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EPIGENETICS IN NASOPHARYNGEAL CARCINOMA

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To my dearest parents

LIST OF PUBLICATIONS

I. **Di Sun***, Zhe Zhang*, Do Nguyen Van, Ingemar Ernberg, Guangwu Huang, Lifu Hu.
Aberrant methylation of CDH13 gene in nasopharyngeal carcinoma could serve as a potential diagnostic biomarker. Oral Oncol. 2006 Jun 24; [Epub ahead of print] PMID: 16807071

II. Zhe Zhang*, **Di Sun***, Do Nguyen Van, Anzhou Tang, Lifu Hu, Guangwu Huang.
Inactivation of RASSF2A by promoter methylation correlates with lymph node metastasis in nasopharyngeal carcinoma. Int J Cancer. 2007 Jan 1;120(1):32-8

III. **Di Sun**, Xiangning Zhang, Do Nguyen Van, Ingemar Ernberg, Lifu Hu
Epigenetic regulation of EBV-encoded latent membrane protein 1 expression in nasopharyngeal carcinoma. (Manuscript)

IV. **Di Sun***, Zhe Zhang*, Do Nguyen Van, Ingemar Ernberg, Guangwu Huang, Lifu Hu
Development of a non-invasive method, multiplex methylation specific PCR (MMSP), for early diagnosis of nasopharyngeal carcinoma (Manuscript)

* Authors contribute equally

ABSTRACT

Nasopharyngeal carcinoma (NPC) shows a remarkably distinctive ethnic and geographic distribution among the world, suggesting that genetic susceptibility backgrounds cooperate with environmental risk factors in the etiology of NPCs. Among the environmental factors, much attention has been focused on a well-documented association with Epstein-Barr virus (EBV), which can be detected in 100% undifferentiated NPCs. During the passing decade, increasing evidence has shown that epigenetic changes play an important role at the early stage of carcinogenesis. Epigenetic changes could also serve as a surrogate for mutations or chromosomal alterations in inactivating tumor suppressor genes (TSGs) in the tumor development. In the present work, we mainly focused on the study of methylation of NPC-related genes, including cellular TSGs and EBV-encoded gene, which may unravel the molecular basis of its tumorigenesis and thereby expand the prospects for the development of diagnostic and prognostic markers.

First, CDH13, which encodes cell adhesion molecule H-cadherin, was found to be methylated in 89.7 % of NPC primary tumors, while only methylated in 10% normal nasopharyngeal epithelia ($p<0.05$). The potential of detecting methylation from nasopharyngeal swabs was then investigated. The high sensitivity (81%) and specificity (0% false positives) of detecting CDH13 methylation from nasopharyngeal swabs suggests it could be utilized as a tentative auxiliary tool for early diagnosis. We next explored another candidate TSG, RASSF2, which can bind directly to K-Ras and function as a negative effector of Ras protein. Promoter methylation of one of RASSF2 isoform, RASSF2A, could be detected in 80% NPC cell lines and 50.9% of primary tumors, but not in any of the normal epithelia. RASSF2A expression was found to be correlated with its promoter methylation. In addition, patients with methylated RASSF2A presented a higher frequency of lymph node metastasis ($p<0.05$). In characterizing the tumor suppressor function of RASSF2A in NPC, we found that RASSF2A could induce cell cycle arrest, and inhibit colony formation and cell migration, which provided further evidence for the tumor suppression function of RASSF2A.

The EBV-encoded latent membrane protein 1 (LMP1) is expressed in about 65% of NPC. In this study, how LMP1 expression is epigenetically regulated in NPC cells was investigated. Our results show that LMP1 promoter was heavily methylated in LMP1-silenced NPCs, while free of methylation in LMP1-expressing NPCs. Methyltransferase inhibitor 5-aza-dC could induce LMP1 mRNA and protein expression in NPC cell line C666-1. Although histone deacetylase inhibitor TSA relieved the repression by deacetylated histones at LMP1 promoter, LMP1 expression was not induced by treating with TSA alone. A synergistic effect on inducing LMP1 expression was observed after treating cells with 5-aza-dC plus TSA, suggesting both epigenetic mechanisms play a role in repressing LMP1 expression. Cytokine IL-4 failed to induce LMP1 expression in C666-1 cells in which LMP1 promoter is heavily methylated. A 15-fold induction on LMP1 expression was achieved by treating cells with 5-aza-dC and IL4 together, compared with 5-aza-dC alone. It suggests that the presence of methylation on LMP1 promoter prevent the transctivation of LMP1 by IL4.

Aiming at developing early diagnostic or prognostic tools for NPC, we developed a powerful technique “multiplex methylation specific-PCR (MMSP)”. MMSP was designed to detect tumor-specific methylation status of several NPC-related genes. It is capable of acquiring multiplex information simultaneously by just single PCR reaction with the tiny tumor DNA derived from nasopharyngeal swabs. This MMSP assay is sensitive enough to detect multiplex information from as few as 10 cells. Among the sixty-nine samples (49 NPCs and 20 normal controls), the detection rate of NPC from NP swabs is 97.9%. The false positive rate of MMSP in detecting NPC is zero.

Key words: NPC, Methylation, TSG, EBV, LMP1, CDH13, RASSF2A, MMSP

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LIST OF ABBREVIATIONS

BL	Burkitt Lymphoma
bp	Base pair
ChIP	Chromatin immunoprecipitation
CpGs	Cytosine-guanosine dinucleotides
CTCF	CCCTC-binding factor
DAP-kinase	Death-associated protein kinase
DNMT	DNA methyltransferase
EA	Early antigen
EBERs	EBV-encoded nuclear RNAs
EBNA1	Epstein-Barr nuclear antigen
EBV	Epstein-Barr Virus
FACS	Fluorescence-Activated Cell Sorting
HAT	Histone acetylase
HDAC	Histone deacetylase
HL	Hodgkin disease
HLA	Human leukocyte antigen
ICR	Imprinting control region
IM	Infectious mononucleosis
LMP1	Latent Membrane protein 1
LOD	Logarithm of the odds
LOH	Loss of heterzygosity
LOI	Loss of imprinting
LRS	LMP1 regulatory sequence
MeCP	Methylcytosine binding protein
MMSP	Multiplex Methylation Specific PCR
MSP	Methylation Specific PCR
NPC	Nasopharyngeal carcinoma
PCR	Polymerase Chain Reaction
RASSF	Ras Association Domain Family
Rb	Retinoblastoma gene
RT	Reverse transcription
SCC	Keratinizing squamous cell carcinoma
SIR	Standardized incidence ratio
SNP	Single nucleotide polymorphism
TSG	Tumor Suppressor Gene
VCA	Viral capsid antigen
WHO	World health organization

1 AIMS OF THE STUDY

Epigenetic changes as an important way of gene transcription regulation have received more and more attention during the past several decades. It is widely accepted that altered epigenetic events could contribute to the carcinogenesis process by disrupting genome stability and aberrantly inactivating tumor suppressor genes (TSGs).

To investigate how differentially methylated cellular or Epstein Barr virus (EBV)-encoded genes may contribute to the development of nasopharyngeal carcinoma (NPC), we did research in the following aspects:

1. Discovering aberrantly methylated cellular genes in NPC tumors and characterizing its properties as a candidate TSG.
2. Exploring the epigenetic mechanism in transcription regulation of EBV encoded oncoprotein, latent membrane protein 1(LMP1), in NPC.
3. Developing a non-invasive diagnosis tool for NPC based on its gene methylation profile.

2 INTRODUCTION

2.1 GENERAL CHARACTERISTICS OF NPC

2.1.1 Anatomy and histopathology

NPC is a tumor arising from the epithelial cells covering the nasopharynx surface. The histological classification of NPC proposed by World Health Organization (WHO) categorized tumors into three histopathological types[1]. Type I: keratinizing squamous cell carcinoma (SCC). Type II: nonkeratinizing carcinoma. Type III: undifferentiated carcinoma. Types II and III carcinoma may also be termed lymphoepithelioma, since they are usually accompanied by infiltration of lymphocytes, plasma cells, and esoinophils. There is different prevalent histological subtype of NPC in non-endemic and endemic regions. In the non-endemic western countries such as U.S., type I keratinizing squamous cell carcinomas comprised 75% of the NPC cases and were found most often in U.S.-born, non-Hispanic whites[2]. Whereas in endemic areas such as southern China, WHO Type III accounts for more than 97% of all the NPCs[3]. WHO types II and III can be considered together as undifferentiated carcinoma of the nasopharyngeal type (UCNT)[4]. The histological types may be of prognostic significance. WHO types II and III patients demonstrated a higher local control rate after treatment with radiotherapy than type I patients[5].

2.1.2 Epidemiology and etiology

For most countries of the world, NPC is an uncommon disease. In the western world, the standard age-adjusted incidence of NPC for both male and female is less than one person per 100000 population[6]. However, it is very frequent in southern China where it is the third most common malignancy amongst male. High-risk provinces include Guangdong, Guangxi, Hunan and Fujian, in which incidence is between 15 and 50 per 100000. However, in the northern part of China, the incidence rates were shown to be only 2-8/100000. Males have always had a higher incidence rate than females. In a recent study in Hong Kong population, the incidence of NPC is 2.5-2.6 fold higher in males than in females. Furthermore, the mortality of male is 3.0-3.5 folds higher[7].

The incidence rate among Chinese people born in North America is lower than in those born in southern China, but still remain high than the local[8, 9]. These findings indicate that genetic, ethnic, and environmental factors could play a role in the etiology of NPC. Among the environmental factors, much attention has been focused on a well-documented association with EBV, which presents in 100% of NPC cells. In the following chapters, the role of genetic factors and EBV infection in the development of

NPC will be illustrated in detail. Here the environmental factors in NPC tumorigenesis are described.

Ingestion of salted fish and other preserved foods containing volatile nitrosamines, especially during childhood is an important carcinogenic factor for NPC[10]. The carcinogenic potential of salted fish is supported by animal studies. Malignant nasal and nasopharyngeal tumors could be induced in rats after feeding salted fish[11, 12]. In Chinese populations, daily consumption of salted fish has been shown to be related to an increased risk of NPC for 1.8-7.5 folds [13]. Herbal medicine use is suggested to be linked to NPC either through its ability to reactivate the EBV or through a direct promoting effect on EBV-transformed cells[14, 15]. Evidence for other factors such as smoking or formaldehyde is weak in the etiology of NPC.

2.1.3 Clinical presentation, diagnosis and therapy

NPC shows a remarkably high cure rate for early-stage disease. Early detection is critical to improve the overall prognosis of these patients. However, NPC is one of the most difficult diseases to be diagnosed at an early stage since it has few early warning signs. Earliest symptoms such as nose bleeding, nasal blockage or stuffiness with bloody drainage, or serious otitis media may frequently be ignored. Bilateral and bulky neck mass is the initial presentation for approximately 70% of patients. Other common symptoms including hearing loss, facial pain, difficulty with opening the mouth, and blurred or double vision, are complications of cranial nerve paralyses when tumor invades into skull base. Histological examination of nasopharyngeal biopsy is the classical standard for NPC diagnosis in clinic. Nevertheless, its invasiveness makes it not suitable as an early screening means in NPC high-risk population.

EBV-specific antibody-based assays such as serum titers of IgA antibodies to viral capsid antigen (VCA), early antigen (EA), nuclear antigen (EBNA) and DNase assay have been commonly used for at least twenty years as standard markers for screening and monitoring the disease. However, the sensitivity and specificity of this serological method are known to be not satisfied[16]. EBV viral load measurement in serum or plasma has been shown to be useful for diagnosis and monitoring in patients affected by NPC. By measuring the BamH1W reiterated DNA in the serum or plasma using quantitative real-time PCR method, it showed an impressively high sensitivity. EBV DNA levels in plasma or serum can reflect tumor stage and overall survival. Furthermore, they are correlated with the residual tumor load after treatment and accurately predict recurrence and prognosis[17-19]. Cell-free EBV DNA becomes one of the potential diagnostic and prognostic molecular markers for NPC. However, most

of these results were based on patients, the predict value in the high-risk populations remains to be explored.

NPC is a relatively radiosensitive tumor and therefore radiotherapy remains the standard treatment for almost all NPC patients. The fields for radiotherapy targeting to the primary tumor and the surrounding anatomical structures at risk of tumor invasion were defined clinically by radiographic imaging. Some pathological types of NPC (WHO types II and III) have been shown to be chemosensitive in all stages of the disease[20]. Several studies have reported the results of the use of chemotherapy in combination with radiotherapy for the management of locoregional advanced cases of nasopharyngeal carcinoma. In spite of encouraging results in terms of response rates, the survival rates remained disappointing[21]. The presence of EBV in tumor cells provides the possibility of targeting EBV-specific therapy for NPC. EBV-targeting immunotherapeutic or gene therapeutic strategies have been attempted [22, 23].Currently several strategies that targeting EBNA1 and the EBV relative oncogene LMP1 for the control of NPC are under investigation.

2.2 NPC AS A GENETIC DISEASE

Cancer has been widely accepted as a genetic disease with inheritable genetic alterations. Among all the tumors, only 5-10% is familial cancers and is caused due to the altered germ line cell inherited from their parental generations. The genes which have a causing role for the familial cancer are always important since they are also implicated in their corresponding sporadic cancers. Most of the cancer are sporadic and are caused by environmental factors. Thus the genetic changes were only found in tumor tissue itself. The progression of tumor to malignancy is a multistage process, involving several genetic events occurred on the same cell. It usually takes many years to accumulate all these changes in one cell, that's why cancer is an age-related disease. Genetic changes like mutations, deletions and amplifications contribute to the development of cancer by deregulating the expression of a group of genes which are key regulators in many cell growth-related pathways, *e.g.* cell cycle, apoptosis, invasion and DNA damage repair. The genes related to cancer cell growth are usually classified into two groups: proto-oncogene and TSG. Proto-oncogenes encode proteins which are key components of cell signaling pathways. Mutations in proto-oncogenes acquire a gain of its function in accelerating cell proliferation. The mutation in proto-oncogene is usually dominant and disruption in anyone of the two alleles will result in deregulated cell signaling pathway. In contrast, TSGs are recessive and can only be silenced when both alleles were destroyed. TSG can inhibit cell growth by blocking its progression through cell cycle and by blocking differentiation.

