a new 2 mL collection tube. The column was capped and centrifuged at full speed for 1 minute. The RNeasy spin

column was placed in a new 1.5 mL collection tube. Then, a volume of 30 μL RNase-free water was added directly to the spin column membrane. The column was capped and centrifuged for 1 minute at 8000 × *g* to elute the RNA. The extracted RNA was stored at − 20°C.

3.2.7 RNA quantification

The concentration of extracted RNA was determined by Nanodrop 2000 Spectrophotometer. "RNA-40" was chosen to measure the concentration of RNA. The purity of eluted RNA was determined by A260/A280. Ratio around 2.0 was considered as pure for RNA.

3.2.8 cDNA synthesis

cDNA was synthesized by Transcriptor High Fidelity cDNA Synthesis Kit (Roche, Basel, Switzerland) following the manufacturer's protocol.

All frozen reagents were thawed and centrifuged before experiment. The template-primer mixture for one 20 μL reaction was prepared by adding the components in the order listed in **Table 3.3** in a sterile, nuclease-free, thin-walled PCR tube. Then, the template-primer mixture was denatured by heating the tube for 10 minutes at 65°C in a block cycler and immediately cooled on ice. The remaining components of the RT mix were added in the order listed in **Table 3.4**. After mix the reagents mixed carefully, the tube was placed in a block cycle and incubated for 10 to 30 minutes at 45°C to 55°C. Transcriptor High Fidelity reverse Transcriptase was

inactivated by heating to 85°C for 5 minutes. Then, the reaction was stopped by placing the tube on ice. The synthesized cDNA was stored at − 20°C.

Table 3.3 Preparation of Template-Primer Mix

Component	Volume
Total RNA (up to 4 µg)	Variable
Anchored-oligo$(dT)_{18}$ primer, 50 pmol/µL	1 µL
Water, PCR-grade	Variable
Total volume	11.4

Table 3.4 Preparation of RT Mix

Component	Volume
Transcriptor High Fidelity Reverse Transcriptase Reaction Buffer, 5× conc.	4 µL
Protector RNase Inhibitor, 40 U/µL	0.5 µL
Deoxynucleotide Mix, 10 mM each	2 µL
DTT	1 µL
Transcriptor High Fidelity Reverse Transcriptase	1.1 µL
Final Volume	20 µL

3.2.9 cDNA quantification

The concentration of bisulfite-modified DNA was determined by Nanodrop 2000 Spectrophotometer. "ssDNA-33" was chosen to measure the concentration of single-stranded DNA. The purity of eluted DNA was determined by A260/A280. Ratio around 1.8 was considered as pure for DNA.

3.2.10 RT-PCR

3.2.10.1 Reagents

(1) HotStarTaq Plus DNA polymerase (5 U/μL), 10× PCR buffer (TrisCl, KCl, $(NH_4)_2SO4$, 15 mM $MgCl_2$: pH 8.7) and 25 mM $MgCl_2$ (Qiagen, Hilden, Germany).

(2) Deoxyribonucleoside triphosphates (dATP, dCTP, dGTP, dTTP each at 20 mM) (GE Healthcare, Buckinghamshire, UK).

(3) Primers for PCR ordered from Invitrogen (Life Technologies, Carlsbad, CA).

(4) Stock Tris-Borate-EDTA (TBE; 10×) buffer was prepared using the same protocol as Chapter 2.

(5) Working TBE buffer (0.5×) was diluted from 10× TBE buffer.

(6) Loading buffer (6×) was prepared using the same protocol as Chapter 2.

(7) 1kb plus DNA Ladder (Life Technologies, Carlsbad, CA).

(8) SeaKem® LE Agarose (Lonza Group, Switzerland).

(9) Ethidium bromide, 20 μg/mL (Sigma, St. Louis, USA).

3.2.10.2 Equipment

(1) 96-well GeneAmp PCR system 9700 (Life Technologies, Carlsbad, CA).

(2) Electrophoresis tank (Jordan scientific Co., Bloominutesgton, USA).

(3) PowerPac™ Basic Power Supply (BioRad, Hercules, CA).

