CHAPTER 5

ACQUIRED GENETIC AND EPIGENETIC ALTERATIONS IN NASOPHARYNGEAL CARCINOMA

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Abstract: Nasopharyngeal carcinoma (NPC) has a distinct geographic distribution and strong association with Epstein-Barr virus (EBV). Recent advances in molecular investigations and bioinformatics have disclosed critical genetic and epigenetic events in NPC. In this chapter, we will focus on important genetic and epigenetic alterations in NPC derived from EBV positive NPC cell lines and human tumoral tissues. Copy number losses on chromosomes 1p, 3p, 9p, 9q, 11q, 13q, 14q and 16q and recurrent gains on chromosome 1q, 3q, 8q, 12p and 12q were frequently observed in NPC. The roles of several important tumor suppressors (e.g., *p16, RASSF1A*) and oncogenes (e.g., *CCND1, LTβR*) have been delineated. However, potential critical cancer associated genes in other chromosomal regions remain to be identified. Frequent wide-spread methylation of cancer related genes is another common phenomenon in NPC and leads to alterations of multiple cellular pathways. The possible mechanisms of NPC tumorigenesis, in particular the roles of EBV latent gene products, have been suggested. There is also emerging information concerning the disruption of various signaling pathways including NF-κB signaling pathways in NPC. NPC serves as a fascinating model to understand the complex interaction among environmental, viral, and genetic factors in human tumorigenesis. Important genetic and epigenetic alterations in NPC are summarized in this chapter. Based on these observations, a hypothetical model of NPC tumorigenesis is proposed and serves as a platform for continuous refinement.

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INTRODUCTION

Nasopharyngeal carcinoma (NPC) is remarkable for its striking geographic and racial distribution. NPC is prevalent in Southern China, Southeast Asia and North Africa. More than 80% of new cases are detected in these endemic regions. In Southern China, the incidence rate is about 25-50 per 100,000 persons per year and is 100-fold higher than that in the Western population.[1,2] The etiology of NPC is multifactorial. Genetic susceptibility and environmental risk factors, including intake of preserved foods (e.g., salted fish) at an early age and Epstein-Barr virus infection, are implicated in NPC tumorigenesis.[3] These factors may directly or indirectly contribute to the acquired genetic and epigenetic alterations that are responsible for initiation and progression of NPC. This distinctive-type of head and neck cancer may serve as an interesting model for molecular carcinogenesis. Recent advances in molecular genetics and bioinformatics have revealed multiple molecular alterations in NPC. In this chapter, we will focus on critical genetic and epigenetic abnormalities in NPC tumorigenesis and their roles in disrupting normal cellular mechanisms and signaling pathways in nasopharyngeal epithelial cells. The possible contribution of environmental and viral factors in inducing somatic genetic changes and transformation of NPC cells are proposed in a new NPC tumorigenesis model.

KARYOTYPIC AND MOLECULAR ANALYSIS OF NPC

According to WHO classification, there are three subtypes of NPC: Type I (keratinizing squamous cell carcinoma), Type II (nonkeratinizing carcinoma) and Type III (undifferentiated carcinoma). In endemic regions, majority of NPC are Type III and II and these subtypes show consistent association with Epstein-Barr virus infection. EBV infection is present in virtually all cancer cells. The monoclonal nature of EBV genome in invasive carcinoma implies that EBV latent infection may occur prior to the expansion of the malignant clone.[4,5] Expression of EBV latent genes might be critical for initiation and progression of NPC through interacting cellular molecules or directly inducing genetic and epigenetic changes. Therefore, in this chapter, we will focus on the findings reported in EBV-positive NPC tumor lines and primary tumors.

