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Epigenetics of Nasopharyngeal Carcinoma

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1. Introduction

Cancer has been previously viewed as a disease exclusively driven by genetic changes, including mutations in tumor suppressor genes and oncogenes, and chromosomal abnormalities. However, recent data have demonstrated that the complexity of human carcinogenesis cannot be accounted for by only genetic machineries, but also involves extensive epigenetic abnormalities. The term "epigenetics" refers to the study of heritable changes in gene regulation that do not involve a change in the DNA sequence or the sequence of the proteins associated with DNA (Egger et al. 2004). Epigenetic machineries plays a fundamental role in several biological processes, such as embryogenesis, imprinting, and X chromosome inactivation, and in disease states such as cancer. Several mechanisms were included in the epigenetic machinery, the most studied of which are DNA methylation; histone modifications; and small, noncoding RNAs (Kargul and Laurent 2009; Jeltsch and Fischle 2011). The molecular mechanisms underlie the epigenetic changes in cancer cells are complicate and only began to be elucidated. The best understood component among which is the transcriptional repression of a growing list of tumor suppressor and candidate tumor suppressor genes (Jones and Laird 1999; Esteller 2007). This suppression is associated with abnormal methylation of DNA at certain CpG islands that often lie in the promoter regions of these genes (Esteller 2006, 2007).

Nasopharyngeal carcinoma (NPC) is a unique head and neck cancer with remarkably distinctive ethnic and geographic distribution among the world. The three major etiologic factors of NPC were well defined as genetic susceptibility, environmental factors and latent infection of the Epstein-Barr Virus (EBV) (Tao and Chan 2007; Lo, To, and Huang 2004). During the passing decade, much attention has been paid to the role of epigenetic alternations occurred in the procedure of tumorigenesis of NPC (Li, Shu, et al. 2011; Tao and Chan 2007).

In this chapter, we will first describe the general mechanisms through which the epigenetic alternations in cancer, then focus on the epigenetic alterations taking place in NPC, with an emphasis on DNA methylation.

2. DNA methylation, histone modifications and chromatin structure

DNA methylation is the only genetically programmed DNA modification in mammals. This postreplication modification is almost exclusively found on the 5' position of the pyrimidine ring of cytosines in the context of the dinucleotide sequence CpG. 5'-methylcytosine accounts for ~1% of all bases, varying slightly in different tissue types and the majority (75%) of CpG dinucleotides throughout mammalian genomes are methylated (Tost 2010). Sequence regions with a high density of CpG residues are termed as CpG islands. A CpG island is defined as a sequence of 200-plus base pairs with a G+C content of more than 50%, and an observed versus expected ratio for the occurrence of CpGs of more than 0.6 (Jones and Takai 2001). These CpG islands are associated with gene promoters in approximately 50% of genes and are generally maintained in an unmethylated state. DNA methylation can interfere with transcription in several ways. It can inhibit the binding of transcriptional activators with their cognate DNA recognition sequence such as Sp1 and Myc through sterical hindrance. The methylation binding proteins and the DNA methyltransferases (DNMTs) bind to methylated DNA and prevent the binding of potentially activating transcription factors. The methylation binding proteins and DNMTs also recruit additional proteins with repressive function such as histone deacetylases and chromatin remodeling complexes to the methylated DNA to establish a repressive chromatin configuration (Bird 2002).

To date, three major cellular enzymic activities associated with DNA methylation have been characterized (DNMT1, DNMT3A, and DNMT3B) (Malik and Brown 2000). They catalyze the transfer of a methyl group from SAM to the cytosine base. DNMT1 is considered as a maintenance methyltransferase, it is located at the replication fork during the S phase of the cell cycle and catalyze the methylation of the newly synthesized DNA strand using the parent strand as a template. The methyltransferases DNMT3A and DNMT3B are responsible for *De novo* methylation. These enzymes not only targeting specific sequences, they also work cooperatively to methylate the genome (Malik and Brown 2000).

Tumor-specific elevation of DNMTs is a causative step in many cancers. All three DNMTs, were observed modestly overexpressed in many types of tumor cells at the mRNA or protein level (Robertson et al. 1999). Furthermore, modest overexpression of exogenous mouse Dnmt1 in NIH 3T3 cells can promote cellular transformation (Wu et al. 1993). Additionally, genetic inactivation of Dnmt1 in mice decreases the development of gastrointestinal tumors in a mouse model of gastrointestinal cancer (Laird et al. 1995). These evidences indicate a possible role for DNMTs in tumorigenesis. However, the mechanisms that underlie such a role in cancer are still not defined.

Genomic DNA is highly folded and packaged into chromosomes or chromatin by histone and nonhistone proteins in the nuclei of all eukaryotic cells (Jenuwein and Allis 2001). The fundamental repeating unit of chromatin is the nucleosome, in which 146 DNA base pairs are wrapped left handed around a core histone protein, which consists of two of each of the four histone protein subunits: H2A, H2B, H3 and H4. Each core histone has an amino-terminal 'tail' of about 25-40 residues long, where they are frequent targets for various posttranslational modifications (Fischle, Wang, and Allis 2003). The state of chromatin is regulated largely by covalent modifications of the histone tails. The major modifications include the acetylation of specific lysine residues by histone acetyltransferases (HATs), the

methylation of lysine and arginine residues by histone methyltransferases (HMTs), and the phosphorylation of specific serine groups by histone kinases (HKs). Other histone modifications include attachment of ubiquitination, and sulmolation. Enzymes responsible for the cleavage of some histone modifications, such as histone deacetylases (HDACs), histone phosphatases (PPs), ubiquitin hydrolases (Ubps) and poly (ADP-ribose)glycohydrolases (PARGs), have already been identified (Biel, Wascholowski, and Giannis 2005).