(4) Gel documentation system-Chemi Genious2 (Syngene, Cambridge, UK).

3.2.10.3 Procedure

Design of primer was based on the DNA sequence of the candidate genes by OLIGO Primer Analysis Software (version 6.65, http://www.oligo.net/).

PCR amplification for *DLEC1*, *DAPK*, *UCHL1* and *GAPDH* was carried out in a 15-μL reaction consisting of each primer (0.5 μM for *DLEC1* and *DAPK*, 0.3 μM for *UCHL1* and 0.1 μM for *GAPDH*), 0.3 unit of HotStarTaq Plus DNA Polymerase (Qiagen), 0.2 mM of each dNTPs, $MgCl_2$ (1.0 mM for *UCHL1* and *GAPDH*) and 1× PCR buffer. The primer sequences and PCR conditions for RT-PCR were shown in **Table 3.5**. One μL (20 ng) of cDNA was added into each PCR reaction. PCR amplification was carried out in a 96-well plate 9700 thermal cycler. PCR conditions were as follows: 95°C for 5 minutes; 27 cycles of 95°C for 30 seconds, specific annealing temperature (60°C for *DAPK* and *UCHL1*; 55°C for *DLEC1*, *GAPDH*) for 30 seconds, and 72°C for 30 seconds; and a final 5 minutes extension at 72°C. *GAPDH* gene was used as internal leading control and molecular grade water were used as blank control, respectively. The PCR products were visualized under UV transillumination in 2% agarose gel stained with ethidium bromide.

3.2.11 Statistical analysis

Cell viability between three groups of cells treated with 0, 1 and 5 μM Aza was analyzed using one way-ANOVA. All statistical analyses were performed using SPSS version 16.0, $P < 0.05$ was considered as statistically significant difference.

Table 3.5 Summary of primer sequences, annealing temperatures, cycles and product sizes for RT-PCR

Gene	Forward primers(5'-3')	Reverse primer(5'-3')	Annealing temp (°C)	Cycles	Product size(bp)
DLEC1	CAG AAG CAG GAG TGT GAG GAG	CCC ATC AGC ATA GTC CAG TAG C	55	27	392
DAPK	TTT CGG TCT GTC CTC CCA TGA	CTG CAC GTC GTA CAC AAA CTG	60	27	122
UCHL1	GAA GGC CAA TGT CGG GTA GAT	AAG GGA AGA GGG GAA ATC AGC	60	27	269
GAPDH	CGG AGT CAA CGG ATT TGG TCG TAT	AGC CTT CTC CAT GGT GGT GAA GAC	55	27	308

3.3 Results

3.3.1 Cell morphology change after Aza treatment

Images of cell morphology changes were taken on the fourth day of Aza treatment before sodium bisulfite treatment and RNA extraction (**Figure 3.3**). In these images, the number of cells decreased, cells shrunk and appeared flattened and elongated with increasing Aza concentration. The observed changes in cell morphology became more pronounced with increasing Aza concentration. This suggested that there were some alterations in genetic or epigenetic regulation of cell growth.