2.2.1 Familial aggregation of NPC

NPC has no recognizable genetic basis while it shows a clear tendency of clustering in families. It is therefore thought that the underlying genetic susceptibility to environmental carcinogens determines the initiation of this tumor. Epidemiological studies have confirmed that the proportion of familial cases on NPC patients in Southern China is significantly higher than that in low-risk areas. Approximately 5-10% of NPC patients have a familial history in this endemic region[24]. The incidence of NPC is still high in the South Chinese population even after emigration from China. Offsprings of mixed marriages between southern Chinese and non-Chinese groups show an intermediate incidence[6]. The relative risk of the first-degree relatives of the NPC patients is 8 times (in Greenland) or 2.09 times (in Cantonese population) of that of the general population in the respective endemic regions, while it drops down as the degree of relative increases[25, 26]. If he/she was affected by NPC in family, the values of standardized incidence ratios (SIRs) for the father, mother, brother or sister were 1.46, 2.76, 2.17, and 2.91, respectively[26].

2.2.2 Susceptibility loci for NPC

Familial aggregations of the disease, together with the findings of migrant studies and case-control studies favor the idea that a genetic susceptibility exists in the endemic region to NPC development. Several studies have showed that genetic polymorphisms for certain modifiers are associated with cancer susceptibility. The relationship of certain human leukocyte antigen (HLA) types with elevated NPC incidence was extensively studied in southern Chinese. An association of NPC with HLA alleles A2, B14, and B46 was well documented[27]. The basis underlying this association is not because HLA is the susceptibility gene for NPC, but rather because a susceptibility locus located in the vicinity of HLA may be responsible for increased risk of NPC. It was further confirmed by an affected sib pair linkage study, which suggested a gene closely linked to the HLA locus at D6S1624 conferred a greatly increased risk of NPC[28]. However, so far no predisposing gene that exhibits clearly inheritable mutations among families has been identified even within this small region. Several other endeavors have been made to locate the region that confers the susceptibility to NPC, either by linkage analysis or by association study. The large-scale whole genome scanning for evidence of linkage once located the susceptibility gene within a 14cm region on 4p15 with the highest LOD score of 3.1 at D4S405[29]. Loss of 3p in almost all the NPC patients suggests that it is worthwhile to identify susceptibility genes in this region on pedigree-based and/or familial case-based analyses to NPC. A Chinese group once reported a strong linkage in this region[30]. In our unpublished study, we showed that a NPC susceptibility locus was linked to chromosome 5. A marker D5S2021 located on 5p13.1 yielded a maximum multipoint lod score of 2.1. No region with significantly increased allele-sharing within 3p or 4p15 was found in our results, suggesting heterogeneity in the origin of NPC carcinogenesis. In addition, certain variant forms of CYP2E1 and GSTM1 gene have been found to be associated with increased risk of NPC[31-34]. The findings implied that polymorphisms of some modifier might lead to different cellular response to environmental carcinogens among different individuals, different degrees of genetic instability or damages in the nasopharyngeal epithelial cells. Interaction of the genetic susceptibility and environmental factors may therefore be a determinant of NPC risk among populations.

2.2.3 Genome-wide allelotyping in NPC

Loss of heterozygosity (LOH) is a common occurrence in cancer. It represents the loss of one parent's contribution to a locus at cell's genome. Usually a tumor suppressor gene is buried in the lost region. Consistent with the Knudson’s two-hit theory, the remaining copy of the tumor suppressor gene was already inactivated by a point mutation. LOH can serve as the second hit and arise via several pathways, including

deletion, gene conversion, mitotic recombination and chromosome loss. LOH can be identified in cancers by using polymorphic markers like SNPs or microsatellites spanning the whole genome. Due to the high polymorphisms, a person rarely inherits the same allele from both parents. The information about LOH can be obtained by comparing germline DNA and cancer DNA.

Two independent whole-genome allelotyping studies in NPC have been conducted by different groups. In one study by a Hong Kong group, 27 cases of microdissection NPC biopsies were used to exclude the contamination of normal tissues. Consistently high frequencies of genetic losses are observed on chromosomes 3p (96.3%), 9p (85.2%), 9q (88.9%), 11q (74.1%), 13q (55.6%), 14q (85.2%), and 16q (55.6%)[35].These results were largely confirmed by the study of a second group[36]. Both studies revealed a high frequency of LOH at chromosome 3p, 9p and 14q, suggest tumor suppressor genes on 3p, 9p, and 14q appears to be a critical event[37-39].

2.2.4 Deregulated cellular pathways in NPC

According to the current findings, many genetic changes have been involved in the tumorigenesis of NPC. Deregulated cellular pathways, such as cell cycle regulation, apoptosis, ras signalling, and metastasis, are though to be critical events in the development of NPC. Altered p53 and Rb pathways are still extensively detected in NPC, even though NPC rarely presents abnormality in the p53 or Rb gene itself[40, 41]. p53 function may be inactivated by either overexpression of ΔN-p63 or loss of p14/ARF. ΔN-p63 is a p53 homolog. It can block p53´s function as transcription factor. p14 functions as a stabilizer of p53 since it can sequester, MDM1, which is a protein responsible for the degradation of p53. The inactivation of p14/ARF may facilitate p53 degradation in NPC cells. Loss of p53 function may affect cell cycle arrest at the G1 or G2/M phase and p53-mediated apoptosis in response to DNA damage[37, 42]. As an inhibitor of CDK4 kinase, p16/INK4A gene is frequently inactivated in 62%–86% of primary NPC tumors[43]. Its inactivation may result in constitutional Rb phosphorylation and thus enhanced NPC cell proliferation[38]. What's more, overexpression of cyclin D1 may contribute to the loss of restriction point control and the G1–S transition in some tumors. Reduced expression of key mitotic checkpoint proteins, CHFR or MAD2 may contribute to the genome instability in NPC[44, 45].

Ras proteins are key signal transducers for various important pathways, such as PI3K, MAPK. By activating a plenty of downstream effectors, Ras exhibits a regulatory role in many aspects of cell properties including cell proliferation, cell survival and cell morphology. Ras gene mutation has been observed in about one third of all human cancers[46]. The mutation frequency of Ras varies greatly among different tumor types, with as high as 90% in pancreatic carcinoma, 30% in non-small cell lung cancer, but

very rare in NPC[47-49]. During the last few years, Ras association domain family (RASSF), were identified as negative effectors of Ras. Six members of RASSF family have been found so far. RASSF1A (Ras-association domain family 1, isoform A) is able to bind to Ras and form heterodimer with Nore1 (also called RASSF5). The Nore1/RASSF1-Ras complex has been shown to be involved in cell cycle regulation, apoptosis, and microtubule instability[50]. Recently, another member of this family, RASSF2 was identified as a new negative effector of Ras protein. It has been shown to bind directly with K-Ras in a GTP dependent manner and its growth inhibition effect could be enhanced in the presence of activated Ras[51]. Inactivation of the Ras effector, RASSF1A in almost all NPCs, and RASSF2A in half of the NPCs, indicated that Ras signaling transduction pathway play an important role in this cancer[37, 39, 52-55].

Normal apoptosis signals were also found to be frequently damaged in NPC. Overexpression of bcl2 is believed to be the major mechanisms for the disruption of apoptosis in this cancer. Consistent upregulation of bcl2 in precancerous suggests that alterations in the apoptotic response are early events in the transformation pathway[56]. Furthermore, DAP kinase, which is a positive mediator of gamma-interferon induced apoptosis, was found to be downregulated by promoter hypermethylated with a high frequency in NPC, suggesting that silencing of this positive mediator of apoptosis may also involve in the development of this cancer[16]. Upregulation of the c-Met tyrosine kinase, metalloproteinases (MMPs) and the hypoxia proteins and the downregulation of E-cadherin and RASSF2A gene were thought to contribute to the metastasis of this cancer[55, 57-61].

2.2.5 Multistage model of NPC tumorigenesis

A LOH study in the histologically normal epithelia, dysplastic lesions and invasive carcinomas of the nasopharynx has suggested that deletions of 3p and 9p were early genetic events during tumor initiation. Since these changes can also detected in dysplastic lesions and even in the histologically normal epithelia from southern Chinese[62, 63]. Furthermore, chromosome 3p and 9p deletions were also more commonly observed in histologically normal nasopharynx epithelia in the high-risk population compared with that in NPC low-risk regions. Disruption of the NPC-associated TSGs on these critical regions, such as p16 and p14 on 9p and RASSF1A, BLU on 3p, may provide growth advantage of the initiated cells to expand[38, 39, 53, 63-66]. In contrast, EBV infection is consistently observed in NPC and high-grade dysplastic lesion but not in low-grade dysplastic lesions, suggesting latent infection of EBV is a critical step for the progression of NPC but not for its initiation. Expression of EBV encoded latent genes may result in the transformation of nasopharyngeal cells which already carry deleted 3p or 9p. The clonal expansion of EBV infected tumor cells may result in its rapid progression to invasive carcinoma. It is believed that only the

dysplastic cell with EBV infection will progress into NPC while the cells without EBV infection may eventually undergo apoptosis. One evidence is that, EBV-encoded LMP1 protein may block p53-mediated apoptosis through the induction of the A20 gene[67, 68]. Other genetic changes such as inactivation of the TSLC1, RASSF2A and E-cadherin genes may be involved in the later steps during development of invasive carcinoma[55, 60, 69-71] (see figure 1).

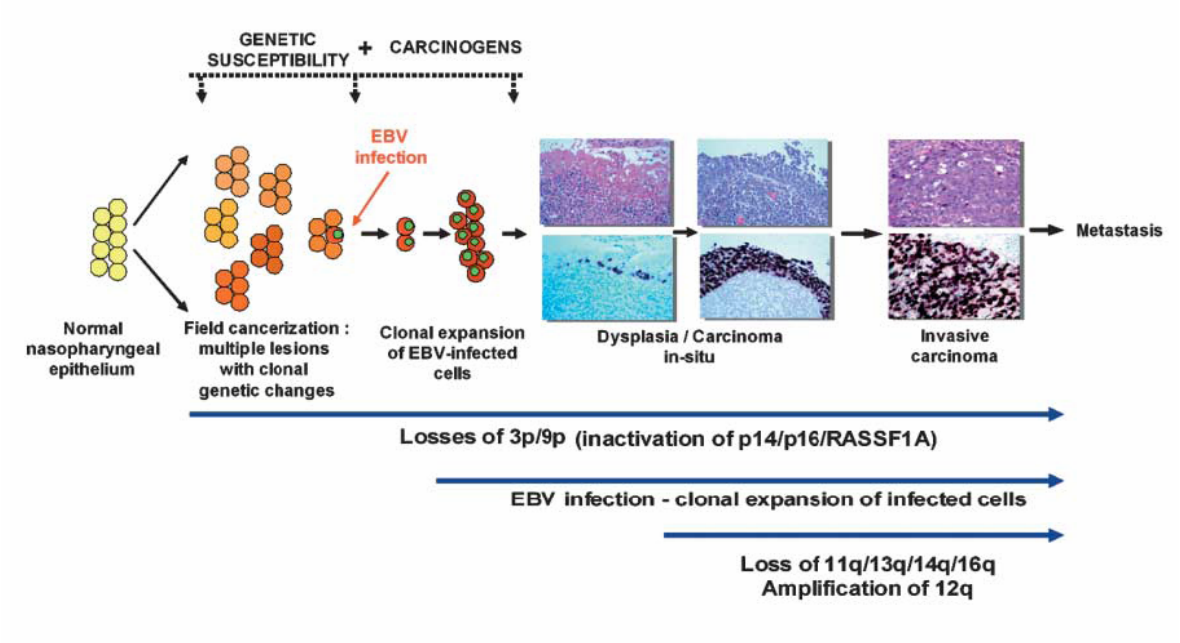

Figure1. Multistep carcinogenesis of nasopharyngeal carcinoma (*cited from Lo KW, To KF, Huang DP. Focus on nasopharyngeal carcinoma. Cancer cell 2004;5:423-8*).

2.3 EPIGENETICS IN NPC

Epigenetics is the study of gene silencing mechanisms that occur without involving changes in gene nucleotide sequence itself. Epigenetic changes are inheritable and can be transmitted from one generation (of cells or organisms) to the next. Widely speaking, any environmental-dependent form of cellular inheritance may be termed 'epigenetic'. Usually, when people talk about epigenetics, three aspects are emphasized: DNA methylation, histone modifications and nucleosome remodeling. It was once thought these epigenetic changes are independent of each other, since DNA methylation occurs at the DNA level, while the other two (histone modification and nucleosome remodeling) occur at the protein level. Now it is increasingly realized all these epigenetic changes are linked together.

Global DNA methylation changes have received attention since half century ago. However, only with the development of research tools targeting specific CpG sites (*e.g.* methylation-specific-PCR, bisulfite sequencing, for details please refer to the methods part of the book.) during the passing decade, the research of DNA methylation in cancer field became prosperous. Apart from DNA methylation, another best studied epigenetic change is histone acetylation. In this thesis, the role of DNA methylation and histone acetylation/deacetylation in general and in cancer is discussed specially.