Posttranslational modifications are closely related to fundamental cellular events like the activation and repression of transcription. In the case of histone H3, in general, acetylation of H3 at lysine 14 (H3-K14), phosphorylation of serine 10 (H3-S10), and methylation of H3-K4 leads to transcriptional activation. In contrast, the repression of certain genes is linked to deacetylation of H3-K14 and methylation of H3-K9. The specific combination of these modifications has been termed the histone code, that determines histone–DNA and histone–histone contacts, which may in turn regulate the on or off state of genes or unfolding/folding state of the chromatin structure (Jenuwein and Allis 2001; Esteller 2007).

Histone modifications and other epigenetic mechanisms such as DNA methylation appear to work together in a coordinated and orderly fashion, to establishing and maintaining gene activity states, thus regulating gene transcription (Fischle, Wang, and Allis 2003; Biel, Wascholowski, and Giannis 2005). In the past decade, more and more attention has been paid on histone modifications, which led to the discovery and characterization of a large number of histone-modifying molecules and protein complexes. Alterations of histone-modifying complexes are believed to disrupt the pattern and levels of histone marks and consequently dysregulate the normal control of chromatin-based cellular processes, ultimately leading to oncogenic transformation and the development of cancer (Esteller 2007).

3. NPC as an epigenetic disease

3.1 Hypermethylation of cellular tumor suppressor genes and the dysregulation of the corresponding cellular pathways

NPC distinguish itself from other malignancies by the number of genes targeted for silencing by promoter methylation. Several classic tumor suppressor genes, such as p53 and Rb, are found to be mutated in more than 50% of all the tumors, but were rarely found to be mutated in NPC (Burgos 2003; Chang et al. 2002; Tao and Chan 2007). 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. The full list of genes which have been found to be aberrantly methylated in NPC was summarized in table 1.

Ras signalling

Activated Ras proteins has been shown to play a key role in the development of human cancers (Bos 1989). Ras proteins serve as a node in the transduction of information from a variety of cell surface receptors to an array of intracellular signaling pathways. Mutated variants of Ras (mutations at residues 12, 13 or 61) are found in 30% of all human cancers

(Bos 1989). Mutations at residues 12, 13 or 61 might lock Ras protein in the active state, which mediate a variety of biological effects associated with enhanced growth and transformation. Ras activity is regulated by cycling between inactive GDP-bound and active GTP-bound forms. When GTP-bound, Ras binds to and activates a plethora of effector molecules. GTPase-activating proteins (GAPs), such as p120GAP and NF1, trigger the hydrolysis of GTP back to the inactive GDP-bound form (Boguski and McCormick 1993). Because Ras GAPs switch off Ras signalling, they have always been considered as potential tumor suppressor genes. Recent study reveal that the Ras GTPase-activating-like protein (RASAL), a Ca2+-regulated Ras GAP that decodes the frequency of Ca2+ oscillations, is silenced through CpG methylation in multiple tumors including NPC (Jin, Wang, Ying, Wong, Cui, et al. 2007). In addition, ectopic expression of catalytically active RASAL leads to growth inhibition of NPC cells by Ras inactivation, thus, epigenetically silencing of RASAL is an alternative mechanism of aberrant Ras activation in NPC (Jin, Wang, Ying, Wong, Cui, et al. 2007).

Although it is widely accepted that Ras functions as an oncoprotein, more and more evidence show that Ras proteins may also induces growth arrest properties of cells, such as senescence, apoptosis, terminal differentiation (Spandidos et al. 2002). The growth inhibitory effects of Ras were induced by a group of proteins with Ras binding domain. These proteins were identified as negative effectors of Ras and designated as Ras association domain family (RASSF). Within this super family, the *RASSF1A* and *RASSF2A* gene are frequently inactivated by promoter hypermethylation (Lo et al. 2001; Zhang et al. 2007), functional studies also support their role as putative tumor suppressors in NPC.

The induction of invasiveness and metastasis by Ras were mediated by downstream effectors which are involved in the regulation of cell adhesion, cell-matrix interaction and cell motility, such as RhoGTPases, RalGEF and components of PI3K pathways (Giehl 2005). Recent studies have further indicated that the Ras/PI3K/AKT pathway is associated in several human cancers. Activation of the Ras/PI3K/AKT pathway can occur by many mechanisms, which include activation of Ras, mutation or amplification of *PI3K*, amplification of *AKT*, and mutation/decreased expression of the tumor-suppressor genes *PTEN* and *HIN-1*. The *HIN-1* gene has various biological functions, including inhibiting cell cycle reentry, suppressing migration and invasion, and inducing apoptosis; these effects are mediated by inhibiting AKT signalling pathway (Krop et al. 2005). *HIN-1* gene is hypermethylated in human NPC. Methylated *HIN-1* promoter was found in 77% of primary NPC tumors and not found in the normal nasopharyngeal biopsies. Moreover, methylated *HIN-1* promoter can be detected in 46% of nasopharyngeal swabs, 19% of throat-rinsing fluids, 18% of plasmas, and 46% of buffy coats of peripheral blood of the NPC patients but was not detectable in all normal controls (Wong, Kwong, et al. 2003).

The Ras family shares at least 30% sequence identity with several other small monomeric G protein families, such as the Rho/Rac/CDC42, Rab/Ypt, Ran, Arf, and Rad families (Adjei 2001). The major 8p22 tumor suppressor Deleted in Liver Cancer 1 (*DLC1*) gene is a homologue of rat p122RhoGAP. It was identified as a major downregulated gene in NPC by expression subtraction. By expression subtraction, Qian Tao's group identified that *DLC1* is an 8p22 TSG as a major downregulated gene in NPC. Their study also demonstrated *DLC1* is hypermethylated not only in NPC, but also in esophageal and cervical carcinomas. Downregulation of *DLC1* contributes to NPC oncogenesis by disrupting

Ras-mediated signalling pathways (Seng et al. 2007). Recently, a novel isoform of the *DLC1* gene was identified, which suppresses tumor growth and frequently silenced in multiple common tumors including NPC. This novel isoform encodes an 1125-aa (amino acid) protein with distinct N-terminus as compared with other known *DLC1* isoforms. Similar to other isoforms, *DLC1-i4* is expressed ubiquitously in normal tissues, and epigenetically inactivated by promoter hypermethylation in NPC. The differential expression of various *DLC1* isoforms suggests interplay in modulating the complex activities of *DLC1* during carcinogenesis (Low et al. 2011).