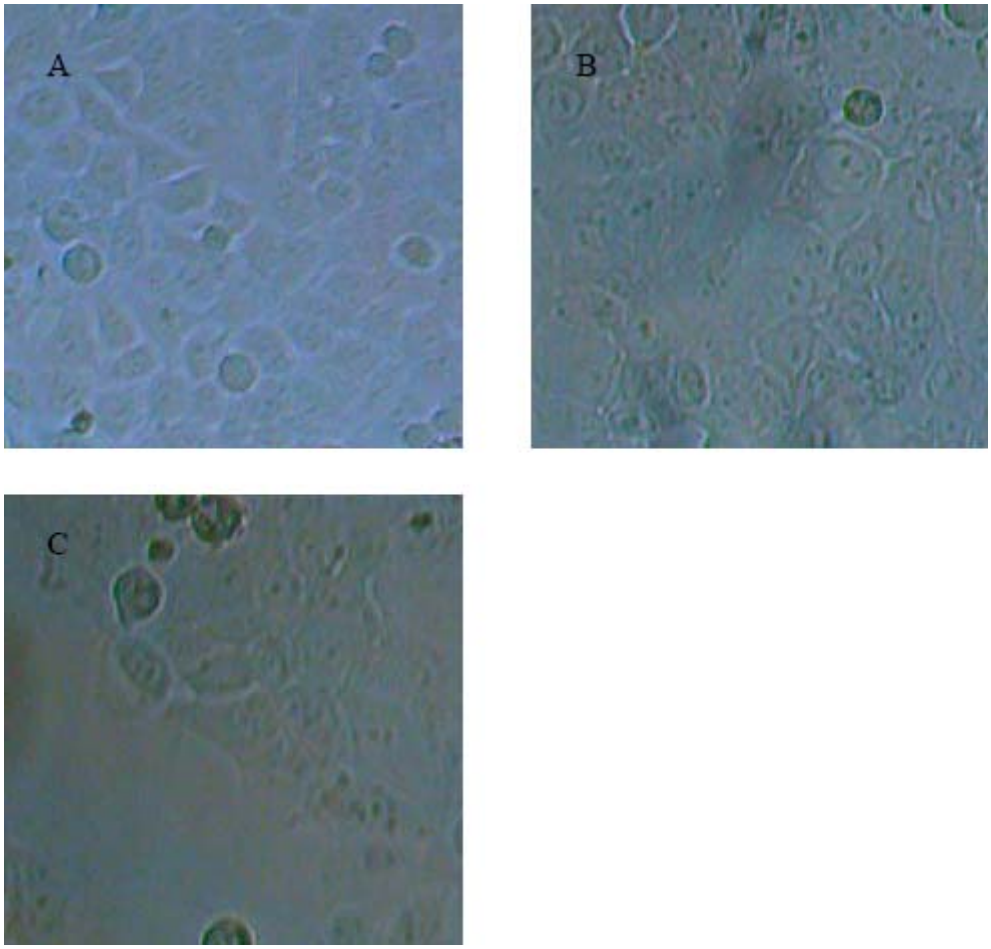

Figure 3.3 Cell morphology change after Aza treatment (100×)

A, cells treated with 0 μM Aza; B, cells treated with 1 μM Aza; C, cells treated with 5 μM Aza.

3.3.2 Cell viability test

The cell number was determined with the use of trypan blue stain. Cell viability equals to the number of live cells (non-stained) divided by the total cell number and multiplied by one hundred percent. From the result of one-way ANOVA, no significant difference of cell viability was found among 0 μM, 1 μM and 5 μM Aza treated cells ($P > 0.05$) (**Figure 3.4**).

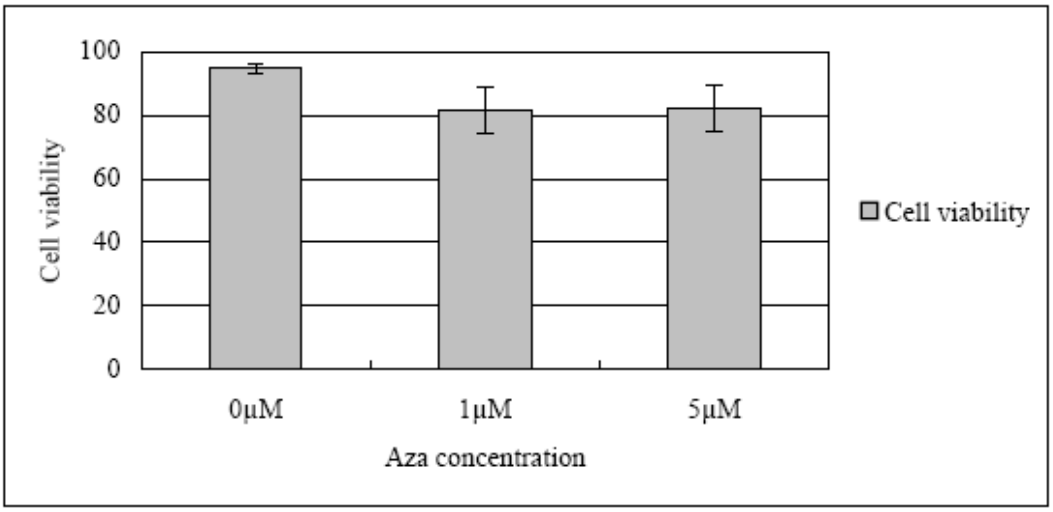

Figure 3.4 Results of cell viability test under different Aza concentrations.