2.3.1 DNA methylation

2.3.1.1 DNA methylation and gene transcription regulation

DNA methylation is a post-replicative event in which a methyl group is covalently added to the 5-carbon of cytosine bases that are located in cytosine–guanosine dinucleotides (CpGs)[72]. The "p" in CpG represents that they are phosphodiester-linked. In mammalian genome, CpG content are usually underrepresented due to the spontaneous mutational deamination of 5-methylcytosine to thymine. However, there are regions called CpG islands which are at least 200bp long and possess a GC percentage that is greater than 50%. About 10~15% of CpG dinucleotides in mammals occur in CpG islands. A CpG island is usually located in the 5′ regions (including promoter, untranslated region and exon1) of approximately 40% of mammalian genes, thus it interferes the transcription initiation of the nearby gene. CpG islands are usually unmethylated in normal tissues. Exceptions are those of imprinted genes and X-chromosome inactivation which are methylated in the normal situation. Approximately 80% of the CpGs in the human genome are located in the nonclustered CpGs in repetitive sequences (*e.g.*, satellite DNA, transposons and endogenous retrovirus). These nonclustered CpGs are usually methylated and therefore serve as a guardian of the genome, since otherwise activated transposons will destabilize the genome by

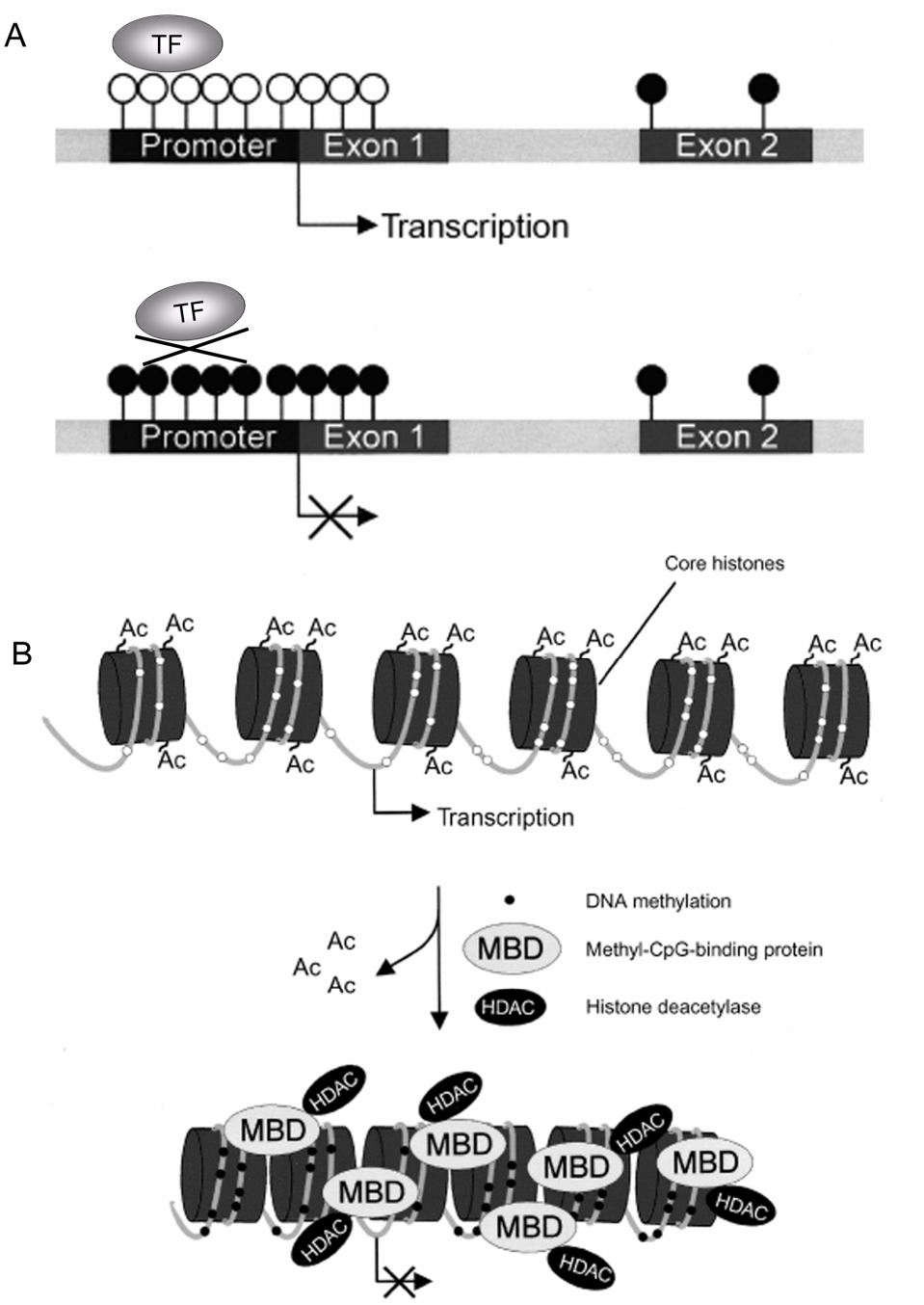

Figure 2. Two mechanisms of gene silencing by DNA methylation. (*adapted from Worm J, Guldberg P. DNA methylation: an epigenetic pathway to cancer and a promising target for anticancer therapy. J Oral Pathol Med 2002;31:443-9.*)

insertional mutagenesis and by favoring rearrangements via recombination between non-allelic repeats[73]. In addition, transcription activation of transposons within the introns of coding genes may interfere the expression of host gene. The above function of methylation suggests its role as a host denfese mechanism of organisms[74]. A direct of evidence of the host-defense hypothesis is that knockout mice of DNA methyltransferase are usually lethal in embryo[75, 76] and demethylation in DNA

methyltransferase-deficient mouse embryos caused transcription of a class of retroposons that were still capable of transposition in mice[77].

Another proposed function of methylation is its role in mammalian development. The DNA methylation level of genome changes in a highly organized way during mammalian development. It starts with a wave of dementylation during cleavage, followed by genome-wide *de novo* methylation after implantation. *De novo* methylation occurs rarely during normal postgastrulation development but is seen frequently during *in vitro* cell culture and in cancer. As a switch for gene expression, DNA methylation may regulate tissue-specific and phase-specific gene expression in mammalian development. However, this opinion about methylation is not out of controversy. A review by Timothy Bestor *et al* proposed their objections by saying that cytosine methylation is not the major regulatory means in the mammalian development, although it does contribute to this process[74, 78]. According to them, there is no single tissue-specific gene has been proved to be regulated by reversible methylation and demethylation. The promoters of tissue-specific genes that are contained within CpG islands are largely unmethylated irrespective of their expression status under normal conditions. They believed that the network which regulates gene expression in mammalian development is rather conservative in metazoa. There must be other mechanism than methylation operating in all the metazoan including lower organisms like *Drosophila Melanogaster* and *Caenorhabditis elegan* in which no methylation machinery was ever detected. It has been in dispute for many years about the causing role of methylation in gene silencing[74, 78]. So far the view suggests that demethylation is probably the consequence of gene activation instead of the cause, whereas methylation is still the cause of gene inactivation but not a consequence.

Usually there are two mechanisms by which CpG methylation could shut down a promoter[79]. First, methylation of cytosine bases can inhibit the association of some DNA-binding factors with their cognate DNA recognition sequences. Such a mechanism by which methylation silence a gene is not widely found among genome. An example is EBV W promoter (Wp) and C promoter (Cp), in which methylation of single CpG within CBF1 or BSAP/Pax5 recognition site blocks the binding of these two proteins to Cp and Wp respectively[80]. Second, binding of methyl-CpG-binding proteins (MeCPs) to the methylated CpG islands could recruit histone deacetylase complexes, which remove the acetyl groups from the N termini of the histones, and make it exhibit a more condensed conformation so that transcription factors can not reach the targeted DNA (see figure2). Most of genes silenced by methylation are mediated through this way. One of the examples is EBV-encoded LMP1 promoter. Data has been shown that repression of LMP1 promoter involves the Max-Mad1-mSin3A multiprotein complex and histone deacetylases[81].

2.3.1.2 DNA methyltransfeases

The unravelment of the mechanism about how DNA methylation happens and how it effectively keeps its pattern during cell division starts from the identification of DNA methyltransferases (DNMTs). So far, four active DNA methyltransferases have been identified in mammals. All the DNMTs can catalyze the transfer of a methyl group to DNA and use S-adenosyl methionine (SAM) as the methyl donor. Mammalian DNA methyltransferase can be classified into two general groups based on their preferred DNA substrate[82-84]. DNMT1 is the most abundant DNA methyltransferase in mammalian cells. DNMT1 is characteristic for being a maintenance methyltransferas, since it predominantly bind to hemimethylated DNA double strands at a rate 5-30 fold more active as compared with unmethylated substrate *in vitro*[85]. With the presence of DNMT1, it is therefore possible for replicating cells to maintain their pattern of gene expression from one cell generation to the next. Although DNMT1 is famous for its methylation maintenance ability, it is still at least as active at *de novo* methylation as other DNMTs. The *de novo* methyltransferases DNMT3a and DNMT3b could methylate hemimethylated and unmethylated CpG at the same rate. They are the main methylatransferase responsible for introducing cytosine methylation at previously unmethylated CpG sites. Unlike maintenance methyltransfereases DNMT1, DNMT3a and 3b may have preferential methylation site throughout genome. For example, mutations in DNMT3b on Chromosome 20 specially release the repression by methylation on pericentric regions of chromosome 1, 9 and 16. The result of this demethylation is ICF (immunodeficiency, centromere instability and facial anomaly) syndrome. There are three tentative ways by which *de novo* methyltransferases can be targeted to their substrate sequences. Firstly, *de novo* methylation may be mediated by specific domain contained in DNMT3[82, 86, 87]. Secondly, certain transcription factors may work as cofactors for DNMT3 in recognization of the target sequence[88, 89]. The third mechanism is said to be mediated by RNA interference[90, 91]. DNMT3L is a DNMT-related protein that is incapable of methylation. But it co-localizes with DNMT3a and DNMT3b and modulates their catalytic activity, by which DNMT3L participates in transcription repression. The loss of DNMT3L leads to bi-allelic expression of genes normally not expressed by the maternal allele. Still there is another possible DNA methyltransferase, DNMT2. However, deletion of DNMT2 causes no change in maintenance and *de novo* methylation in embryonic stem cells[82].

2.3.1.3 Genomic imprinting

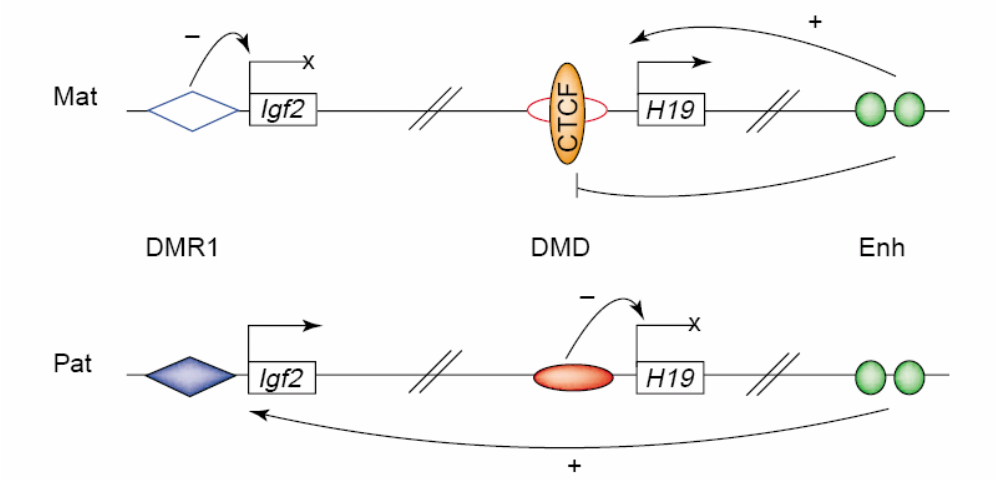

Figure3. Mechanism of imprinting at H19/Igf2 (*adapted from Arney KL. H19 and Igf2--enhancing the confusion? Trends Genet 2003;19:17-23*)

It is increasingly realized that the maternal and paternal genomes in an individual are not equivalent. A major difference is embodied in the pattern of DNA methylation in specific DNA sequence classes. This phenomenon of parent-of-origin specific silencing of a small proportion of genes in the genome is called genomic imprinting. In other words, some imprinted genes are expressed from a maternally inherited chromosome and silenced on the paternal chromosome; while other imprinted genes show the opposite expression pattern and are only expressed from a paternally inherited chromosome. The exact mechanism of genomic imprinting is not clear yet. DNA methylation seems to play an important role in it. Also there is evidence showing the existence of methylation-independent imprinting[92]. A canonical example of genomic imprinting is Igf2/ H19 genes which are expressed by different parental alleles. Igf2 and H19 are located on chromosome 11[93, 94]. Igf2 encodes a growth factor, and the H19 gene encodes a nonbonding, polypore-associated transcript that is implicated in the translational control of Igf2 mRNA. H19 is specially expressed from maternally inherited allele, while as Igf2 is specially expressed from paternally inherited allele. The elucidation of the mechanism of this differential imprinting starts from the identification of the H19 imprinting control region (ICR), which function as a chromatin insulator. It is located in the 5′-flank of the H19 gene and 90 kb downstream of the Igf2 gene. ICR is maternally unmethylated, thus permit the binding of insulator protein CTCF, which presumably blocks the communication between the Igf2 promoters and downstream enhancers and thereby silenced Igf2 gene with H19 gene expression unaffected. In the paternally inherited allele where ICR is methylated, expression of H19 gene is silenced by DNA methylation. On the other side, CTCF cannot bind to the methylated ICR which lead to the activation of Igf2 by the downstream enhancer in the paternal allele[95, 96].

Currently, about 41 genes or transcription units were reported to be imprinted in human[92]. The loss of imprinting (LOI) contributes to the development of many human diseases. Prader-Willi syndrome (characterized by hypotonia, obesity, and hypogonadism) and Angelman syndrome (characterized by epilepsy, tremors, and a perpetually smiling facial expression) are examples of such[97,98]. Usually 5q13 is differently imprinted in maternal and paternal chromosomes, and both imprintings are needed for normal development. If neither copy of 15q13 has paternal imprinting, the result is Prader-Willi syndrome. If neither copy has maternal imprinting, the result is Angelman syndrome. In addition, abnormal methylation of a cluster of imprinted genes on 11p15 like L1T1, H19 or $p57^{KIP2}$ was shown to be the cause of Beckwith-Wiedemann syndrome (BWS), a congenital overgrowth syndrome[99-102].