Cytogenetic and molecular alterations in NPC genome have been explored since late 1980s. Cytogenetic information is limited since primary tumors grow poorly in vitro and only a few EBV-positive NPC lines are available. The pioneering cytogenetic works in EBV-positive undifferentiated NPC xenografts from Huang et al[6] and Bernheim et al[7] provided the first piece of information concerning chromosomal abnormalities in NPC. These EBV-positive tumor lines remain important models for molecular and functional analysis for this viral-associated malignant disease. Despite the many complex re-arrangement found, recurrent structural abnormalities on chromosomes 1, 3p, 11q, 12 and 17 were observed.[6-8] Deletion of chromosome 3p was consistently found in NPC cell line, xenografts, and primary tumor biopsies in these studies. Strikingly, the modern molecular cytogenetic and genetic studies have subsequently proven that inactivation of tumor suppressor gene on this chromosomal region is one of most critical molecular events in NPC tumorigenesis.[8-12] By genome-wide screening approaches, including allelotyping/LOH analysis and comparative genome hybridization (CGH), detailed "genome map" for cataloguing genetic alterations in NPC has been established (Fig. 1). A number of recurrent chromosomal abnormalities identified by CGH studies suggested

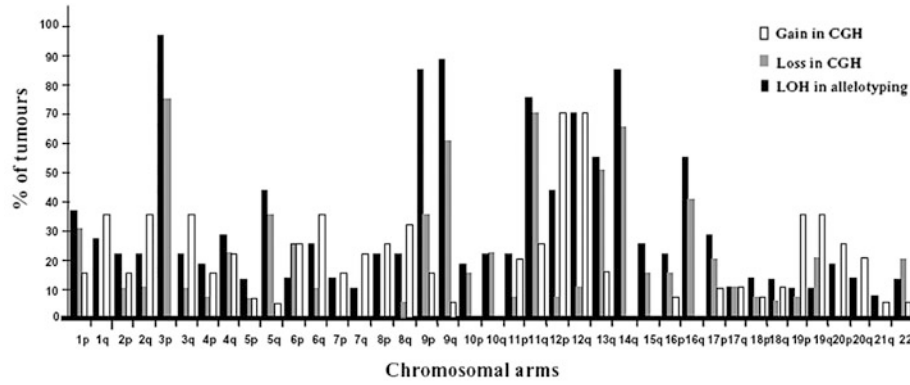

Figure 1. Frequencies of LOH, CGH gain and loss in microdissected NPC tumors.

the involvement of multiple genetic defects in NPC tumorigenesis. High incidences of copy number losses were detected on chromosomes 1p, 3p, 9p, 9q, 11q, 13q, 14q and 16q, while recurrent gains on chromosome 1q, 3q, 8q, 12p and 12q were observed in Chinese NPC patients.[8,9,11,12] Interestingly, distinct regions of gain at 11q13 and 12p12-13 were identified in 53% and 59% primary tumors respectively in a study of Taiwan NPC patients.[11] The findings were confirmed in a recent high-resolution array-based CGH analysis.[13] The tree models constructed by multiple sets of CGH data predicted 3p deletion and chromosome 12p gain as important early events.[14,15] By high-resolution allelotyping study, genome abnormalities on similar chromosomal regions were also demonstrated in a panel of microdissected primary NPCs. Moreover, allelic losses on chromosomes 3p, 9p and 14q were consistently detected in more than 85% of primary tumors.[10] Importantly, high incidence of 3p/9p LOH was also found in the precancerous lesions.[16,17] These findings suggested that the inactivation of tumor suppressor genes in these regions is critical events for transformation of nasopharyngeal epithelial cells.

In addition to deciphering the global genetic changes, these CGH and LOH studies have defined multiple minimal regions (e.g., 3p21, 9p21.3, 11q13.3, and 12p13.3) in which a number of candidate NPC-associated tumor suppressor genes and oncogenes were identified (Table 1).

ONCOGENES

Recurrent copy number gain and amplification of chromosomal regions are commonly associated with activation of oncogenes reside in these regions. Our array-based CGH (aCGH) studies have identified several novel amplicons in NPC.[13] Two most common amplicons in NPC were delineated at chromosome 11q13 and 12p12-13. The incidences of copy number gain of 11q13 and 12p12-13 were 57% and 62% respectively. Fine mapping and detail analysis showed that *CCND1* gene within the 11q13 amplicon is amplified and highly expressed in NPC cell lines, xenografts and primary tumors.[13] *CCND1* encodes the cell cycle regulating protein cyclin D1, which interacts with cyclin dependent kinases (CDK4 and CDK6) in G1 to S-phase transition of cell cycle, initiating DNA synthesis. Knockdown of *CCND1* in NPC cell lines by siRNA showed a dramatic