3.3.3 MSP

The MSP results revealed that the level of methylation of *DLEC1*, *DAPK* and *UCHL1* gradually decreased and the level of demethylation gradually increased with increasing Aza concentration (**Figure 3.5**).

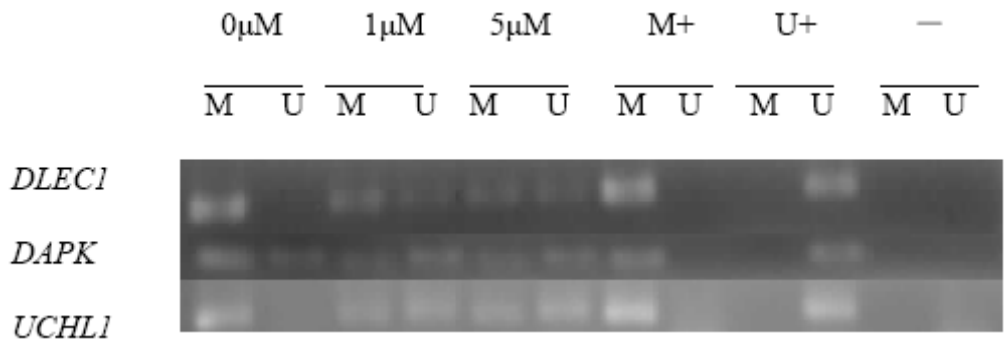

Figure 3.5 MSP results for *DLEC1*, *DAPK* and *UCHL1*

M+, positive control for methylated primers; U+, positive control for unmethylated primers; —, blank control.

3.3.4 RT-PCR

Transcription of *GAPDH* did not change in 0, 1 and 5 μM Aza-treated cells, indicating that gene transcription of 0, 1 and 5 μM Aza-treated cells were comparable. Transcription of *DLEC1*, *DAPK* and *UCHL1* was gradually up-regulated with increasing Aza concentration (**Figure 3.6**).

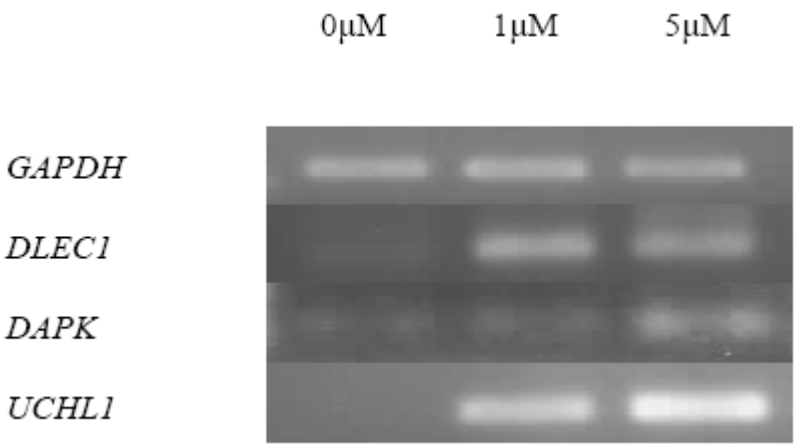

Figure 3.6 RT-PCR result for *DLEC1*, *DAPK* and *UCHL1*

3.4 Discussion

It is known that the inactivation of tumor suppressor genes play a key role in the initiation and progression of tumors (Lo and Huang, 2002). Based on the fact that tumor suppressor genes *DLEC1*, *DAPK* and *UCHL1* were frequently methylated in the primary tumor of NPC (Kwong et al., 2007; Kwong et al., 2002; Li et al., 2010), we hypothesized that the aberrant hypermethylation of gene promoter may inhibit the transcription of tumor suppressor genes in NPC. This study investigated the association of DNA hypermethylation with transcriptional silencing of *DLEC1*, *DAPK* and *UCHL1* in NPC cell line HNE1. The results from this study may lay a foundation for future exploration on the roles of *DLEC1*, *DAPK* and *UCHL1* in the development of NPC.