P53 signalling

Altered p53 pathway is common detected in NPC, even though NPC rarely presents abnormality in the p53 gene itself, 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 as it can interact with, and sequester, MDM1, a protein responsible for the degradation of p53 (Ozenne et al. 2010). *p14* is methylated in 20% on NPC, the epigenetic inactivation of *p14/ARF* may facilitate p53 degradation in NPC cells (Kwong et al. 2002). 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 (Kwong et al. 2002; Crook et al. 2000).

Recently, Qian Tao et al. found that *UCHL1* was frequently silenced by promoter CpG methylation in nasopharyngeal carcinoma; and acts as a functional tumor suppressor gene for NPC through stabilizing p53 through deubiquitinating p53 and p14ARF and ubiquitinating MDM2, which is mediated by its hydrolase and ligase activities, further resulting in the induction of tumor cell apoptosis (Li et al. 2010).

Wnt signalling

The Wnt signalling pathway is important for normal development and is frequently aberrantly activated in a variety of cancers. Although the role of the Wnt pathway in NPC has not been fully explored, there is abundant evidence that aberrant Wnt signalling plays a role in NPC development. In a recent study by gene expression profiling, the aberrant expression of the Wnt signalling pathway components, such as wingless-type MMTV integration site family, member 5A, Frizzled homolog 7, casein kinase II beta, β-catenin, CREB-binding protein, and dishevelled-associated activator of morphogenesis 2 was identified and further validated on NPC tissue microarrays (Zeng et al. 2007). Furthermore, most NPC tumors exhibit Wnt pathway protein dysregulation: 93% have increased Wnt protein expression and 75% have decreased expression of Wnt inhibitory factor (WIF), an endogenous Wnt antagonist (Shi et al. 2006; Zeng et al. 2007). These results indicate that aberrant Wnt signalling is a critical component of NPC.

The Wnt inhibitory factor 1 (*WIF1*) gene acts as a Wnt antagonist factor by direct binding to Wnt ligands. In NPC, methylation was frequently observed in 85% of NPC primary tumors, with *WIF1* expressed and unmethylated in normal cell lines and normal tissues. Ectopic expression of WIF1 in NPC cells resulted in significant inhibition of tumor cell colony formation, and significant downregulation of β-catenin protein level in NPC cells. Indicates that epigenetic inactivation of *WIF1* contributes to the aberrant activation of Wnt pathway and is involved in the pathogenesis of NPC (Chan et al. 2007).

Cell cycle and DNA repair

Aberrant apoptosis, as in all malignancies, is also required for NPC development. Inhibition of apoptosis seems to be critical to NPC tumorigenesis. Death-associated protein kinase (*DAPK*) is a Ca/calmodulin-regulated serine/threonine kinase and a positive mediator of apoptosis. Loss of *DAPK* expression was shown to be associated with promoter region methylation in NPC. Methylation of the promoter was found in 76% of NPC, as well as plasma of patients with NPC (Chang et al. 2003). A demethylating agent, 5-aza-2'-deoxycytidine, might slow the growth of NPC cells in vitro and in vivo by reactivating the *DAPK* gene silenced by de novo methylation (Kong et al. 2006).

Like all cancers, development of NPC requires the derangement of the normal cell cycle. Several classical CDK inhibitors in G1 -S checkpoint, such as p16/INK4A, p15/INK4A, and p14/ARF, were demonstrated to be hypermethylated in NPC and act as tumor suppressors during NPC development (Li, Shu, et al. 2011).

Dysregulation of the DNA repair system by DNA methylation is also an essential event in NPC development (Lo, To, and Huang 2004; Tao and Chan 2007). MGMT is a DNA repair protein that removes mutagenic and cytotoxic adducts from O6-guanine in DNA. Frequent methylation of *MGMT* associated with gene silencing occurs in human cancers. However, only a small portion (28%) of primary NPC were *MGMT* hypermethylated (Wong, Tang, et al. 2003). A rather high frequency (40%) of hypermethylation of the DNA mismatch repair gene *hMLH1* was observed in NPC primary tumors (Wong, Tang, et al. 2003). But methylation of *hMLH1* cannot be detected in the plasma of NPC patients (Wong et al. 2004).

Chromosomal instability (CIN) is a cytogenetic hallmark of human cancers (Cheung et al. 2005; Lengauer, Kinzler, and Vogelstein 1998). Increasing evidence suggests that impairment of mitotic checkpoint is causally associated with CIN. Several chromosomal aberrations have been identified in NPC. Some sites correspond to proteins key to NPC development, including p16, RASSF1A, and CKIs, while a number of sites do not correspond to any known tumor suppressors or oncogenes (Li, Shu, et al. 2011). CHFR is one of the mitotic checkpoint regulators and it delays chromosome condensation in response to mitotic stress. *CHFR* mRNA was significantly decreased or undetectable in NPC cell lines as well as human NPC xenografts, hypermethylation of *CHFR* promoter was strongly correlated with decreased CHFR expression in NPC cell lines and xenografts (Cheung et al. 2005). And hypermethylation of *CHFR* promoter region was detected in 61.1% (22 out of 36) of primary NPC tumors while it was absent in non-malignant tissues (Cheung et al. 2005).

Cell adhesion

Multiple cell adhesion molecules involve in intercellular and cell-extracellular matrix interactions of cancer. Cancer progression is a multi-step process in which some adhesion molecules play a pivotal role in the development of recurrent, invasion, and metastasis. Alterations in the adhesion properties of cancer cells play an essential role in the development and progression of cancer. Loss of intercellular adhesion allows malignant cells to escape from their site of origin, degrade the extracellular matrix, acquire a more motile and invasion phenotype, and finally, invade and metastasize. In NPC, epigenetic mechanism was involved in the abnormal cell adhesion, a diverse of molecules such as

cadherins, connexins, and other components of cell adhesion are dysregulated (Du et al. 2011; Sun et al. 2007; Ying et al. 2006; Huang et al. 2001; Lou, Chen, Lin, et al. 1999; Xiang et al. 2002).