2.3.2 Histone modifications and 'histone code'

Genomic DNA is highly folded and compacted by histone and nonhistone proteins in the form of chromatin in the nuclei of all eukaryotic cells. The basic unit of chromatin is called nucleosome, which is typically composed of an octamer of the four core histones H2A, H2B, H3 and H4 with 146 base pairs of DNA wrapped around the histones. Each core histone has an amino-terminal 'tail' of about 25-40 residues long. It is the histone tail that can be subjected to various posttranslational modifications, including acetylation, phosphorylation, methylation, and ubiquitination *etc*. It is now clear that histones are integral and dynamic components of the machinery responsible for regulating gene transcription. Modifications of histone tails determine histone-DNA and histone-histone contacts, which may in turn regulate chromatin structure. Among the various histone modifications, acetylation/deacetylation of lysine residues presented on histone tails is the one has been most intensively studied and was thought to be linked to transcriptional activity. The idea was validated by the discovery of enzymes responsible for bringing about the steady-state balance of histone acetylation /deacetylation: histone acetyltransferases (HATs) and histone deacetylase (HDACs). Their transcription activating/repressing function has long been recognized before their acetylation enzymatic function. Acetylated histones are usually associated with transcriptionally active chromatin which is permissive for transcription factors binding to the cognate sites. While deacetylated histones can reverse this process and thereby are associated with inactive chromatin. The mechanism underpinning this chromatin switch is that acetylation could neutralize the basic charge of the histone tails which may reduce the affinity between histone-histone and histone-other regulatory proteins. Certain amino acid such as lysine or serine at H3 or H4 tails are preferred sites for various modification, *e.g.* the highly conserved histone H3 lysines at amino-terminal amino-acid positions 9, 14, 18 and 23, and H4 lysines 5, 8, 12 and 16, are frequently

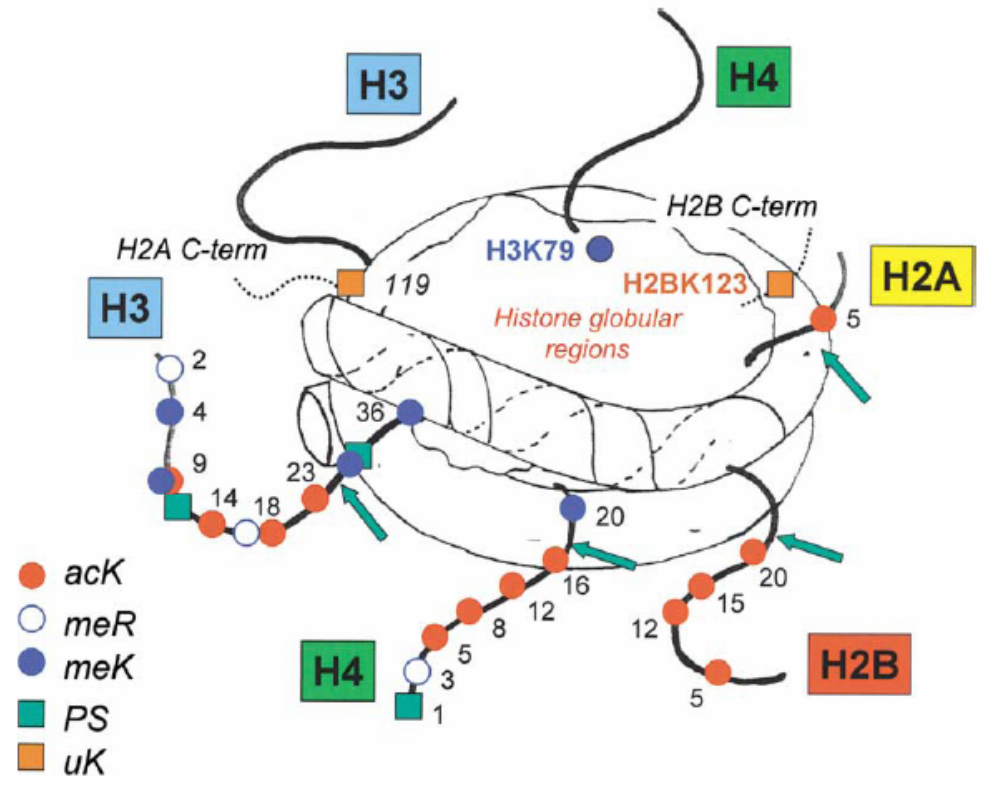

Figure4. The theory of histone code *(cited from Turner BM. Cellular memory and the histone code. Cell 2002;111:285-91).*

targeted for modification. Distinct H3 and H4 tail modifications act sequentially or in combination contribute to the remarkable diversity and biological specificity of organisms. The distinct patterns of covalent histone marks suggest that there exists a 'histone code' which is read by other transcription related proteins (see figure4)[103-105]. The existence of "histone code" may considerably extend the information potential which stored in the genetic code. The characteristic of replicating cells that they are capable of maintaining their identity from one cell generation to the next has been called cellular memory. It is not difficult to understand any more that DNA methylation pattern can be maintained during cell mitosis since the identification of DNMT1, which has the characteristic of specially binding to hemi-methylated DNA and catalyze the unmethylated daughter strand by adding a methyl-group on the corresponding sites. Now there comes the question of how histone code can be faithfully passed through cell division. The disclosure of this enigma will benefit a lot to the etiology of many diseases, *e.g.* cancer. As an important mechanism of regulating gene expression, chromatin-based epigenetics, histone modification may have similar scope of function as DNA methylation and may have an impact on X inactivation, imprinting, developmental reprogramming of cell lineages, and the plasticity of stem cells. The implications of translating the 'histone code' for human biology and disease, including cancer and aging, are far-reaching.

2.3.3 Epigenetics in tumor initiation and progression

2.3.3.1 The 'epigenetic progenitor model' of tumor initiation

During the past several decades cancer has been thought to stem from a single cell, in which a series of genetic alterations could be found on key TSGs and oncogenes. It is the deregulated expression of TSGs and oncogenes that are responsible for continued clonal selection and tumor cell heterogeneity. This 'monoclonal genetic model' of tumor initiation was well exampled in the case of colorectal cancer in which sequentially happened genetic events such as loss of APC, activation of oncogene K-RAS, inactivation of TSG p53 altogether contribute to the final phenotype of this tumor[106].

Now it was increasingly realized that epigenetic alterations are essential for not only the maintenance but also the initiation of many human tumor types. Epigenetic silencing of TSGs by promoter methylation is an early event in the multi-step process of carcinogenesis. Aberrant methylation of p16 and/or O^6-methyl-guanine-DNA methyltransferase can already be detected in the DNA from sputum of all the patients with squamous cell lung carcinoma even three years before clinical diagnosis[107]. Other examples are that LOI at specific imprinting locus might contribute to Wilms tumor, colorectal cancer and other cancers[108-110]. Increasing evidence support that epigenetic changes are more early than genetic changes during tumor initiation and responsible for the clonal expansion of cancer cells. One important evidence is that demethylation and re-expression of secreted frizzled-relatedprotein (SFRP), which is an antagonizer of Wnt signalling pathway[111, 112], would induce apoptosis of HCT166 cell line probably through blocking the abnormal expansion of epithelial stem or progenitor cells that rely on the Wnt signalling, even though the downstream factors of this pathway are still mutationally activated[113]. A recent review by Stephen Baylin mentioned a model of tumor initiation called 'epigenetic sensitization'[114]. In his model, epigenetic events may repress the transcription of a key regulator which can activate cellular signals that foster stem-or progenitor cell expansion during cell renewal incited by events such as chronic inflammation. The subsequent progression to malignancy would involve both genetic and epigenetic alterations. Epigenetic changes may 'addict' cancer cells to deregulated signal-transduction pathways during the very early phase of tumor development. It is because of these pathways which are involved in regulation of cell proliferation or survival that cancer cells acquire further genetic mutations, providing the cell with selective advantages that promote tumor development[114]. Andrew Feinberg *et al* also raised a similar opinion that cancer may initiate from a few of stem-like cells or progenitor cells, which are called 'cancer stem cell'. In their 'epigenetic progenitor model' of tumor initiation, cancer arises in three steps. The first step is aberrant regulation of 'tumor-progenitor genes' in progenitor cells, which lead to an enlarged

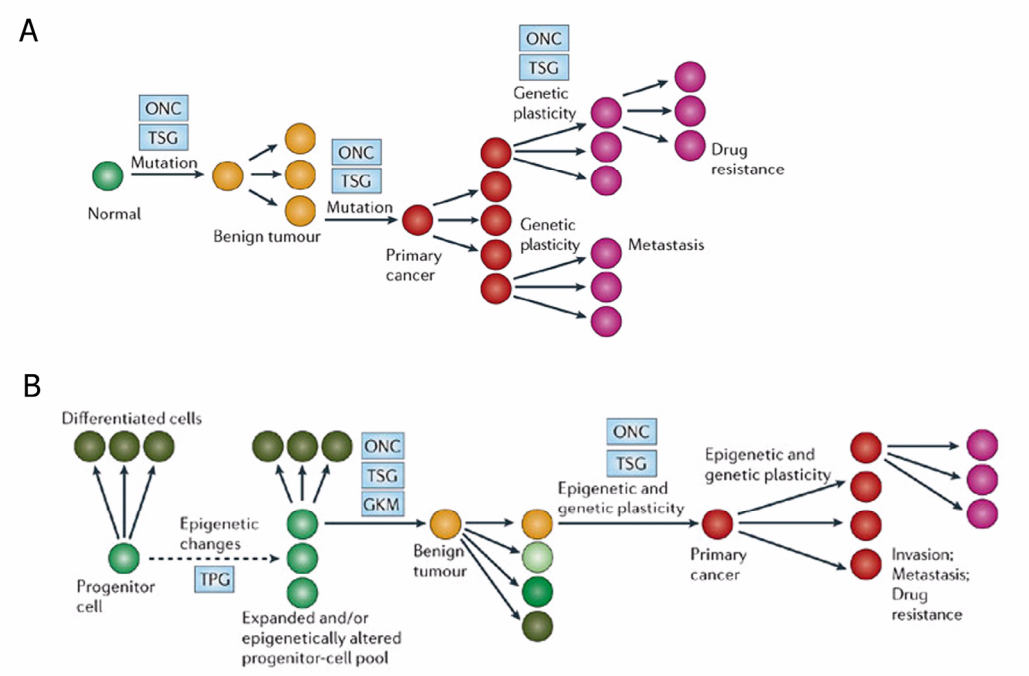

Figure5. A. The monoclonal genetic model of cancer. B. The epigenetic progenitor model of cancer. (*Cited from Feinberg AP et al. (2006) The epigenetic progenitor origin of human cancer. Nat Rev genet. 7: 21 – 33*)

polyclonal population of neoplasia-ready cells. The second step is mutation on a gatekeeper gene in the epigenetically disrupted progenitor cells from the first step. Thirdly, cells with the above epigenetic and genetic alterations must acquire an enhanced ability to stably evolve its phenotype[115] (see figure5). Thus, although epigenetic changes are reversible, reversible epigenetic changes could give rise to irreversible genetic alterations.

2.3.3.2 Epigenetic changes as a surrogate for genetic changes in tumor development

As a main mechanism of switching on/ off gene expression, the role of epigenetic changes in silencing important tumor-suppressor function has been widely accepted for many years. According to the revised Knudson two-hit theory[79], methylation could be an alternative mechanism to mutation or deletion involved in TSG silencing. As a substitute for mutation or chromosomal alterations, epigenetic inactivation by methylation occurs as frequently as those in the progression of tumor. It is found in virtually in every type of human neoplasm and is associated with gene silencing. There is bulky evidence showing that methylation could act as one of or even both of the two hits in silencing a TSG in a somatic cancer (see figure6). Also, enough evidence was shown that methylation could serve as the second hit in a familial cancer. Nearly 50% of the genes that cause familial forms of cancer when mutated in the germ line are known to undergo methylation-associated silencing in various sporadic forms of cancer.

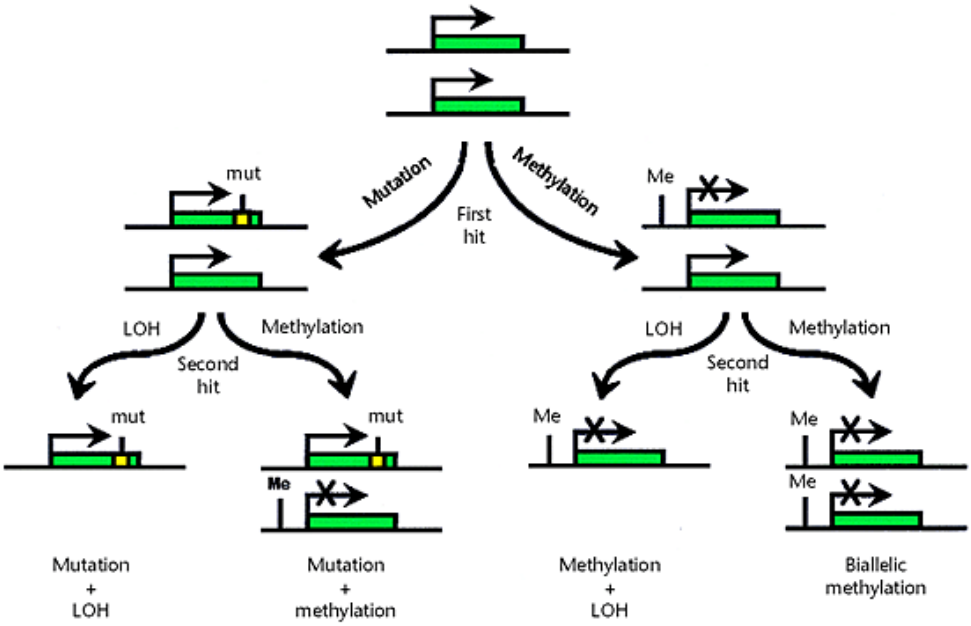

Figure6. Revised Knudson's two−hit hypothesis. DNA methylation can serve as the first hit, the second hit, or both hits in silencing TSGs. (cited *from Jones PA, Laird PW. Cancer epigenetics comes of age. Nature genetics 1999;21:163-7*）

For some genes, promoter methylation is the only form of gene silencing, such as O^6-methylguanine-DNA methyltransferase (MGMT), which encodes an important DNA-repair gene; cyclin-dependent kinase inhibitor 2B (CDKN2B), which encodes p15, a cell-cycle regulator[116, 117]. So far there is only limited report about the role of methylation as the first hit of silencing a susceptibility gene for a familial cancer. An example is MLH gene in hereditary colorectal cancer. Such kind of methylation is also named 'epimutation', suggesting its role as a surrogate for mutation[118-121].

The importance of epigenetic gene silencing in cancer is also highlighted by the growing awareness that such changes can actually predispose to mutational events during tumor progression. Aberrant methylation of caretakers of genome, like the DNA repair genes MLH1 and MGMT can result in microsatellite instability and increased frequency of mutations, respectively, thus promotes the progression of tumor[122, 123]. In addition, gene specific hypomethylation of some oncogene, which is supposed to be silenced in a specific tissue, could upregulate the expression of proto-oncogene and therefore contribute to the development of cancer (*e.g.* R-RAS in gastric carcinoma[124]). A global decrease in genomic methylation is well documented in malignant cells. Hypomethylation in malignant cell has been shown to be associated with increased levels of recombination and mutation[125].

2.3.4 Epigenetic changes in NPC

NPC also distinguishes itself from other tumors by the number of genes targeted for silencing by promoter methylation. The key tumor suppressor genes like p53 or Rb which are found to be mutated in 50% of all the tumors were rarely found to be mutated in NPC[40, 41]. On the contrary, hypermethylation of known or candidate tumor suppressor genes involved in various fundamental pathways has been reported in NPC, such as apoptosis, DNA damage repair, tumor invasion and metastasis. Many TSGs were aberrantly methylated in their 5′CpG islands in NPC: 84% of the RASSF1A, 80% of the RARβ2, 76% of the DAP-kinase, 46% of the p16, 89,7% of CDH13, 65% of the CHFR, 50.9% of the RASSF2A[37, 44, 55, 126]. The full list of genes which have been found to be aberrantly methylated in NPC was summarized in Table1.