In this study, the effect of Aza on the methylation status of the promoter region of *DLEC1*, *DAPK* and *UCHL1* has been detected in NPC cell line HNE1. After Aza treatment, morphology changes were observed in HNE1 cells. With the increase of Aza concentration, these morphology changes became more pronounced. There was a decrease of cell number in Aza treated cells, but no significant difference in cell viability between cells treated with Aza of different concentrations was found. Since Aza is a demethylation reagent, the changes on cell morphology might result from the demethylation of the promoter regions of the genes. To test this hypothesis, MSP was done to explore the change of methylation status for the three genes in HNE1. Results showed that Aza could effectively demethylate the promoter region of *DLEC1*, *DAPK* and *UCHL1* in a concentration-dependent manner. We also analyzed the transcription of these three genes by RT-PCR to test whether the changes of promoter methylation could lead to the changes of gene transcription. Results

showed that with increasing Aza concentration, the transcription level of these three genes was gradually increased, suggesting that there might be a relationship between DNA methylation and transcriptional silencing.

This study might be improved from the following aspects. First, the pictures of cells could be taken in greater detail. So the changes on cell remembrance, cytoplasm and nuclear could be observed more clearly. Secondly, real-time PCR could be performed to detect the changes in methylation status of the three genes. Although a decreasing tendency of promoter methylation was identified by MSP, the more sensitive and reproducible real-time PCR might provide better results for analyzing the subtle changes in promoter methylation. Taking pictures for DNA products running on polyacrylamide gel stained with SYBR Green instead of agarose gel stained with ethidium bromide could improve the resolution of the picture.

Our results only indicated that DNA hypermethylation might contribute to the transcriptional inactivation of *DLEC1*, *DAPK* and *UCHL1* in undifferentiated NPC cell line HNE1. The methylation status of *DLEC1*, *DAPK* and *UCHL1* could be examined in other NPC cell lines. To further investigate the roles of *DLEC1*, *DAPK* and *UCHL1* in NPC carcinogenesis, over-expression vectors carrying relevant genes could be constructed and transfected into cell line HNE1. As proliferation and metastasis are important factors in evaluating the biological behavior of tumor cells, cellular proliferation essay and invasion essay could be performed in transfected HNE1 cell line. The expression of *DLEC1*, *DAPK* and *UCHL1* could be measured by RT-PCR after transfection. Western blot could also be performed to determine the amount of proteins expressed. Co-Immunopreciptation may be performed to identify

other proteins that might interact with *DLEC1*, *DAPK* and *UCHL1*. Other functional studies may be conducted based on the results of Co-Immunopreciptation.

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Chapter 4: Conclusions

The concentration of cell-free circulating DNA in the serum of nasopharyngeal carcinoma patients was significantly higher than that in normal controls. The five tumor suppressor genes *RASSF1A*, *CDKN2A*, *DLEC1*, *DAPK* and *UCHL1* selected in our study were found to be methylated in 17.5%, 22.5%, 25%, 51.4% and 64.9% patients, respectively. The combination of four-gene biomarker -*CDKN2A*, *DLEC1*, *DAPK* and *UCHL1*- had the highest sensitivity and specificity in predicting NPC. Our results suggested that screening of DNA hypermethylation of tumor suppressor genes in serum may be a promising approach for the diagnosis of nasopharyngeal carcinoma. These results may provide a useful reference in the detection and treatment of NPC in clinical practice.

After Aza treatment, the methylation level of *DLEC1*, *DAPK* and *UCHL1* decreased with increasing Aza concentration. However, the transcription of *DLEC1*, *DAPK* and *UCHL1* increased with increasing Aza concentration. Promoter hypermethylation could lead to transcriptional silencing of *DLEC1*, *DAPK* and *UCHL1* in nasopharyngeal carcinoma cell line HNE1.