Cadherins have strong implications in tumorigenesis through cadherin-mediated cell–cell adhesion, which maintains tissue integrity and homeostasis. Disruption of this organized adhesion by genetic and epigenetic mechanisms during carcinogenesis might result in changes in signal transduction, loss of contact inhibition, and altered cell migration and stromal interactions. Some of the cadherins, such as E-cadherin and H-cadherin, were characterized as TGSs, which inhibit tumor invasion and metastasis (Berx and van Roy 2009; Jeanes, Gottardi, and Yap 2008). Disruption of cadherin expression and inappropriate switching among cadherin family members by genetic or epigenetic mechanisms are key events in the acquisition of the invasive phenotype for many tumors. The E-cadherin gene is silenced by promoter hypermethylation in human NPC because of aberrant expression of DNMT induced by the Epstein-Barr virus-encoded oncoprotein latent membrane 1 (Tsai et al. 2002). Moreover, loss of E-cadherin expression is significantly associated with histological grade, intracranial invasion and lymph node and distant metastasis (Lou, Chen, Sheen, et al. 1999). Three other members of the cadherin family: *CDH13*, *CDH4* and *PCDH10*, are involved in NPC owing to promoter methylation (Sun et al. 2007; Ying et al. 2006; Du et al. 2011). This evidence indicates a deep involvement of epigenetic regulation of the cadherin family in the carcinogenesis of NPC.

Intercellular communication through gap junction (GJIC) have a significant role in maintaining tissue homeostasis and has long been proposed as a mechanism to regulate growth control, development and differentiation. Reduced GJIC activity has long been implicated in carcinogenesis. Loss of GJIC leads to aberrant proliferation and an enhanced neoplastic phenotype. Reduced expression of the connexin (Cx) genes dysregulation of GJIC activity were observed in a series of human cancers. Thus, some Cx genes have been suggested as tumor suppressor genes (Pointis et al. 2007). Down-regulation of connexin 43 (*Cx43*) expression and dysfunctional GJIC were demonstrated in NPC tissues and cells, suggesting that dysfunctional GJIC plays a key role in nasopharyngeal carcinogenesis (Shen et al. 2002; Xiang et al. 2002). Further study revealed that inactivation of *Cx43* gene was mediated by epigenetic mechanism of promoter hypermethylation in NPC. Treatment of DNA methyltransferase inhibitor 5-aza-2'-deoxycytidine could induce restoration of GJIC and an inhibition of tumor phenotype of CNE-1 cells (Yi et al. 2007).

MMPs are type IV collagenases whose overexpression has been implicated in a number of cancers. MMPs can not only degrade basement membranes and extracellular matrices to allow for tumor invasion, they are also involved in activation of growth factors to promote cell growth and angiogenesis, and also protect tumor cells from apoptotic signals (Gialeli, Theocharis, and Karamanos 2011). In NPC, MMP1, MMP3 and MMP9 were shown to be up-regulated by LMP1 (Stevenson, Charalambous, and Wilson 2005; Kondo et al. 2005; Lee et al. 2007). While MMP19 appears to be down-regulated in 69.7% of primary NPC specimens (Chan et al. 2010). Allelic deletion and promoter hypermethylation contribute to MMP19 down-regulation. The catalytic activity of MMP19 plays an important role in anti-tumor and anti-angiogenesis activities (Chan et al. 2010).

OPCML (opioid binding protein/cell adhesion molecule-like gene), also known as *OBCAM* (opioid binding cell adhesion molecule), belonging to the IgLON family of glycosylphosphatidylinositol (GPI)-anchored cell adhesion molecules involved in cell adhesion and cell-cell recognition. Located at 11q25, *OPCML* was the first IgLON member linked to tumorigenesis. In NPC, the *OPCML-v1* were observed to be epigenetically inactivated, what's more, the methylation was detected in a remarkable frequency: 98% of NPC tumor tissues. The high incidence of epigenetic inactivation of *OPCML* in NPC indicates that *OPCML* methylation could be an epigenetic biomarker for the molecular diagnosis of NPC (Cui et al. 2008).

Cancer-related process	Gene	Full name	Chromo-somal location	Function	Refs
Cell cycle	*CDKN2B/ P15/MTS2/T P15/ INK4B*	Cyclin-dependent kinase inhibitor 2B	9p21	Cyclin-dependent kinase inhibitor for CDK4 and CDK6, a cell growth regulator of cell cycle G1 progression	(Wong, Tang, et al. 2003; Chang et al. 2003; Wong et al. 2004; Li, Shu, et al. 2011)
	CDKN2A/ P16/INK4A/ MTS1/ CDK4I/ CDKN2	Cyclin-dependent kinase inhibitor 2A	9p21	Cell cycle regulation	(Wong et al. 2004; Chang et al. 2003; Wong, Tang, et al. 2003; Lo et al. 1996; Li, Shu, et al. 2011)
	CHFR/ RNF116/ RNF196	Checkpoint with forkhead and ring finger domains	12q24.33	Mitotic checkpoint regulator early in G2-M transition	(Cheung et al. 2005; Li, Shu, et al. 2011)
	BRD7	Bromodomain containing 7	16q12	Transcriptional regulation, inhibits G1-S transition	(Liu et al. 2008)
	FHIT/ FRA3B/ 3P3Aase	Fragile histidine triad gene	3P14.2	Cell-cycle regulation, G1-S phase checkpoint, DNA-damage response, nucleotide and nucleic acid metabolism	(Loyo et al. 2011)
	GADD45G	Growth arrest and DNA-damage-inducible, gamma	9q22	Inhibits G1-S and G2-M transition, apoptosis	(Ying et al. 2005)
	DLEC1	Deleted in lung and esophageal cancer1	3p22-21.3	G1 cell cycle arrest	(Ayadi et al. 2008)
	ZMYND10/ BLU	Zinc finger, MYND-type containing 10	3P21.3	Cell cycle	(Liu et al. 2003)
	MIPOL1	Mirror-image polydactyly1	14q13.1	Negative regulator of G1 progression	(Cheung et al. 2009)
	PRDM2/ PRDM2	PR domain containing 2, with ZNF domain	1p36.21	G2-M cell cycle arrest	(Chang et al. 2003)