The role of EBV in the development of a methylator phenotype for EBV-related tumor cells has received increasing attention during the past several years. EBV encoded protein, LMP1, has been shown to induce E-cadherin promoter methylation through interacting with methyltransferase, DNMT[127]. In vivo study of this kind for NPC, a 100% EBV associated tumor, still faces certain difficulties. It is difficult to find EBV-negative NPCs as control group in the endemic region. The problem may be solved by establishing normal nasopharyngeal carcinoma cell lines with and without *in vitro* infection by EBV. In addition, DNA methylation may also play an important role in the maintenance of specific EBV latency programmes in the NPC cells. Methylation of both viral and cellular genes may thus be involved in the transformation of nasopharyngeal epithelial cells. Induction of epigenetic alterations in certain cellular genes was proposed as one of the mechanisms for enhancing the transformation of nasopharyngeal epithelial cells by EBV infection.

Frequent aberrantly methylated TSGs in tumors have been used as molecular markers for the detection of malignant cells in many kinds of clinical materials[128]. It provides possibilities of both cancer early detection and dynamic monitoring of cancer patients during treatment[129]. Tumor specific methlated DNA has been detected from swab of NPC patients. Such an approach allows us to directly investigate the tumor progression in the nasopharynx[126]. By combining with detecting EBV DNA and multiple gene methylation status in the swab form NPC patients, we have reached a high diagnostic sensitivity of 97.9%, and zero false positive (unpublished data).

Table1. TSGs or candidate TSGs silenced by promoter methylation in NPC.

Cancer-related process	Gene	Chromosomal location	Function
Cell cycle control	P14[37]	9p21	CDK inhibitor
	P15[37]	9p21	CDK inhibitor
	P16[38]	9p21	CDK inhibitor
	CHFR[44]	12q24.3	Mitotic checkpoint regulator
Apoptosis	DAP-kinase[16]	9p34.1	Proapoptosis
	RIZ1[130]	1p36	Apoptosis
Invasion and metastasis	CDH1[131]	16q22.1	Cell adhesion
	CDH13[126]	16q24.2-24.3	Cell adhesion
	PCDH10[132]	4q28.3	Cell adhesion
	THY1 [133]	11q22-23	Invasion and metastasis
DNA repair	MGMT[134]	10q26	Repair alkylated guanine
Signal transduction	RASSF1A[39]	3p21.3	Regulate Ras signaling pathway
	RASSF2A[55]	20p	Regulate Ras signaling pathway
	RASSF4[135]	10q11	Regulate Ras signaling pathway
	RAR β2[136]	3q24	Retinoic acid receptor
	EDNRB[137]	13q22	Endothelin receptor
	WIF-1[138]	Chr12	Antagonist of Wnt signaling
	DLC-1[139]	8p21.3-22	GTPase-activating protein specific for RhoA and Cdc42
	HIN1[140]	5q35	An autocrin cytokine
	TIG1[141]	3q25	Retinoic acid target gene
	CRBPI [136]	3q21-22	Cellular Retinol Binding Protein 1
	CRBPIV[136]	1p36.22	Cellular Retinol Binding Protein 4
Transcription regulation	RIZ1[130]	1p36.21	Histone/DNA methyltransferases superfamily member
Unknown	TSLC1 [69]	11q22-23	Unknown
	BLU[66]	3p21.3	Unknown

2.4 EBV IN NPC

2.4.1 Introduction to EBV

EBV is a prototype of gamma herpes virus which infects >90% of the world's adult population[142]. Humans are the only natural host for EBV. Once infected with EBV, the individual remains a lifelong carrier of the virus. Primary infection with EBV normally occurs in early childhood and is usually asymptomatic in most underdeveloped countries. But when exposure to EBV is delayed until adolescence, such as in the cases in developed countries, it occasionally presents as infection mononucleosis. Long-term EBV coexists with most human hosts without overt serious consequences. However, in some individuals, the virus is implicated in the development of malignancy. EBV has a strong tropism for human lymphocytes and for the epithelium of the upper respiratory tract[143]. EBV was the first human virus identified to be associated with human lymphomas as well as epithelial tumors, such as post-transplant lymphoma, AIDS-associated lymphomas, Burkitt lymphoma, Hodgkin's disease, T-cell lymphoma, NPC, parotid gland carcinoma, and gastric carcinoma[144]. In NPC, EBV shows the strongest worldwide association due to the fact that EBV genome presents in virtually all NPC cells[145]. Tumorigenesis of NPC is a multistep process. EBV may play an important role

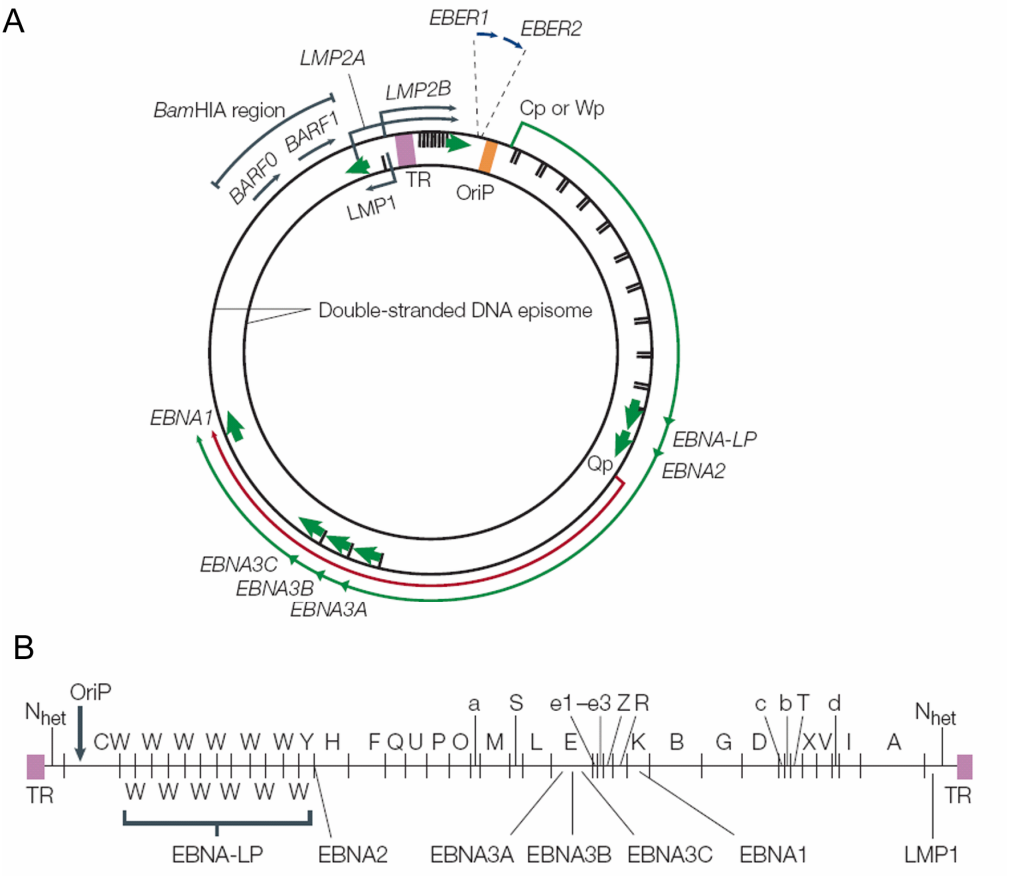

Figure 7. The Epstein-Barr Virus genome. (*adapted from Young LS, Rickinson AB. Epstein-Barr virus: 40 years on. Nat Rev Cancer 2004;4:757-68.*)

in the progression of NPC, involving activation of oncogenes and/or the inactivation of tumor suppressor genes. Early genetic changes may predispose the epithelial cells to EBV infection or persistent maintenance of latent cycle.

2.4.2 EBV-encoded proteins and latency programs

In EBV-infected cells, virus replication with production of infectious virus is a rare event. Typically, EBV establishes a latent infection. This is characterized by the expression of a restricted set of 'latent' genes, including six EBV-encoded nuclear antigens (EBNA1, 2, 3A, 3B, 3C, -LP), three latent membrane proteins (LMP1, 2A, 2B) and two EBV-encoded nuclear RNAs (EBER1, EBER2). Four different latent viral gene expression programmes have been identified[146]. In peripheral blood of healthy person, EBERs and LMP2 are expressed. This latency is called latency 0. In Burkitt Lymphoma, only EBERs and EBNA1 could be detected (latency I). In HL and NPC, LMP1 and LMP2 are also expressed besides EBERs and EBNA1 (latency II). The type of latency associated with the expression of all latent genes is referred to as latency III and is observed in immunosuppression-associated lymphoproliferations and in infectious mononucleosis. Three promoters are responsible for the transcription of EBV nuclear antigens. In establishing latency III, ENBA transcription after viral infection is initiated from Wp, followed by a switch to Cp with concomitant downregulation of Wp activity. In latency I and II, EBNA2, 3 and LP are not expressed. EBNA1 expression is initiated neither from Wp or Cp. Instead, another promoter Qp is used. LMP1 and LMP2B transcription is controlled by a bidirectional promoter close to terminal repeats, with LMP1 is transcribed from the left direction and LMP2B from the right direction. LMP2A is transcribed from a promoter about 3000 bp upstream of the promoter of LMP1 and LMP2B.

EBNA-1 is a sequence-specific DNA binding phosphoprotein that is expressed in all transformed lymphoid cell lines and EBV associated tumors. EBNA1 is required for the replication and maintenance of the EBV genome. It binds to the origin of replication for the plasmid form of the viral genome and enables the viral episome to segregate with the host chromosomes during mitosis[147]. This is the single viral gene product that must be expressed to enable the viral genome to be transmitted to the daughter cells.
EBNA-2 is one of the first two latent proteins detected after EBV infection[148]. It is a transcriptional coactivator of many viral proteins expressed in latency III. It also plays a critical role in cell immortalization by transactivating many cellular genes. Among these are CD23 (a surface marker of activated B-cells), c-myc (a cellular proto-oncogene), and viral EBNA-C promoter[149-151]. EBNA2 cannot bind directly to DNA sequence but rather mediated by, mostly, the viral Cp binding factor 1(CBF1), but also by other transcription factor[152]. EBNA-2 is also known for its ability of mimicking transcription

factors involved in the Notch signalling pathway, which may play a role in development of T-cell lymphoma in humans[152, 153].

EBNA-LP , also known as EBNA-5, is one of the first viral proteins produced during EBV infection of B cells[154]. EBNA-LP interacts with EBNA-2 to drive resting B lymphocytes into the G_1 phase of the cell cycle by binding and inactivating cellular p53 and retinoblastoma protein tumor suppressor gene products[155,156].

EBNA-3A, EBNA-3B, and EBNA-3C are transcriptional regulators[148]. EBNA-3A and EBNA-3C are crucial for *in vitro* B-cell transformation, whereas EBNA-3B is not indispensible[148].

LMP1 is a latent membrane protein with six transmembrane domains. Among EBV-encoded proteins, LMP1 is of equal importance as EBNA2, since both can transform B cells into LCL *in vitro*.

LMP2A and 2B differs only in that LMP2A has a 119 amino acid N-terminal cytoplasmic domain. Expression of LMP2A helps maintain a latent infection in B lymphocyte. In epithelial cell, LMP2A is probably also transforming by activating Akt. The exact function of LMP2B is not clarified yet. It may function as a blocker of LMP2A function.

EBERs are expressed abundantly, with about 10^7 copies in each cell. The presence of EBERs can be consistently detected in all EBV-associated tumors except in oral hairy leukoplakia. Thus staining of EBERs could serve as an evidence of EBV infection.

Latent EBV genome has been shown to exhibit a host cell-dependent expression of its proteins under the regulation by DNA methylation. In EBV genome, Wp is the first promoter to be activated immediatly after EBV infection of human B cells, but it undergoes progressively methylation and switches off in established lymphoblastoid cell lines. In parallel, an unmethylated promoter, Cp, is switched on. Cp is subjected to methylation and keeps silent in BL cell lines, which use Qp for the initiation of EBNA1 messages instead. Qp is a methylation-free promoter irrespective of its activation status[157].

2.4.3 LMP1 is the main EBV-encoded transforming protein in NPC

As mentioned above, EBV exhibits a restricted expression program in NPC, with only EBNA1, LMP1 and LMP2 proteins could be detected. Thus, LMP1 receives most attention as an oncoprotein in NPC. LMP1 is an integral membrane protein with a complex molecular structure containing a cytoplasmic amino terminus, six transmembrane domains, and a long cytoplasmic carboxy terminal portion.LMP1 has shown an unequivocal expression in approximately 65% of NPC biopsies[158, 159]. LMP1 receives most attention because it inhibits the differentiation of human keratinocytes in vitro with morphological changes[160] and transforms established rodent fibroblasts and

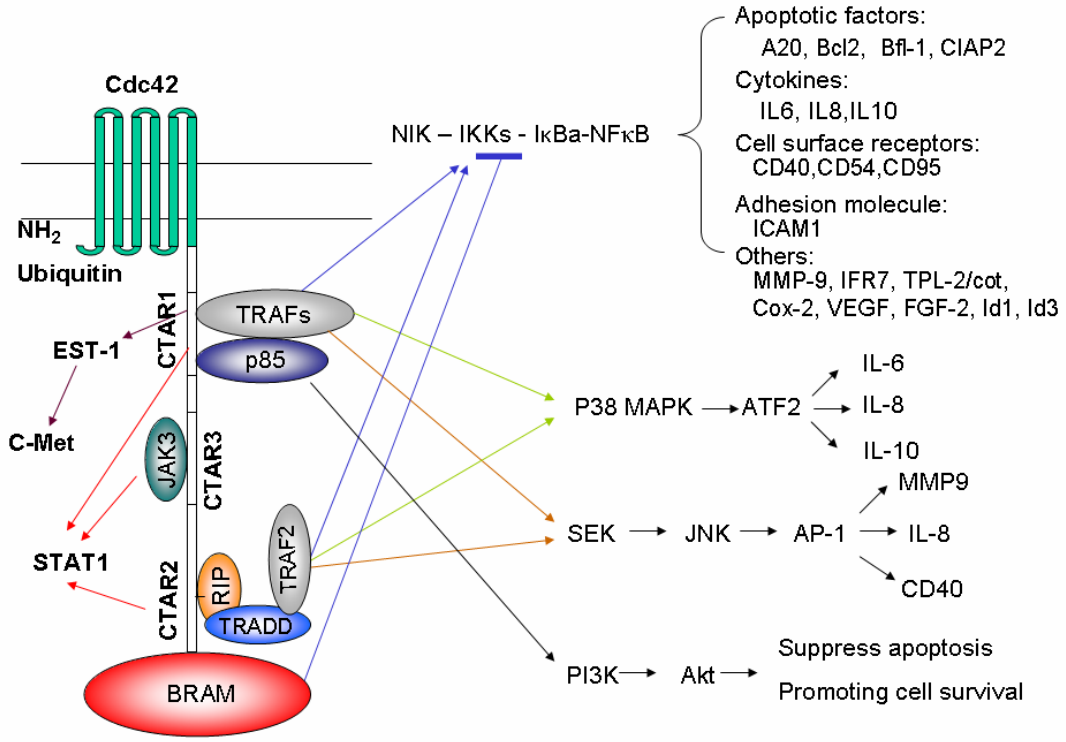

Figure8. LMP1 properties as an oncoprotein.

human epithelial cells in vitro[161, 162]. By activating several important cellular signalling pathways like NF-κB, JNK, JAK/STAT and PI-3K pathway, LMP1 could upregulate anti-apoptotic gene products, such as bcl2, A20, AP-1, CD40, CD54 and also cytokines IL-6 and IL-8; thereby exhibit its oncogenic characteristics[163](see figure 8). LMP1 also can enhance the expression of MMP9, EGFR and VEGF[164-166]. Thus LMP1 is also involved in the process of angiogenesis in NPC. According to clinical and follow-up study, LMP1-expressing NPCs show different growth pattern and prognosis from those without LMP1 expression[167]. However, the mechanism underlying LMP1 differential expression in NPC is not clarified yet.