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Appendices

Appendix Ⅰ TNM classification of nasopharyngeal carcinoma (2010)

Classification		Description
Nasopharynx (T)	T1	Tumor confined to nasopharynx, with or without extension to oropharynx, nasal cavity but without parapharyngeal extension
	T2	Tumor with parapharyngeal extension
	T2a	Tumor extension to oropharynx and/or nasal cavity without parapharyngeal extension
	T2b	Tumor with parapharyngeal extension
	T3	Tumor invades bony structures of skull and/or paranasal sinuses
	T4	Tumor with intracranial extension and/or involvement of cranial nerves, infratemporal fossa, hypopharynx, orbit or masticator space
Regional lymph nodes (N)	N1	Unilateral cervical, unilatered or bilateral retropharyngeal lymph node(s), 6cm or less in greatest dimension, above supraclavicular fossa
	N2	Bilateral cervica lymph nodes, ≤ 6cm in greatest dimension, above supraclavicular fossa
	N3	Metastasis in lymph node(s), > 6cm in greatest dimension (N3a) or in the supraclavicular fossa (N3b)
Distant Metastasis (M)	M0	No distant metastasis
	M1	Distant metastasis

AJCC stage group (2010)

Staging	T	N	M
Stage 0	T in situ	N0	M0
Stage I	T1	N0	M0
Stage II	T1	N1	M0
	T2	N0. N1	M0
Stage III	T1,T2	N2	M0
	T3	N0, N1, N2	M0
Stage IVA	T4	N0, N1, N2	M0
Stage IVB	Any T	N3	M0
Stage IVC	Any T	Any T	M1

Appendix Ⅱ Consent form and information sheet

THE HONG KONG
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Research Study Consent Form

Project Title: A study on the hypermethylation of tumour suppressor genes (TSG) in nasopharyngeal carcinoma (NPC)

I, _____ (HKID No._____), hereby consent to take part in the above study.

I confirm that I have read and understood the information sheet for the above study and have had the opportunity to ask questions.

I understand that the data of the study will be kept in a safe place and will only be accessed by the investigators. I also realise that the data will be kept for one more year after the completion of study for producing research reports and publications.

I realise that I can withdraw from the study at any time, and may contact the Secretary of the Human Subjects Ethics Sub-committee of The Hong Kong Polytechnic University with any enquiries or complaints.

_____	_____
Investigator's Signature	Patient's Signature
_____	_____
Investigator's Name	Patient's Name
_____	_____
Date	Date

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香港理工大學

II. 研究同意書

研究題目：鼻咽癌腫瘤抑制基因的甲基化研究

本人_____(香港身分證號碼:_____)茲同意接受此上述研究。

我已詳細閱讀這份研究資料及同意書，並且已有足夠的時間對這項研究提出詢問。

本人知道此研究之數據將會存放在一安全地方及只會給此研究之研究員使用。本人也明悉研究數據將會在此研究結束後儲存一年作選寫研究報告之用。

本人更明悉，本人有權隨時拒絕接受此研究和向香港理工大學道德委員會祕書提出咨詢或投訴。

_____	_____
研究員簽署	病人簽署
_____	_____
研究員姓名	病人姓名
_____	_____
日期	日期

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POLYTECHNIC UNIVERSITY
香港理工大學

Research Study Information Sheet

Title of Project:
A study on the hypermethylation of tumour suppressor genes (TSGs) in nasopharyngeal carcinoma (NPC)

Principle Investigator:
Dr. Vincent Wing-Cheung Wu
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Tel: 2255 4519

Aims of the Project:

The objectives of this study are:

a) To evaluate the potential of single and panels of methylated TSGs in early detection of NPC in serum
b) To verify the tumour suppression role of methylated TSGs in NPC cell line by functional studies.

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What do volunteers for the study have to do?