Cancer-related process	Gene	Full name	Chromosomal location	Function	Refs
	LTF	Lactoferrin	3p21.3	Cell cycle regulation	(Yi et al. 2006; Zhang et al. 2011)
	CCNA1	Cyclin A1	13q12.3-q13	An important regulator of the cell cycle required for S phase and passage through G_2	(Yanatatsaneejit et al. 2008)
	PTPRG	Receptor-type tyrosine-protein phosphatase gamma	3p14-21	Cell cycle regulator via inhibition of pRB phosphorylation through down-regulation of cyclin D1	(Cheung et al. 2008)
	TP73	Tumor protein p73	1p36.3	Cell cycle, DNA damage response, apoptosis, transcription factor	(Wong, Tang, et al. 2003)
Apoptosis	DAPK	Death-associated protein kinase	9p34.1	Positive mediator of gamma-interferon induced apoptosis	(Wong et al. 2004; Chang et al. 2003; Kwong et al. 2002; Li, Shu, et al. 2011)
	CASP8/CAP4/MACH/MCH5/FLICE	Caspase 8, apoptosis-related cysteine peptidase	2q33-q34	Apoptosis	(Li, Shu, et al. 2011; Wong, Tang, et al. 2003)
	GSTP1/DFN7/GST3	Glutathione S-transferase pi 1	11q13	Apoptosis, metobolism, energy pathways	(Kwong et al. 2002; Li, Shu, et al. 2011)
	CMTM3	CKLF like MARVEL transmembrane domain-containing member 3	16q21	Induces apoptosis with caspase-3 activation	(Wang et al. 2009)
	CMTM5	CKLF like MARVEL transmembrane domain-containing member 5	14q11.2	Induces apoptosis with activation of caspase 3, 8 and 9, synergistic effects with TNF-α.	(Shao et al. 2007)
	ZNF382	Zinc finger protein 382	19q13.12	Key regulator of cell proliferation, differentiation, and apoptosis, repress NF-kB and AP-1 signaling	(Cheng et al. 2010)
	TNFRSF11B/OPG	Tumor necrosis factor receptor superfamily, member 11b	8q24	Induced apoptosis, inhibits tumor growth specifically in bones	(Lu et al. 2009)
	PLA2G16/HRASLS3	Phospholipase A2, group XVI	11q12.3	Proapoptotic function through the inhibition of PP2A	(Yanatatsaneejit et al. 2008)

Cancer-related process	Gene	Full name	Chromosomal location	Function	Refs
Invasion and metastasis	*CDH1*	Cadherin 1, type 1, E-cadherin (epithelial)	16q22.1	Calcium-dependent adhesion and cell migration	(Wong, Tang, et al. 2003; Wong et al. 2004)
	CDH13	Cadherin 13, H-cadherin (heart)	16q23.3	Calcium-dependent adhesion and cell migration	(Sun et al. 2007)
	PCDH10 /OL-PCDH /KIAA1400	Protocadherin 10	4q28.3	Calcium-dependent adhesion and cell migration	(Ying et al. 2006)
	CDH4	Cadherin 4, type 1, R-cadherin (retinal)	20q13.3	Calcium-dependent adhesion and cell migration	(Du et al. 2011)
	OPCML	Opioid binding protein/cell adhesion molecule	11q25	Cell adhesion, cell-cell recognition	(Cui et al. 2008)
	TFPI-2	Tissue factor pathway inhibitor-2	7q22	Serine protease inhibitor	(Wang et al. 2010)
	MMP19	Matrix metalloproteinase-19	12q14	Extra cellular matrix	(Chan et al. 2010)
	THBS1	Thrombospondin 1	15q15	An adhesive glycoprotein that mediates cell-to-cell and cell-to-matrix interactions	(Wong, Tang, et al. 2003)
	Cx43	Connexin 43	20q11	Gap junction and intercellular communication	(Yi et al. 2007)
	TSLC1 /CADM1	Tumor suppressor in lung cancer 1	11q23	Cell adhesion molecules, mediate cell-cell interaction	(Hui et al. 2003; Lung et al. 2004)
	ADAMTS18	ADAM metallopeptidase with thrombospondin type 1 motif, 18	16q23.1	Cell adhesion modulator, inhibits growth factor-independent cell proliferation	(Jin, Wang, Ying, Wong, Li, et al. 2007; Wei et al. 2010)
	THY1/CD90	Thy-1 cell surface antigen	11q23.3	Regulates cytoskeletal organization, focal adhesion and migration by modulating the activity of p190 RhoGAP and Rho GTPase	(Lung et al. 2005)
DNA repair	*MGMT*	O-6-methyoguanine-DNA methyltransferase	10q26	Repair alkylated guanine	(Kwong et al. 2002)
	MLH1 /hMLH1 /HNPCC /FCC2	MutL homolog 1, colon cancer, nonpolyposis type 2 (E. coli)	3p21.3	DNA mismatch repair protein, cell cycle G2-M arrest	(Wong, Tang, et al. 2003; Wong et al. 2004)