2.4.4 Transcription regulation of LMP1 expression

2.4.4.1 Promoter usage of LMP1

The transcription of LMP1 was found to be initiated from three promoters (see figure 9). The transcript from ED-L1 promoter encodes a 2.8kb mRNA. It is the main transcript expressed by LCL cells. The transcription starting site of ED-L1 promoter was located at 169,546 of EBV genome. A DNA fragment spanning residues +40 to -634 relative to the transcription initiation site was defined as the LMP1 transcription regulatory sequence (LRS)[168]. The LMP1 gene is transcribed in a leftward direction under the regulation of LRS. In human epithelial cell lines, C-33A and NPC-TW076, it was

found that the region between -643 and -496 contained a promoter activity that was approximately five-fold higher than ED-L1 promoter. This region between -643 and -496 was designated as ED-L1E[169]. In a nude mouse-passaged NPC, C15, two 3' coterminal LMP1 transcripts of 2.8 and 3.5 kb have been detected by northern blotting. B95.8 mainly expressed the 2.8kb transcript, but also a low abundance 3.7kb mRNA[170]. The transcription starting site of 3.5 kb transcript was mapped to 170,099 by RNase protection assay. The 3.5kb transcript initiates from heterogeneous start sites within the first terminal repeat (TR) of viral genome. So the promoter for initiating long LMP1 transcript is also named L1-TR. The expression of the 3.5 kb transcript was consistently detected in nasopharyngeal carcinoma samples, also detected in Hodgkin's disease.
In addition, an alternatively spliced mRNA is produced by transcription from the ED-L1A promoter within the first intron of LMP1 gene. It encoded a truncated protein of 28kD corresponding to the C-terminal two-thirds of the 42-kilodalton protein. This promoter is active in the late lytic phase in B cell. It is not yet clear how different patterns of promoter activation are determined by different cell types. The availability of specific transcription activators in a cell may play a role.

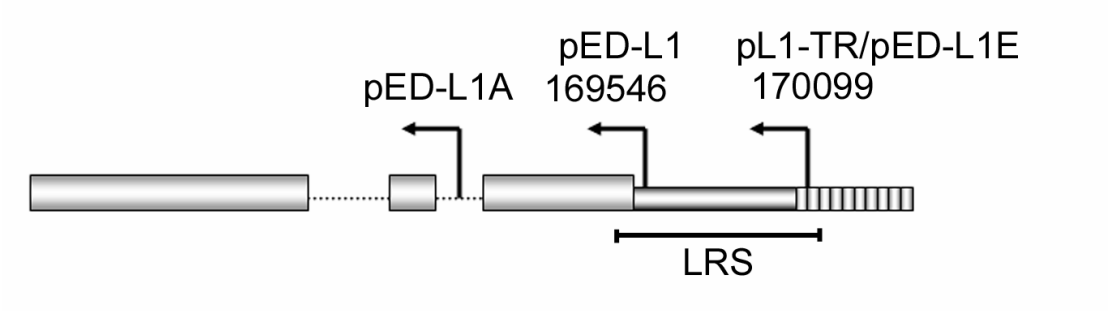

Figure9. LMP1 promoter usage.

2.4.4.2 Transcription regulation of LMP1 by cellular factors and virus encoded proteins

LRS is composed of both positive and negative transcription *cis* elements. In B cell, the expression of LMP1 can be induced by both EBV encoded proteins and celluar transcription factors. Among EBV encoded proteins, EBNA2 is the most powerful transactivator of LMP1 transcription. Three regions within LRS are required for EBNA2-dependent transactivation of LMP1 promoter. They are ATF/CRE element (-45/-39), the octamer motif and PU-box site (-165/-141) and the RBP-Jk sites (-223/-217). EBNA2 is able to interact with ATF2/c-Jun heterodimer, the POU domain protein, PU.1 and RBP-Jk, to transactivate the LMP1 promoter. The expression of LMP1 can be induced by both EBNA2-dependent and EBNA2-independent mechanisms. Sp1, ATF-1/CREB-1, USF, AP-2 and IRF7 transcription factors can transactivate the LMP1 promoter in the absence of EBNA2, through Sp, ATF/CRE, Ebox , AP-2 and ISRE

elements respectively[81, 171-175]. The expression of LMP1 in epithelial cell is induced through EBNA2-independent mechanisms. In NPC cells, the long 3.5kb transcript of LMP1 is preferentially expressed. This transcript started from a region within the first terminal repeat, which is TATA-less, but contains multiple GC-rich elements. The transcription of LMP1 3.5 kb transcript is EBNA2-independent. Several important *cis* element has been identified in this region. The -48 to-33 region (relative to 170,099) was essential for L1-TR (ED-L1E) promoter activity. Sp1 and Sp3 could bind to the -48 to -33 region as examined by EMSA[175]. Examination of the L1-TR and the standard ED-L1 promoters by EMSA revealed that both promoters contained STAT binding sites and responded in reporter assays to activation of JAK-STAT signalling. However, the two LMP1 promoters are regulated by different STAT family members. STAT3 is likely to be responsible for biological LMP1 L1-TR promoter regulation, but not for ED-L1 promoter. A potential model is that EBV infection of an epithelial cell containing activated STATs would permit LMP1 expression, which in turn establishes a positive feedback loop of IL-6-induced STAT activation and LMP1 expression[176, 177].

3 METHODS

3.1 MEANS FOR STUDYING EPIGENETIC CHANGES

3.1.1 Bisulfite treatment

Sample DNA was chemically modified by sodium bisulfite, which converts unmethylated cytosine into uracil, while methylated cytosing does not react. This reaction constitutes the basis for discriminating between methylated and unmethylated DNA. Bisulfite transformation methods can be followed by several methods, including sequencing, combined bisulfite restriction assay (COBRA) and others. Bisulfite modification of DNA requires prior DNA denaturation because only methylcytosine that are located in single strands are susceptible to attack.

3.1.2 Bisulfite sequencing

Sequencing bisulfite-altered DNA is the most straightforward means of detecting cytosine methylation. In general, after the denaturation and bisulfite modification, double strand DNA is obtained by primer extension, and the fragment of interest is amplified by PCR. Methylcytosine can then be detected by standard DNA sequencing of the PCR products. Cloning PCR products into plasmid vectors followed by the sequencing of individual clones is an alternative method that, although slower, could provide methylation maps of single DNA molecules, instead of the average values of the methylation status in the population of molecules provided by direct sequencing of the PCR products.

3.1.3 Methylation specific PCR (MSP)

Methylation-specific PCR is the most widely used technique for studying the methylation of CpG islands, which are common in the promoter regions of many genes. Cytosines in CpG islands are usually unmethylated in normal tissues, whereas they become methylated in the promoter sequences of genes associated with certain abnormal cellular processes such as cancer. The differences between methylated and unmethylated alleles that arise from sodium bisulfite treatment are the basis of methylation-specific PCR and are especially valuable in CpG islands because of the abundance of CpG sites. Primer design is a critical and complex component of the procedure. Bisulfite-converted DNA strands are no longer complementary, so primer design must be customized for each DNA chain. Methylation patterns of all sequences must be determined in separate reactions. To optimize the PCR amplification step, the

following critical requirements must be considered when designing the primers: (i) the annealing temperature of both primers is similar and always between 55°C and 65°C; (ii) the size of PCR product is usually between 80 and175 bp; (iii) each primer should contain at least two CpG pairs with at least one CpG pair at the 3′-end; (iv) to avoid false positives (amplification of unmethylated or unmodified DNA), primers should contain non-CpG cytosines. The great sensitivity of the method allows the methylation status of small samples of DNA, including those from paraffin-embedded or microdissected tissues.

3.1.4 Multiplex Methylation Specific PCR (MMSP)

Multiplex MSP is a less material-consuming method for detecting several biomarkers for dynamical methylation pattern. It is principally the same as classical MSP, excepting that it involves several pairs of PCR methylation primers for different genes in one reaction tube, and therefore the readout provides methylation information for several genes at the same time. This kind of method is especially suitable for those with limited source of starting material. *e.g.* DNA from serum or body fluid, like sputum from lung cancer patients, urine from bladder or prostate cancer patients, nasopharyngeal swab and mouth washing fluid from NPC, etc.
In our pilot study, the panel of markers for NPC diagnostic analysis is: a. EBV encoded LMP1 gene, an oncogene which expressed in about 65% of all NPCs and its expression is strictly related by promoter methylation. b. EBV encoded EBNA1 gene, which serves as a marker for existence of EBV infection released by the elapsed tumor cells to the body fluid and also for EBV load, which is correlated with the stage of NPC. c. DAP-Kinase: A positive mediator of IFN-γ-induced programmed cell death, suppressing tumor growth and metastasis by increasing the occurrence of apoptosis in vivo. This gene is methylated in 76% of NPC tumors. d. RASSF1A: one of putative tumor suppressor gene and the RAS-association domain family gene. It was shown to be methylated in 84% of NPC tumors.

3.1.5 Chromatin Immunoprecipitation (ChIP)

ChIP is a procedure used to determine whether a given protein binds to or is localized to a specific DNA sequence *in vivo*. The principle underpinning this assay is that DNA-bound proteins (including transcription factors) in living cells can be cross-linked to the chromatin on which they are situated. This is usually accomplished by a gentle formaldehyde fixation. Following fixation, the cells are lysed and the DNA is broken into pieces 0.2-1 kb in length by sonication. Once the proteins are immobilized on the chromatin and the chromatin is fragmented, whole protein-DNA complexes can be immunoprecipitated using an antibody specific for the protein in question. The DNA

from the isolated protein/DNA fraction can then be purified. The identity of the DNA fragments isolated in complex with the protein of interest can then be determined by PCR using primers specific for the DNA regions that the protein in question is hypothesized to bind.

3.2 METHODS FOR DETECTING RNA OR PROTEIN EXPRESSION

3.2.1 Quantitative real-time PCR

PCR is a method that allows logarithmic amplification of short DNA sequences within a longer double stranded DNA molecule using a DNA polymerase enzyme that is tolerant to elevated temperatures. mRNA is copied to cDNA by reverse transcriptase using an oligo dT primer or random oligomers. The gene whose expression levels we wish to analyze is then further amplified from the cDNA mixture together with a "housekeeping" gene. The basic idea behind real-time polymerase chain reaction is that the more abundant a particular cDNA (and thus mRNA) is in a sample, the earlier it will be detected during repeated cycles of amplification. Various systems exist which allow the amplification of DNA to be followed and they often involve the use of a fluorescent dye which is incorporated into newly synthesized DNA molecules during real-time amplification. Real-time PCR machines, which control the thermocycling process, can then detect the abundance of fluorescent DNA and thus the amplification progress of a given sample.

3.2.2 Western blot

Western blot is a method to detect protein in a given sample of tissue homogenate or extract. It uses gel electrophoresis to separate denatured proteins by mass. The proteins are then transferred onto a membrane (typically nitrocellulose), where they are "probed" using antibodies specific to the protein. As a result, we can examine the amount of protein in a given sample and compare levels between several groups.

3.3 FUNCTIONAL ASSAYS OF CANDIDATE TSG

3.3.1 Transfection

Transfection is the process of introducing DNA into eukaryotic cells, such as human cells. For most applications of transfection, it is sufficient if the transfected gene is only transiently expressed. Since the DNA introduced in the transfection process is usually not inserted into the nuclear genome, the foreign DNA is lost at the latest when the

cells undergo mitosis. If it is desired that the transfected gene actually remains in the genome of the cell and its daughter cells, a stable transfection must occur.
To accomplish this, another gene is co-transfected, which gives the cell some selection advantage, such as resistance against a certain toxin. Some (very few) of the transfected cells will, by chance, have inserted the foreign genetic material into their genome. If the toxin, towards which the co-transfected gene offers resistance, is then added to the cell culture, only those few cells with the foreign genes inserted into their genome will be able to proliferate, while other cells will die. After applying this selection pressure for some time, only the cells with a stable transfection remain and can be cultivated further. A common agent for stable transfection is Geneticin, also known as G418, which is a toxin that can be neutralized by the product of the neomycin resistance gene

3.3.2 Cell cycle analysis by flow cytometry

The measurement of the DNA content of cells was one of the first major applications of flow cytometry. The DNA content of the cell can provide a great deal of information about the cell cycle, and consequently the effect on the cell cycle of added stimuli, *e.g.* transfected genes or drug treatment. We can also combine the measurement of DNA content with the quantitation of an antigen, so that we can assess its expression during the cell cycle, or alternatively look at the DNA profile of a subset of cells defined by antigens. Ideally all cells in the G1 phase will take up the same amount of dye and will all fluoresce in a single channel. Unfortunately, flow cytometers are not that accurate and there will be minor conformational variations in the DNA leading slightly different amounts of dye being taken up - we quantify this by using the coefficient of variation (CV) of the G1 peak. The lower the CV the better we can assess DNA changes especially aneuploidy. We usually measure fluorescence from a DNA-binding fluorochrome and infer that this is the same as DNA content.