Table 1. Cancer-related genes involved in NPC

	Location	Function	Abnormalities in NPC
Oncogenes			
PIK3CA	3q26.1	Lipid kinase	Gene amplification
Cyclin D1	11q13	Cell cycle progression	Gene amplification, overexpression
Bmi-1	10p11.23	Polycomb protein	Overexpression
Bcl2	18q21.3	Anti-apoptosis	Overexpression
Tumour suppressor genes			
RASSF1A	3p21.3	Cell cycle arrest, signal transduction, cell adhesion, microtubule stability	Promoter methylation, mutation
BLU/ZMYD10	3p21.3	Stress responsive gene	Promoter hypermethlyation
DLEC1	3p21.3	Unknown	Promoter hypermethylation, histone deacetylation
p16	9p21.3	Cell cycle, G1 control	Homozygous deletion, promoter methylation
ARF	9p21.3	Stabilizer of p53 by sequestering MDM2	Homozygous deletion, promoter methylation
TSLC1	11q22-23	Immunoglobulin superfamily cell adhesion molecule, cell-cell interaction	Deletion, methylation
THY1	11q22-23	Cellular adhesion, proliferation, survival, and cytokine/growth factor responses	Deletion, down-regulation

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Table 1. Continued

	Location	Function	Abnormalities in NPC
Others			
RIZ1/RDM2	1p36.2	Histone methyltransferase	Promoter hypermethylation
CRBP4	Ip36.2	Retinoid signaling pathway	Promoter hypermethylation
RARB2	3p24	Retinoid signaling pathway	Promoter hypermethylation
CRBP1	3q23	Retinoid signaling pathway	Promoter hypermethylation
TIG1	3q25.32-q25.33	Retinoid signaling pathway	Promoter hypermethylation
UCHL1	4p14	p53 stabilization	Promoter hypermethylation
PCDH10	4q28.3	Cell-cell connection	Promoter hypermethylation
DAB2	5p13	RAS GTPase pathway	Promoter hypermethylation
HIN-1/SCGB3A1	5q35-qter	AKT signaling pathway	Promoter hypermethylation
GADD45G	9q22.1-q22.2	DNA-damage response	Promoter hypermethylation
DAPK1	9q34.1	Mediator of gamma-interferon induced programmed cell death	Promoter hypermethylation
WIF1	12q14.3	Wnt signaling pathway	Promoter hypermethylation
RASAL1	12q23-24	RAS GTPase pathway	Promoter hypermethyoation
CHFR	12q24.3	Mitotic checkpoint control	Promoter hypermethylation
ENDRB	13q22	G protein-coupled receptor, Endothelin-1 signaling	Promoter hypermethylation
CDH1	16q22.1	Calcium dependent cell-cell adhesion glycoprotein	Promoter hypermethylation
IRF8	16q24.1	Response to IFN-gamma stimulation	Promoter hypermethylation
CDH13	16q24.2-q24.3	Calcium dependent cell-cell adhesion glycoprotein	Promoter hypermethylation
RASSF2A	20pter-p12.1	Ras signaling regulation	Promoter hypermethylation

decrease in cell proliferation. This finding supported the critical role of *CCND1* in growth of NPC cells. Progression of cells from G1 to S phase, on the other hand, is blocked by a tumor suppressor, p16, which acts to disrupt the cyclinD1/CDK4/6 complex. Concurrent overexpression of cyclin D1 and downregulation of p16 has been reported in NPC, suggesting an altered cell cycle control in NPC tumorigenesis.

Another highly amplified region in NPC was chromosome 12p12-13. Using high density oligonucleotide aCGH, we have defined a 2.77 MB novel region of gain at 12p13.31.[18] This amplicon is a gene-rich region, harboring more than 10 genes. We found that several genes in this region were overexpressed in an expression array study. Among the overexpressed genes identified, Lymphotoxin-β receptor (*LTβR*) showed the highest expression level. LTβR was overexpressed in 76% primary NPC tumors with 54% showing amplification of *LTβR* gene. LTβR is a member of the tumor necrosis factor receptor (TNFR) family and is activated by two members of the TNF family, LTα1β2 and LIGHT, which then activates multiple downstream signaling pathways including NFκB and c-Jun N-terminal kinase. Activation of NFκB has been demonstrated in NPC cell lines, xenografts and primary tumors.[19-22] Ectopic expression of *LTBR* highly induced NFκB activity in immortalized nasopharyngeal epithelial cells.[18] This indicates a possible involvement of LTβR overexpression in NFκB activation in NPC tumorigenesis.