If you volunteer for the study, you will be asked:

1. to sign an informed consent form that states you understand the information presented on this sheet and willing to participate in this study
2. to give some personal information (such as name, age) and information of your medical history. All information, results, and data will be kept confidential, and will only be accessed by the investigators
3. to donate 3 ml blood for DNA analysis and related studies

Duration of examination

The blood extraction will take about 5 minutes.

Any medication involves in the study?

No

Is there any discomfort during the blood extraction?

No. The potential hazards include pain and occasional bruising following venepuncture, but without serious consequences.

Can a volunteer withdraw from the study?

Yes, you can stop participating in the study at any time with no penalty.

Any benefit to the volunteer?

There is no intended clinical benefit to you from taking part in this study. Your participation will generate useful data for identifying patients with high risk of suffering the same disease and provide better patient care to these patients in future.

Can I get more information on the study?

Yes, contact Dr. Vincent Wu and he will try to answer any questions you may have.

This study was approved by the Ethics Sub-Committee, Department of Health Technology and Informatics, the Hong Kong Polytechnic University. However, if you think there are any procedures that seem to be unethical, please do not hesitate to contact the Secretary of the Human Subjects Ethics Sub-committee of the Hong Kong Polytechnic University in writing c/o Human Resources Office of the University.

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研究資料詳情及同意書

研究題目：
鼻咽癌腫瘤抑制基因的甲基化研究

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研究目的：

研究旨在探討將血清中腫瘤抑制基因的甲基化檢測用于鼻咽癌早期診斷的可能性及某些腫瘤抑制基因在鼻咽癌中的功能分析。

自願參加者需要怎樣做？

如你願意參加這項研究，你將會需要：

1. 簽署一張研究同意書以表示你明白本研究資料詳情和願意參加這項研究。

2. 提供一些個人資料，所有資料和研究結果將會保密和只許上列研究員參閱。

3. 接受一次抽血化驗(大約3毫升血液)作爲有關你的基因分析及有關的研究。

所需時間

整個抽血過程大概需時5分鐘。

這項研究需要使用藥物嗎？

不需要。

抽血會引起身體不適嗎？

不會，但有機會引致疼痛，及因靜脈穿刺後之瘀傷，但不會造成嚴重後果。

參加者可否中途退出這項研究計劃？

你可以隨時退出這項研究而不會有任何懲罰。

參加者會否得到任何利益?

您並不會因參與研究而得到直接利益。這個研究將有利於研究人員在未來找出高危患者並在作出供更好的醫護服務。

我可以得到更多此項研究的資料嗎？

你可以聯絡胡永祥博士。他會盡量解答你的問題。

本研究是得到香港理工大學醫療科技及資訊學系道德委員會批準。如你覺得任何程序侵犯你的個人權益，你可以書面向香港理工大學道德委員會祕書提出投訴及轉交理工大學人力資源部。

Appendix Ⅲ Correlations analysis (Spearman's rho)

		RASSF1A	*CDKN2A*	*DLEC1*	*DAPK*	*UCHL1*
RASSF1A	Correlation Coefficient	1.000	-0.013	0.273*	0.049	0.116
	Sig. (2-tailed)	.	0.906	0.013	0.664	0.302
	N	81	81	81	81	81
CDKN2A	Correlation Coefficient	-0.013	1.000	-0.062	0.108	0.234*
	Sig. (2-tailed)	0.906	.	0.583	0.336	0.035
	N	81	81	81	81	81
DLEC1	Correlation Coefficient	0.273*	-0.062	1.000	0.035	0.128
	Sig. (2-tailed)	0.013	0.583	.	0.753	0.254
	N	81	81	81	81	81
DAPK	Correlation Coefficient	0.049	0.108	0.035	1.000	0.301**
	Sig. (2-tailed)	0.664	0.336	0.753	.	0.006
	N	81	81	81	81	81
UCHL1	Correlation Coefficient	0.116	0.234*	0.128	0.301**	1.000
	Sig. (2-tailed)	0.302	0.035	0.254	0.006	.
	N	81	81	81	81	81

*. Correlation is significant at the 0.05 level (2-tailed).

**. Correlation is significant at the 0.01 level (2-tailed).