3.3.3 Wound healing assay

The wound-healing assay is simple, inexpensive, and one of the earliest developed methods to study directional cell migration in vitro. This method mimics cell migration during wound healing *in vivo*. The basic steps involve creating a "wound" in a cell monolayer, capturing the images at the beginning and at regular intervals during cell migration to close the wound, and comparing the images to quantify the migration rate of the cells. It is particularly suitable for studies on the effects of cell-matrix and cell-cell interactions on cell migration.

4 RESULTS AND DISCUSSIONS

4.1 ABERRANT METHYLATION OF CELLULAR CANDIDATE TSGS (PAPER I AND II)

4.1.1 Detection of CDH13 gene promoter hypermethylation in NPC

The gene CDH13 encodes a cell adhesion molecule superfamily member, which has been found to be epigenetically silenced by 5'CpG methylation of the promoter region in various tumors[176]. Ectopic expression of CDH13 in breast cancer cell lines is capable of inhibiting tumor growth in nude mice, suggesting that CDH13 may act as a tumor suppressor gene. In the present study, we examined this gene in NPC, a tumor where this was not investigated before. We found that CDH13 expression correlated with promoter methylation both in cell lines and biopsies. In CDH13 expression-silenced cell line C666-1, its expression could be dramatically restored by demethylation with 5-aza-dC, inferring that DNA methylation is a major regulator of CDH13 expression. In NPC biopsies, there is a strikingly high frequency of CDH13 methylation (89.7%), while only one of the 10 (10%) normal nasopharyngeal epithelia detected is methylated (p<0.05). This suggests that CDH13 is a common target for methylation and epigenetic gene silencing in NPC. The MSP primers we used cover the most frequently methylated CpG sites in other tumor types. We also confirmed this for NPC by bisulfite sequencing. Since CDH13 could convert the breast cancer cells from an invasive morphology to a normal-like morphology, we explored whether CDH13 methylation status correlated with NPC metastasis features. However, we failed to see such a correlation with the TNM stages according to UICC in our samples. CDH13 may have a role early in the pathogenesis of NPC, which would be consistent with the studies in colorectal cancer and adenomas which have shown that CDH13 methylation occurs at an early stage in the multistage tumorgenic process. A lack of correlation of CDH13 methylation with metastasis in NPC *in vivo* is not paradoxical, since tumor metastasis *in vivo* is a complicated process and many genes functioning in diverse pathways are involved. Single gene may contribute only partially to the final step of metastasis.

Detection of molecular alterations in body fluids of cancer patients is more and more used for early detection and diagnosis, *e.g.* detection of bladder cancer in urine, lung cancer by sputum or ovarian cancer by peritoneal fluid. Detection of tumor DNA from peripheral circulation and mouth washing fluids is not very sensitive according to our own experiments and reports from several other groups[178]. Nasopharyngeal swabs take the advantage of NPC as a surface mucosal tumor and its tangible location. Tumor cells

can be released from the tumor mass more easily than normal epithelial cells, especially when the cell-cell adhesion is subverted. MSP is a powerful technique and could detect two copies of methylated alleles against the background of unmethylated alleles. MSP's sensitivity further improved when combined with our modified protocol of Alexander Olek's bisulfite treatment.

The specificity of detecting CDH13 methylation from NP swabs was 81% and the rate of false positives was 0%. Such a high correlation of detecting CDH13 promoter methylation when comparing primary tumors and paired NP swabs further confirmed that NP swabs could be used to assist the detection and diagnosis of NPC.

4.1.2 Inactivation of RASSF2A by promoter methylation correlates with lymph node metastasis in NPC

RASSF2 can bind directly to K-Ras and function as a negative effector of Ras protein. RASSF2A is the only isoform of RASSF2 that contains CpG island in its promoter and it has been reported to be inactivated by its promoter methylation in several human cancers. In the present study, we investigated the correlation of RASSF2A expression with its promoter methylation in nasopharyngeal carcinoma (NPC). Expression of RASSF2A was down-regulated in 80% (4/5) of NPC cell lines. Decreased RASSF2A expression was also observed in NPC primary tumors compared with normal nasopharyngeal epithelia. Promoter methylation of RASSF2A could be detected in all the RASSF2A-silenced cell lines (4/5) and 50.9% (27/53) of primary tumors, but not in any of the normal epithelia. RASSF2A-methylated cases showed a significantly lower level of RASSF2A expression than unmethylated cases ($p<0.05$, Mann-Whitney's U test). Loss of RASSF2A expression can be greatly restored by methyltransferase inhibitor 5-aza-dC in NPC cell lines. Statistical analyses of the correlation between RASSF2A promoter hypermethylation and clinicopathological parameters are summarized in table 2. Patients with methylated RASSF2A presented a higher frequency of lymph node metastasis ($p<0.05$). No significant differences were observed between methylated and unmethylated patients with regard to age, gender, staging, and pathological subtypes.

In the present study, we demonstrated that RASSF2A mRNA expression was down-regulated in NPC cell lines and primary tumors. The MSP and real-time RT-PCR data suggested a correlation between mRNA expression level and methylation status in NPC cell lines and tumors. In the revised Knudson two-hit theory, besides deletion and mutation, DNA methylation was included as an alternative mechanism for the inactivation of tumor suppressor genes. So far, no evidence of loss of heterozygosity (LOH) on 20p was reported in NPC[35, 36] and no somatic mutations other than

polymorphism of RASSF2A could be found in human cancers. The methylation of RASSF2A in NPC seems to be bi-allelic, since a methylation degree of 60% to 100% was observed for most of the CpG pairs in the RASSF2A-silenced NPC cell lines CNE1 and CNE2. Heavy degree of methylation was also observed in NPC tumor biopsies despite of the possibility of normal tissue contamination. In this context, epigenetic inactivation appears to be a major mechanism for the loss of RASSF2A expression. Restoration of RASSF2A expression by the demethylating agent 5-aza-dc further raised this possibility. Hypermethylated DNA can serve as a potential molecular tumor marker because of its high specificity in distinguishing cancers from normal tissues[128, 129]. Our MSP results showed that RASSF2A promoter region was subject to methylation in 80% of the NPC cell lines and 50.9% of the primary tumors. Therefore, RASSF2A methylation was frequent in NPC and this is not just a phenomenon associated with *in vitro* cell culture. In addition, our findings of a lack of RASSF2A methylation in normal nasopharyngeal epithelia support the fact that epigenetic silencing of RASSF2A is a tumor-specific process. This tumor-specific hypermethylation of RASSF2A can be detected in both early-stage and advanced NPC tumors, suggesting that RASSF2A gene promoter methylation might play an important role in the early development of nasopharyngeal carcinogenesis. Hence, RASSF2A methylation could serve as a potential biomarker for early diagnosis of NPC.

4.1.3 Characterization of the TSG properties of RASSF2A

To characterize the tumor suppressor function of RASSF2A in NPC, we examined the effects of RASSF2A on cell cycle regulation, cell growth, clonogenicity and cell mobility. To elucidate the possible effect of RASSF2A on the cell cycle regulation, the CNE2 cells transiently transfected with RASSF2A-expressing vector or empty vector were subjected to flow cytometry cell cycle assay. Twenty-four hours after transfection, the CNE2-RASSF2A cells showed a 12% increase in G0/G1 cell population than that of CNE2-empty vector cells.

The proliferation rate of CNE2 parental cells, CNE2-empty vectors cells and CNE2-RASSF2A cells were estimated by trypan blue dye exclusion assay. CNE2-RASSF2A cells grew significantly slower compared to CNE2 parental cells and CNE2-empty vector cells.

The colony formation efficiencies were evaluated by monolayer culture. After selection by G418 for 2 weeks, the number of colonies formed by CNE2-RASSF2A cells was obviously less than that of CNE2-empty vector cells.
As a measurement of cell mobility, wound-healing assay was carried out by comparing the scractching healing efficiency of CNE2- RASSF2A cells with that of CNE2-empty

vector cells. The CNE2-RASSF2A cells moved slower into cell free area than CNE2-empty vector control cells, suggesting that restoration of RASSF2A inhibits the motility of NPC cells.

RASSF2A has been observed to be epigenetically inactivated in several human cancers[179-181]. Although the exact mechanisms about how RASSF2 functions in human cancers were largely unknown, inactivation of RASSF2 in kidney cells showed an advanced growth during transformation[181], and *in vitro* expression of RASSF2 could induce apoptosis and cell cycle arrest in lung cancer cells[51]. In our study, we further confirmed the ability of RASSF2 to induce cell cycle arrest and inhibit colony formation in NPC cells. What's more, RASSF2 was found to be capable of inhibiting cell migration by scratch wound healing assay. All these clues suggested a potential role of RASSF2 as a tumor suppressor gene and its inactivation may be supportive for the survival and migration of tumor cells by reducing Ras-induced oncogenic signals.
In our study, *in vitro* expression of RASSF2A in cells was able to inhibit cell mobility when compared with cells transfected with empty vector, suggesting RASSF2A was involved in tumor invasion and metastasis led by Ras. This was further supported by our result that RASSF2A methylation was correlated with lymph node metastases in primary NPC tumors.

Lymph node metastasis is one of the most important clinical features of NPC. It strongly reduces the possibility of cure and the survival time. Identification of molecular markers for lymph node metastasis would be very helpful in designing optimized and individualized therapeutic regimens for NPC patients. Our current study revealed an obvious correlation between RASSF2A methylation and lymph node metastasis in NPC, suggesting that RASSF2A methylation can be considered as such an indicator of the propensity of lymph node metastasis, and would have a great potential in clinical application. In addition, methylation-mediated inactivation is potentially a reversible phenomenon. Turning this process around and up-regulating RASSF2A may probably prevent or reverse the malignant and metastatic phenotype, therefore become a novel therapeutic target in NPC treatment.

4.2 EPIGENETIC REGULATION OF LMP1 EXPRESSION IN NPC (PAPER III).

Gene transcriptional regulation in response to cellular signalling may happen in many forms. In brief, the binding of appropriate transcription factors to the corresponding *cis*-regulatory elements on the promoter of a target gene determines whether it is actively transcribed in a specific cell. Thereafter, all the factors affecting this binding may influence gene expression, *e.g.*, the presence of activated transcription factors needed

for the regulation of promoter in the nuclear; the elements which influence the access of transcription factors to the specific regulatory sequence. Many endeavours have been made in identifying transactivators for LMP1 expression both in lymphoid cells and in epithelial cells[173-175, 177, 182]. In the present study, we mainly focused on how the access of transactivators to LMP1 promoter was epigenetically regulated in NPC cells.

4.2.1 LMP1 promoter methylation correlates with LMP1 protein expression in NPC biopsies

Aiming at detecting the role of promoter methylation in regulation LMP1 expression in NPCs, we first divided our NPC samples into two groups by western blot: LMP1-expressing group and LMP1-silenced group. The methylation status of LMP1 promoter (EBV genome 169,546-170,110, Genbank access number: V01555) was compared between these two groups of NPCs by bisulfite sequencing. The results show that 45 CpG pairs within the region investigated were highly methylated in the NPC samples of LMP1-silenced group, whereas LMP1 promoter was free of methylation in the NPC biopsies which belong to LMP1-expressing NPC groups. No single CpG site was ever found to receive preferential methylation within the examined region. The above data suggest that LMP1 protein expression tightly correlated with its promoter methylation status, and methylation is a repression mechanism influencing LMP1 expression in NPC. DNA methylation could also silence a gene through interfering directly the association of transcription factor with their cognate recognition site. So far no evidence of this kind was ever found for LMP1p. On the other side, certain repressive elements within promoter can recruit histone deacetylase complex without the need of methylated CpG sites. There is evidence that HDACs could be targeted to LMP1 promoter through interaction with Max-Mad1-mSin3A bound to the E-Box and with Ikaros-NuRD bound to the Ikaros site[81].

4.2.2 Induction of LMP1 mRNA and protein by 5-aza-dc in NPC cell line with methylated LMP1 promoter

In order to further confirm that methylation plays an important role in LMP1 silencing, we used an EBV-positive NPC cell line as a model to study if LMP1 expression could be restored by demethylating agent, 5-aza-dC. C666-1 is the only NPC cell lines carrying naturally infected EBV so far. The LMP1 promoter of C666-1 cells is heavily methylated, as shown by bisulfite sequencing results. After incubating the NPC cell lines with 5μM 5-aza-dC for 96 h, the expression of both LMP1 mRNA and protein was significantly induced in C666-1 cells according to the results of RT-PCR and Western Blot. Bisulfite sequencing of the LMP1 promoter after 5-aza-dC treatment in

C666-1 cells revealed a successful demethylation effect of the drug. More than half (3 out of 5) of the clones being sequenced showed largely umenthylated CpGs after 5-aza-dC treatment. This result suggests that transcription repression of LMP1 gene could be at least partly relieved by a demethylation process in C666-1cells.

4.2.3 Histone hyperacetylation alone cannot release the repression on LMP1 promoter

Next we investigated if histone deacetylation also takes a hand in regulation of LMP1 expression, as it usually does in other cells such as TW03/EBV, P3HR-1, Daudi, *etc*[81, 183]. C666-1 cells were treated with a HDAC inhibitor, TSA, for 24h at a concentration of 200ng/ml. However, no elevated LMP1 mRNA or protein expression could be observed in C666-1 cells. Then the acetylation status of histone 3 and histone 4 was examined by chromatin immunoprecipitation. Two different primer sets covering LMP1 promoter region +1 to -238 and -431 to -651 relative to the transcription starting site designated as +1 were used to amplify the precipitated DNA. Quantitative real-time PCR results show that acetylated H3 and H4 protein could hardly be detected before TSA treatment, whereas the level of both acetylated H3 and H4 significantly increase after TSA treatment. In order to examine the relationship of DNA methylation with histone deacetylation, methylation status of the C666-1 LMP1 promoter after TSA treatment was further examined. We found LMP1 promoter still remained methylated after TSA treatment, suggesting that histone hyperacetylation can not induce DNA demethylation in this case. On the contrary, when we come back to the study of H3 and H4 acetylation status after cells were treated by 5-aza-dC, we surprisingly found the acetylation level was elevated by simply a demethylation process, suggesting DNA methylation may be the cause of regional chromatin packaging. Although histone deacetylase inhibitor alone cannot overcome the repression exhibited on LMP1 promoter, a jointed treatment of cells by TSA and 5-aza-dC could induce LMP1 expression at least five fold of that of 5-aza-dC alone, suggesting both epigenetic mechanisms play a role in LMP1 expression. The repression effect executed by histone deacetylation was at least partly methylation-dependent. Promoter methylation plays an overriding role in the event of LMP1 silencing. A previously study about regulation LMP1 transcription by histone deacetylation using another NPC cell line TW03, which was infected *in vitro* with Akata EBV strain, presented an opposite result: 5-aza-dC could not induce LMP1 expression in TW03/EBV, while histone deacetylation is the main factor in LMP1 silencing in this cell[183]. C666-1 cell and TW03/EBV cell are both epithelial cells and supposed to express similar pattern of tissues-specific genes. The discrepancy may be due to the difference of EBV strains infected these two NPC cells. It has been shown that LMP1 promoter is highly polymorphic and gene variation happens at an average frequency of 1/20 base pair between strains[184]. It is worth

thinking that newly created or lost cognate sites for certain transcription factor caused by gene variation may have different affinity for *de novo* methyltransferases, which may explain the different behaviours by cells of the same type.