Cancer-related process	Gene	Full name	Chromosomal location	Function	Refs
Signal transduction	*ARF/P14*	Alternate open reading frame	9p21	Stabilizes p53, interacts with MDM2	(Li, Shu, et al. 2011; Wong, Tang, et al. 2003)
	RASSF1A	Ras association (RalGDS/AF-6) domain family member 1A	3p21.3	Regulate Ras signaling pathway	(Chow et al. 2004; Zhou et al. 2005)
	RASFF2A	Ras association (RalGDS/AF-6) domain family member 2A	20p12.1	Regulate Ras signaling pathway	(Zhang et al. 2007)
	WIF-1	Wnt inhibitory factor-1	12q14	Antagonist of Wnt signaling	(Lin et al. 2006; Chan et al. 2007)
	DLC-1	Deleted in liver cancer-1	8p21.3-22	GTPase-activating protein specific for RhoA and Cdc42	(Peng et al. 2006)
	DAB2	Disabled homolog 2, mitogen-responsive phosphoprotein (Drosophila)	5p13	Adaptor molecule involved in multiple receptor-mediated signaling pathways	(Tong et al. 2010)
	RASAL1	RAS protein activator like 1 (GAP1 like)	12q23-q24	Ras GTPase-activating protein, negatively regulates RAS signaling	(Jin, Wang, Ying, Wong, Cui, et al. 2007)
	UCHL1	Ubiquitin carboxyl-terminal esterase L1	4p14	Stabilize p53 and activate the p14^{ARF}-p53 signaling pathway	(Li et al. 2010)
	SFN/14-3-3 σ	Stratifin	1p36.11	Downstream target of p53, negative regulator of G2-M phase checkpoint	(Yi et al. 2009)
Angio-genesis	*EDNRB*	Endothelin receptor type B	13q22	Negative regulator of ET/ETAR pathway	(Lo et al. 2002; Zhou et al. 2007)
	ADAMTS9	A disintegrin-like and metallopeptidase with thrombospondin type 1 motif 9	3p14.1	Anti-angiogenesis	(Lung, Lo, Xie, et al. 2008)
	FBLN2	Fibulin 2	3p25.1	Angiogenesis suppression via concomitant downregulation of vascular endothelial growth factor and matrix metalloproteinase 2	(Law et al. 2011)

Cancer-related process	Gene	Full name	Chromo-somal location	Function	Refs
Vitamin response	*RARβ2*	Retinoic acid receptor beta 2	3q24	Binds retinoic acid to mediates cellular signaling during embryonic morphogenesis, cell growth and differentiation	(Kwong, Lo, Chow, To, et al. 2005; Kwong et al. 2002; Seo, Kim, and Jang 2008)
	RARRES1 /TIG1	Retinoic acid receptor responder (tazarotene induced) 1	3q25	Retinoic acid target gene	(Yanatatsaneejit et al. 2008; Kwong, Lo, Chow, Chan, et al. 2005; Kwok et al. 2009)
	CRBP I /RBP1	Cellular retinol binding protein 1	3q23	Draws retinol from blood stream into cells, solubilizes retinol and retinal, protects cells from membranolytic retinoid action	(Kwong, Lo, Chow, To, et al. 2005)
	CRBP IV	Cellular retinol binding protein 4	1p36.22	Similar to CRBP1	(Kwong, Lo, Chow, To, et al. 2005)
Tissue develo-pment and differe-ntiation	*Myocd*	Myocardin	17p11.2	Transcription factor, involved in smooth muscle cell differentiation	(Chen et al. 2011)
	HIN1 /SCGB3A1	High-in-normal-1	5q35	Involved in epithelial cell differentiation, cell-cycle reentry regulator, suppresses tumor cell migration and invasion, induces apoptosis	(Wong, Kwong, et al. 2003)
Others	*NOR1*	Oxidored-nitro domain-contrining protein 1	1p34.3	Interaction partner of the mitochondrial ATP synthase subunit OSCP/ATP5O protein, a stress-responsive gene	(Li, Li, et al. 2011)
	LARS2	Leucyl-tRNA synthetase 2, mitochondrial	3p21.3	Essential roles in group I intron RNA splicing and protein synthesis within the mitochondria, indirectly required for mitochondrial genome maintenance	(Zhou et al. 2009)
	CRYAB	Crystallin,alpha B	11q23.1	An important nuclear role in maintaining genomic integrity	(Lung, Lo, Wong, et al. 2008)

Table 1. List of methylated tumor suppressor genes involved in nasopharyngeal carcinoma (NPC)

3.2 Epstein-Barr virus and DNA methylation

EBV is a prototype of gamma herpes virus which was discovered more than 40 years ago from Burkitt's lymphoma, a childhood tumor that is common in sub-Saharan Africa. Further studies reveal that EBV was widespread in all human populations, which infects more than 90% of the world's adult population. Human are the only natural host for EBV. Once infected with EBV, the individual remains a lifelong asymptomatic carrier of the virus (Young and Rickinson 2004).

EBV was implicated in a variety of human malignancies, such as post-transplant lymphoma, AIDS-associated lymphomas, Burkitt lymohoma, Hodgkin's disease, T-cell lymphoma, NPC, parotid gland carcinoma and gastric carcinoma (Young and Rickinson 2004; Pattle and Farrell 2006). The association between EBV infection and NPC was well documented by the fact that EBV genome presents in virtually all the NPC cells (Lo and Huang 2002; Lo, To, and Huang 2004). Tumorigenesis of NPC is proposed to be a multistep process. EBV may play an important role in the etiology of the 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. Expression of latent genes in the EBV-infected cells may enhance its transformation capacities, and subsequently, clonal expansion may result in the rapid progression to invasive carcinoma.

There are two alternative states of EBV infection: lytic and latent (Young and Rickinson 2004; Fernandez et al. 2009). 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 limited set of viral products, 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). Expression of different panels of latent gene transcripts is controlled by usage of three distinct EBV nuclear antigen (EBNA) promoters (Wp, Cp, and Qp). In established lymphoblastoid cell lines (LCLs), the EBNA transcripts are initiated at the C promoter, Cp, located to the BamHI C fragment of the viral genome. In EBV genome, W promoter (Wp) is the first promoter to be activated immediately after EBV infection of human B cells, but it undergoes progressively methylation and switches off in LCLs. In parallel, an unmethylated promoter, Cp, is switched on. In other EBV-carrying cell types, Cp is switched off. These include memory B cells, Burkitt's lymphomas (BLs), EBV-associated carcinomas (NPC, gastric carcinoma) and Hodgkin's lymphomas; these cells typically use the Q promoter (Qp) for expression of EBNA1 transcripts, but not the transcripts coding for the other five EBNAs, and may differ from each other regarding the expression of LMPs, BARTs (BARF0 and BARF1) and EBV-encoded microRNAs (Li and Minarovits 2003). LMP1 is the major EBV oncoprotein in NPC (Tao and Chan 2007; Lo, To, and Huang 2004). 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 (Eliopoulos and Young 2001). LMP1-expressing NPCs show different growth pattern and prognosis from those without LMP1 expression (Hu et al. 1995). Although EBV genome presents in virtually all the NPC cells, expression of LMP1 is variable in NPC: LMP1 is expressed in only approximately 65% of NPC biopsies (Fahraeus et al. 1988; Young et al. 1988). This variability can be related to the

methylation status of the regulatory sequences (LRS, LMP1 regulatory sequence) located 5′ from LMP1p, as LMP1 is expressed in NPCs with unmethylated LRS but is absent from NPCs with highly methylated LRS. A good correlation exists between LRS methylation and silencing of LMP1p in EBV-carrying lymphoid cell lines and tumors as well (Li and Minarovits 2003)).

On the other hand, EBV regulates the expression of critical cellular genes using cellular DNA methylation machinery. LMP1 has been shown to interacting with methyltransferase and further induce the cellular gene E-cadherin (*CDH1*) promoter methylation. Increased methylation may occur through the activity of DNA methyltransferases 1, 3a, and 3b that in turn are induced through JNK/AP1 signalling by LMP1. Transfection of LMP1 into cancer cells suppressed E-cadherin expression, thereby facilitating a more invasive growth of NPC cells (Tsai et al. 2006). It will be interesting to discover novel target genes regulated by epigenetic mechanism of EBV.

3.3 MicroRNAs in the development of NPC

MicroRNAs (miRNAs) are short non-coding RNA molecules of about 20-23 nucleotides in length, involved in post-transcriptional gene regulation. In animals, miRNAs control the expression of target genes by inhibiting translation or degradating target mRNAs through binding to their 3′UTR. MicroRNAs are involved in regulating a broad range of biological processes, such as development, differentiation, proliferation, apoptosis, and signal transduction pathways often deregulated in cancers. Some miRNAs can function as tumor suppressors or oncogenes (McManus 2003; Ventura and Jacks 2009).

Several biological pathways that are well characterised in cancer are significantly targeted by the downregulated miRNAs. These pathways include TGF-Wnt pathways, G1-S cell cycle progression, VEGF signalling pathways, apoptosis and survival pathways, and IP3 signalling pathways (Chen et al. 2009). Several known oncogenic miRNAs, such as miR-141 (Zhang et al. 2010) miR-17-92 cluster and miR-155 (Chen et al. 2009)were found to significantly up-regulated in NPC tumors. While some tumor suppressive miRNAs, including miR-34 family, miR-143, and miR-145, miR-218 (Alajez et al. 2011), mir-29c, miR-200a, miR-26a and let-7 (Wong et al. 2011) are significantly down-regulated in NPC. Among them, let-7 inhibits cell proliferation through down-regulation of c-Myc expression while miR-26a inhibits cell growth and tumorigenesis through repression of another oncogene: *EZH2* (Lu et al. 2011).

EBV is reported to be present in almost all NPCs and can transform cells, which subsequently induces cell proliferation and tumor growth. In addition to EBV-encoded protein-coding genes such as EBNA1 and LMP1, NPC cells and tissues also express high levels of non-coding EBV RNAs, including EBER1, EBER2 and multiple microRNAs (miRNAs). EBV was the first human virus found to encode microRNAs (Barth, Meister, and Grasser 2011). By small RNA cloning and sequencing, Zhu JY et al. characterized the miRNA expression profile of NPC tissues. Their study revealed an NPC-specific miRNA signature. EBV expresses all miRNAs from the BART cluster in NPC tissues, while no miRNA originating from the BHRF1 region of the EBV genome was found. Their study suggested that BART-derived miRNAs may have an important function in maintaining the virus in NPC tissues, whereas BHRF1 origin miRNAs might not be required for NPC

pathogenesis. In the same study, they also identified two novel and highly abundant EBV miRNA genes, namely, miR-BART21 and miR-BART22 (Zhu et al. 2009). A parallel study demonstrated that LMP2A is the putative target of miR-BART22 in NPC. LMP2A is a potent immunogenic viral antigen that is recognized by the cytotoxic T cells, down-modulation of LMP2A expression by miR-BART22 may permit escape of EBV-infected cells from host immune surveillance (Lung et al. 2009). Similar regulations were also addressed on LMP1: EBV-encoded BART miRNAs target the 3′ UTR of the LMP1 gene and negatively regulate LMP1 protein expression. These miRNAs also modulate LMP1-induced NF-κB signalling and alleviate the cisplatin sensitivity of LMP1-expressing NPC cells (Lo et al. 2007).

4. Epigenetic alternations in relation to clinical parameters of NPC, and their roles as biomarkers

Frequent aberrantly methylated TSGs in tumors have been used as molecular markers for the detection of malignant cells from various clinical materials. It provides possibilities of both cancer early detection and dynamic monitoring of cancer patients after treatment (Schulz 2005).

DNA methylation biomarkers hold a number of advantages over other biomarker types, such as proteins, gene expression and DNA mutations (Balch et al. 2009; Laird 2003). Methylated DNA sequences are more chemically and biologically stable, and more easier to be amplified, thus greatly enhancing detection sensitivity. DNA methylation are often cancer specific, and restriction to limited regions of DNA in the CpG islands. Compared to genetic alternations such as gene mutation or amplification, aberrant methylation on TSG promoters is rather prevalent and tumor-specific among NPCs. As mentioned above, NPC tumor progression is well characterized by a number of combinatorial epigenetic aberrations distinct to other malignancy, including DNA methylation of more than 30 genes. Consequently, these methylated DNA sequences represent potential biomarkers for diagnosis, staging, prognosis and monitoring of response to therapy or tumor recurrence (Balch et al. 2009; Laird 2003).