4.2.4 Requirement of 5-aza-dc for induction of LMP1 expression by IL4

IL4 is a cytokine which has multiple biological functions in regulation of immune response. STAT6 is the crucial element in IL4-mediated functions[185]. There were several functional STAT binding sites were identified in LMP1 promoters, which also responded in reporter assays to activation of JAK-STAT signalling[176, 177]. IL4 has been shown to induce LMP1 expression in various EBV-positive cell lines through JAK/STAT pathways. However, IL4 cannot induce LMP1 expression in C666-1 cells, as shown in RT-PCR and western blot. To investigate if the discrepancy is due to the heavily methylated promoter of LMP1 in C666-1, we treated C666-1 cells with IL4 together with 5-aza-dC. A more than 15-fold increase of LMP1 expression was observed when compared to cells treated by 5-aza-dC alone. This provides evidence that IL4 needs demethylation before inducing LMP1 expression at least in C666-1 cell. In other words, hypomethylation of LMP1 promoter was a prerequisite for IL4 to activate LMP1 promoter. This could be supportive evidence that a methylated CpG site within or around transcription binding site may prevent the binding. Although *in vivo* analysis in cells with lymphoid origin has disclosed an equal binding ability of various transcription factors binding to the unmethylated and methylated LMP1 promoter, it is still worth further investigation that if the methylation of CpG within STAT binding site decide the binding under an epithelial cell context. Since LMP1 expression regulation is suffered from the regulation by different groups of transcription factors in B-cells and epithelial cells. There is no such a synergistic effect in C666-1 by treating cells with IL4 plus TSA. Histone deacetylation is not the key factor in this event since IL4 can not induce LMP1 expression even if the chromatin structure in the vicinity of LMP1 promoter was relaxed. This data support the above result that LMP1 promoter methylation overrides the presence of histone hyperacetylation.

4.3 DEVELOPMENT OF A NON-INVASIVE METHOD, MMSP, FOR DIAGNOSIS OF NPC (PAPER IV)

4.3.1 The feasibility of MMSP

In order to test the feasibility of MMSP to achieve the same readout as those from five single MSPs, we used Namalwa cell line as a positive control. All the markers in our MMSP system are supposed to show positive bands for bisulfite-treated namalwa DNA. We compared the specificity and efficiency of MMSP assay using the corresponding

single MSPs as control. According to our result, all the markers demonstrated highly specific bands in this MMSP assay. And there was an excellent concordance with the relative density of the five bands between MMSP and separate PCR reactions. These results suggest that MMSP assay is tenable both in principle and in reality.

Several technical approaches are now available for analyzing DNA methylation status, such as Southern blot analysis, bisulfite genomic sequencing, MSP, and methylation-sensitive restriction enzyme digestion. Although considerable amounts of DNA are incorporated in these methods, the number of genes investigated in a single reaction is still limited. Recently, some approaches that allow high-throughput analyses of multiple CpG islands were developed, *e.g.* methylation specific oligonucleotide microarray, restriction landmark genomic scanning, and differential methylation hybridization[186-188]. However, these techniques require sophisticated equipments for bioinformatic analyses, which limits their prevalence in the clinic. The MMSP assay demonstrated here is capable of simultaneously detecting methylation status of multiple genes, which are key regulators of diverse tumor-related pathways. The informativeness and amenability of MMSP make it suitable for clinical use.

A study by Esteller *et al*. has shown that a panel of three to four markers could define an abnormality in 70–90% of each cancer type through detecting their aberrant methylation[189]. In the present study, two cellular TSG markers (RASSF1A and DAPK), two EBV related markers (EBNA1 and LMP1), and one DNA quality control marker (β-Actin) were included in this NPC-specific MMSP assay. RASSF1A is a putative TSG. Loss or altered expression of this gene has been shown to be a key event in the development of NPC. The RASSF1A protein could interact with DNA repair system and also induce cell cycle arrest. The aberrant methylation is tightly correlated with loss of expression of RASSF1A in NPC [39]. DAP-kinase is a positive mediator of the programmed cell death induced by gamma-interferon. DAP kinase methylation is one of the most frequent epigenetic inactivation events detected in NPC. It has also been demonstrated to be associated with aggressive and metastatic phenotype in many human cancers[190]. LMP1 is the main EBV transforming gene in NPC. Its protein is expressed in 65% of NPC. By judging LMP1 expression or not in a specific NPC according to its methylation status, it will further increase the specificity of MMSP and provide tentative information about the prognosis of NPC. EBNA1 is a nuclear antigen encoded by EBV. We included EBNA1 as a marker for the presence of EBV infection. The intensity of the marker for EBV presence in MMSP has a potential to be semi-quantified for EBV load using namalwa cell as reference. EBV presence marker EBNA1 and DNA quality marker β-Actin were both amplified from bisulfite-treated tumor DNA with specific primers which do not distinguish methylated or unmethylated allele. This is different from the situation for the other three markers included in the MMSP, in which either methylated alleles (RASSF1A and DAPK) or unmethylated alleles (LMP1) were specially amplified

4.3.2 Determining the sensitivity of MMSP assay

Frequent aberrantly methylated TSGs in tumors have been used as molecular markers for the detection of malignant cells in many kinds of clinical materials. It provides possibilities of both cancer early detection and dynamic monitoring of cancer patients during treatment[129]. Gaining sufficient and high-quality tumor DNA is critical for diagnosis. A variety of body fluid and sampling methods have been attempted for the non-invasive diagnosis of NPC, such as serum or plasma from peripheral circulation, buffy coat, and NP swab. The quantity and integrity of cell-free DNA fragments from peripheral circulation specimens such as serum and plasma are usually compromised. Thus, the detection rates of methylated DNA from serum or plasma had been proved to be very low[178]. Buffy coat yields DNA of higher quantity and quality; however the DNA is mainly from peripheral lymphocyte instead of from elapsed tumor cells. NP swab achieved the highest specificity among the above body fluid samples, since it gathers tumor cells by contact the primary tumor directly[178]. There are also many other advantages of NP swab, such as ease of application and swiftness. These attributes make NP swab extremely amenable for the diagnostic use of NPC.

To evaluate the sensitivity of the MMSP assay, we prepared a serial dilution of DNA from certain number of Namalwa cells. The serial diluted DNA equals to DNA extracted from 640 cells, 160 cells, 40 cells, 10 cells, 2.5 cells and 1.25 cells was then bisulfite modified respectively and used as templates for MMSP assays. The known MMSP pattern with five markers could be clearly and faithfully exhibited with one single reaction of MMSP from as few as 10 Namalwa cells. Using the conversion estimate of 5 pg/copy of genome DNA, our data demonstrated an extremely high sensitivity of detecting multiple biomarkers from a minimum starting material of 50 pg tumor DNA.

4.3.3 Detection of MMSP patterns in NPC cell lines, NP biopsies and corresponding swabs

A series of NPC cell lines and xenograft were included in this study to further validate the reproducibility of MMSP assay. C15 is an African-origin, EBV-positive and LMP1-expressing NPC xenograft. Among the six NPC cell lines included in this study, C666-1 is the only EBV-positive one. It is also a LMP1 non-expressing cell line with heavily-methylated LMP1 promoters (unpublished manuscript). The other five cell lines: CNE1, CNE2, HONE1, SUNE1 and HK1, are all EBV negative NPC cell lines.The hallmark for the presence of EBV in our MMSP assay, EBNA1, can be detected in the C666-1 cell line and the EBV-positive xenograft C15. Unmethylated-LMP1 can be only detected in LMP1 expressing xenograft, C15. All the rest of cell

lines demonstrated negative bands for EBNA1 and LMP1 markers. But they all showed aberrant methylation on at least one of the two TSGs included in the MMSP assay. The pattern for these five markers in separate MSP reactions was invariably reproduced in all the cells tested by the MMSP system, which further validate the robustness of MMSP assay (data not shown).

Forty-nine NPC biopsies and their corresponding nasopharynx swabs were included in this study to examine the reliability of NP swabs as the origin of material for MMSP test. 20 paired NP biopsies and swabs from noncancerous volunteers were also included as normal control. All swab samples from NPC patients and noncancerous controls showed easily detectable bands of the β-Actin gene in our MMSP analysis, indicating sufficient yields of genomic DNA and successful bisulfite conversion. Presence of EBV latent infection in the biopsy and swab samples was confirmed by amplifying EBV-encoded EBNA1 gene in the MMSP reaction. EBV DNA was detected in 100% (49 of 49) of NPC primary tumors and 97.9% (48 of 49) of their paired swab samples, but in none of the noncancerous controls. 63.3% of NPC primary tumors and 55.1% of their corresponding swab samples showed unmethylated-LMP1 (U-LMP1) bands in the MMSP assay, suggesting LMP1 is expressed in these samples. The ratio of U-LMP1 in NPC samples is quite close to the reported ratio of 65% for LMP1 expression in NPC, which further confirm the validity of unmethylated LMP1 allele as the marker for LMP1 expression. The frequencies of promoter hypermethylation of RASSF1A and DAP-kinase were 79.6% (39 of 49) and 67.3% (33 of 49) in NPC biopsy samples, while methylated in 55.1% (27 of 49) and 57.2% (29 of 49) respectively in the corresponding swabs.

In swab samples of NPC, 18.4% (9 of 49) exhibited tumor suggestive signal (positive PCR band) from all the four markers; 34.70% (17 of 49) from three of the four markers, 30.6 %(15 of 49) from two markers and 14.3 %(7 of 49) from one marker only. The MMSP pattern of 42.9% NP swabs (21 of 49) matched exactly with those of corresponding biopsies. If we regard the presence of EBV DNA with tumor suggestive information from any one of the other three markers as a diagnostic criterion for NPC, we reached a sensitivity of 97.9% in detecting NPC, which covers all the stages of NPC patients. No EBV or methylated markers were ever found in swabs of the noncancerous controls, suggesting a specificity of 100% (zero false-positive). Taking EBV presence marker (EBNA1) plus tumor suggestive signal from at least one of the other three markers as a judging criteria, we achieved a diagnostic rate of 97.9% in 49 NPCs. Noticeably, we successfully detected all of eight T1 NPC patients included in this MMSP study, suggesting that the alternation of panel markers reflects changes on the early stage of nasopharyngeal carcinogenesis. It will pave the way for our future plan of developing MMSP as a population-based screening tool. Since pattern and timing of methylation status in certain genes are demonstrated to be associated with defined biological behaviors[191], MMSP method will not only provide the diagnostic

information, but also has the potential of predicting the specific behavior of individual tumors. Both EBV load and methylation level of certain gene have been shown to be capable of monitoring disease relapse during treatment, suggesting MMSP may serve as a way of long-term guardian during NPC treatment in the clinic. Considering the flexibility of replacing certain tumor-specific markers in the MMSP panel, it may also be possible to extend the usage of MMSP assay for the diagnosis of other types of cancer by evaluating MMSP pattern of tumor DNA from other body fluids, such as detecting prostate cancer or bladder cancer from urine, cervical cancer by cervical swab, *etc*.

5 CONCLUDING REMARKS AND FUTURE PERSPECTIVES

In this work, we have tried to access the epigenetic mechanisms that contribute to the development of NPC. We got the following conclusions based on our findings:

1. CDH13 promoter is aberrantly methylated in NPC both *in vitro* and *in vivo*. Its promoter methylation plays a pivotal role in the silencing of H-cadherin transcription. Furthermore, the high sensitivity (81%) and specificity (0% false positives) of detecting CDH13 methylation from nasopharyngeal swabs suggests it could be utilized as a tool for early diagnosis.

2 .Candidate TSG, RASSF2A, is frequently inactivated by its promoter methylation in NPC. Its aberrant methylation correlates with lymph node metastasis in NPC. Its TSG function was further confirmed by its ability of inhibiting cell cycle progression, colony formation and cell migration in NPC cells.

3. Both DNA methylation and histone hypoacetylation executed repression on LMP1 promoter in NPC cells. DNA methylation is the overriding mechanism in this event. The presence of DNA methylation on LMP1 promoter may prevent the binding of certain transcription factor.

4. We developed a method, MMSP, which can simultaneously detect EBV load and assess multiple virus and cellular gene methylation status using only picograms of tumor DNA from NP swabs. The sensitivity and specificity of MMSP in detecting 49 NPC which include 19 early-stage patients are 97.9% and 100% respectively. Being characterized by its noninvasiveness, reproducibility and informativeness, MMSP assay is a reliable and potential diagnostic tool for NPC. It paves the way for the development of population screening and early diagnosis approaches for various tumor

In future, we would like to lead ourselves to the study of how genetic factors, epigenetic factors and environmental factors interact with each other. Unlike those in other tumor types, the main changes happened to NPC at the molecular level is rather ´epi-genotypic´ than being ´genotypic´. Very rare genetic events have been reported in this special kind of cancer. On the contrary, dozens of TSGs or candidate TSGs have been found to be aberrantly methylated in NPC. And the list is still growing. As an alternative mechanism of TSG silencing to mutation and deletion, DNA methylation

could function as the first hit event in the Knudson model. However, very little attention has ever been paid to this property of methylation in NPC.

In addition, the role of EBV in the development of a ´methylator´ phenotype for EBV-related tumor cells has received increasing attention during the past several years, however, this kind of study for NPC, a 100% EBV associated tumor, still faces certain difficulties. We will try to solve this problem by establishing normal nasopharyngeal carcinoma cell lines with and without *in vitro* infection by EBV.

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