4.1 DNA methylation, results from tumor tissues

It has been shown that some genes are high frequently methylated in tumor tissue DNA obtained from NPC primary tumors, but not in normal tissues (Pan et al. 2005; Sun et al. 2007; Zhang et al. 2007; Li, Shu, et al. 2011). These genes are ideal candidate to serve as biomarkers for detection of NPC. Some of these TSGs are not only methylated in NPC, but also commonly methylated in other cancers. So methylation assessment of single genes lacks sufficient specificity for NPC diagnosis. It is believed that panels of multiple methylation biomarkers may achieve higher accuracy required for discriminate NPC from other cancers (Kwong et al. 2002; Hutajulu et al. 2011). This notion was supported by a study of Esteller et al, which showed that a panel of three to four markers could define an abnormality in 70–90% of each cancer type through detecting their aberrant methylation (Esteller et al. 2001). Some studies have been conducted using different combination of gene panels, though there is overlap among them. Combination of methylation markers not only improved the discrimination between NPC and non-NPC diseases, but also the sensitivity of cancer

detection. The detection rate can reach 98% when combined analysis of five methylation markers (*RASSF1A, p16, WIF1, CHFR* and *RIZ1*) in a recent study (Hutajulu et al. 2011).

4.2 Methylation markers in circulating DNA

Cancer specific DNA methylation can be detected in tumor-derived free DNA in the bloodstream, e.g. in serum or plasma. High frequency of methylated *DAPK* gene were found not only in NPC tumors, but also could be detected in plasma and buffy coat of NPC patients (Wong et al. 2002). Methylated DNA was detectable in plasma of NPC patients before treatment including 46% for *CDH1*,42% for *CDH1*, 42% for *p16*, 20% for *DAPK* ,20% for *p15 and 5%* for *RASSF1A*. Aberrantly hypermethylated promoter DNA of at least one of the five genes was detectable in 71% of plasma of NPC patients before treatment. Hypermethylated promoter DNA of at least one of the three genes (*CDH1*, *DAPK1*, and p16) was detectable in post-treatment plasma of 38% recurrent NPC patients and none of the patients in remission. Suggesting that cell-free circulating methylated DNA might be a useful serological marker in assisting in screening of primary and potentially salvageable local or regional recurrent NPC (Wong et al. 2004).

4.3 Methylation markers in other body fluids and nasopharyngeal swabs

In addition to tissue analysis, methylated DNA has been detected in the mouth and throat rinsing fluid, saliva and nasopharyngeal swabs of NPC patients. Methylated DNA found in cancer patient serum correlated reasonably well with methylation levels in tumor tissue, and it is also believed that the source of serum DNA is necrotic tumor cells. Hypermethylated *RIZ1* gene was detected in 60% of NPC primary tumors, but not in any of the normal controls. Of 30 matched body fluid samples, methylated *RIZ1* DNA was found in 37% of NP swabs, 30% of rinsing fluid, 23% of plasma, and 10% of buffy coat samples. The results in NPC tumor and NP swab samples from the same patients show good concordance. Our early study also reported that the high sensitivity (81%) and specificity (0% false positives) of detecting aberrant methylation of *CDH13* (encoded a cell adhesion molecule H-cadherin) from nasopharyngeal swabs suggested it could be utilized as a tool for early diagnosis.

5. DNA methylation modification as therapeutic targets in NPC

DNA methylation plays important roles in NPC carcinogenesis, including the silencing of cellular TSGs and some EBV encoded genes. The EBV encoded oncoprotein, LMP1, has been shown to interacting with methyltransferase (DNMT) and further induce the cellular gene E-cadherin promoter methylation (Tsai et al. 2006). And DNA methylation also suppresse EBV encoded genes, including the LMP1, immediate-early lytic antigens Zta and Rta, and some EBV immunodominant antigens (EBNA2,3A, 3B, 3C) (Paulson and Speck 1999; Tierney et al. 2000; Salamon et al. 2001). Thus, DNA methylation also plays an important role in the maintenance of specific EBV latency programmers and regulating EBV lifecycle and latency in NPC cells.

DNA methylation is a reversible phenomenon. Reactivating methylated and silenced cellular tumor suppressor genes and immunodominant tumor/viral antigens by

demethylating agents might restore normal cell growth control, or induce cell immunity against cancer cells. Demethylating agents would also reactivate the expression of EBV early and lytic genes in latently infected NPC cells, which will lead to further tumor cell death.

Epigenetic therapeutic agents include DNA methyltransferase inhibitors and histone deacetylase (HDAC) inhibitors. 5-Azacytidine and 5-aza-2′-deoxycytidine are the most widely studied DNMT inhibitors. Clinical trials using such agents have been carried out on a series of cancer patients. In several phase I/II/III studies, decitabine (5-aza-2′-deoxycytidine) has also shown promising data in patients with MDS and AML (Kantarjian et al. 2007; Issa et al. 2004). In patients with NPC and EBV-positive AIDS-associated Burkitt lymphoma, azacitidine effectively induces demethylation of all the latent and early lytic EBV promoters and some viral antigens, indicated the potential of epigenetic therapy for NPC (Chan et al. 2004).

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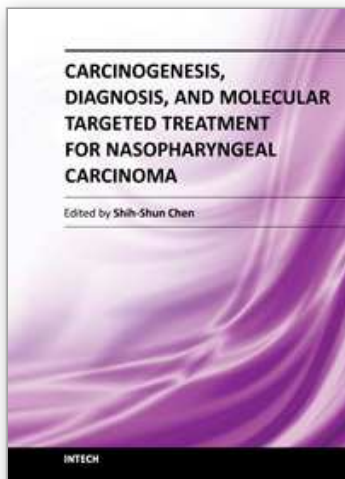
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http://www.intechopen.com/books/carcinogenesis-diagnosis-and-molecular-targeted-treatment-for-nasopharyngeal-carcinoma/epigenetics-of-nasopharyngeal-carcinoma

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