Chromosome 3q, in addition to chromosome 11q and 12p, is another region showing consistent high copy number gain and amplification.[9,23] *PIK3CA* residing at 3q26.1 was frequently amplified and overexpressed in NPC cell lines and xenografts. *PIK3CA* encodes the 110-kDa catalytic subunit of phosphatidylinositol 3-kinase (PI3K), which coupled with the 85-kDa subunit activates protein tyrosine kinases and generates second messenger phosphatidylinositol-3,4,5-triphosphate (PIP3). PIP3 in turn activates a wide range of downstream targets involved in cell proliferation, survival, membrane trafficking and cytoskeletal re-organization. Despite that activating somatic mutations of *PIK3CA* are common in breast, liver and colon cancer,[24,25] no mutation has been found in NPC.[26] Thus, copy number gain/amplification of *PIK3CA*, instead of gain-of-function mutation, is a common mechanism in NPC tumorigenesis.

Recent evidences suggest that polycomb group (PcG) genes can act as oncogenes, in addition to their epigenetic gene silencing property. Bmi-1 is one of the polycomb group proteins and was first identified to co-operate with c-Myc in murine lymphoma.[27,28] Song et al[29] found that Bmi-1 was overexpressed at both the mRNA and protein level in NPC cell lines. Overexpression of the Bmi-1 protein was further demonstrated in 38.7% primary NPC tumors. The oncogenic potential of Bmi-1 was revealed by its ability to immortalize normal nasopharyngeal epithelial cells (NPEC). Song et al demonstrated that overexpressing Bmi-1 in NPEC by retrovirus transfection could bypass senescence and result in immortalization. More importantly, overexpression of Bmi-1 resulted in down regulation of p16 and up regulation of telomerase activity. Recent study showed that overexpression of telomerase could also lead to immortalization of nasopharyngeal epithelial cells.[30] In Bmi-1 immortalized NPEC, down regulation of p16 leading to hyperphosphorylation of Rb, resulted in an uncontrolled cell growth. EZH2 is another member of the PcG family that is commonly overexpressed in NPC. It mediates several important cellular processes, such as differentiation, response to ROS and DNA repair. Knockdown of EZH2 inhibited cell cycle progression and induced apoptosis. Expression of EZH2 suppressed FOXM1, Bcl-2, and multiple cell cycle regulators, such as c-Myc, CDK4, CDK6, CCND3 and CCNE2 in NPC cells.[31]

Overexpression of Bcl-2 was found in over 60% of NPC tumors.[32-34] *Bcl-2* is located on chromosome 18q21.3. It is commonly activated by translocation into juxtaposition of immunoglobin heavy chain loci at 14q32 in lymphoma and leukemia.[35] In contrast, no structural abnormality of *bcl2* has been reported in NPC. It has been shown that Bcl-2 expression was closely associated with the presence of EBV.[31] Latent membrane protein 1 (LMP1), one of the EBV latent gene product has been shown to up-regulate and co-operate with Bcl-2 to induce epithelial cell transformation.[36] This suggests that the LMP1 expression together with Bcl-2 overexpression may have an important role in the early step of NPC tumorigenesis.

TUMOR SUPPRESSOR GENES

Since allelic losses at 3p and 9p are critical events in multistep tumorigenesis of NPC, identification of the target tumor suppressor genes in these chromosomal regions is important in understanding the molecular basis of this cancer. By LOH and southern blotting analysis, we have delineated a tumor suppressor locus at 9p21.3 in which homozygous deletion was consistently detected in NPC xenografts and 40% of primary tumors.[37,38] The minimal region of homozygous deletion appears to center on *INK4/ARF* locus encoding the *p15* (*INK4b*), *p16* (*INK4a*) and *ARF* tumor suppressor genes (Fig. 2). Except *p15*, inactivation of the *p16* and *ARF* genes by promoter hypermethylation was also commonly found in tumors without 9p21 homozygous deletion.[39,40] Overall, p16 and ARF inactivation was found in 62-86% and 54% of NPC respectively. Loss of these two tumor suppressors may lead to Rb and p53 dysfunction in the cancer cells. The p16 protein is an important cell cycle regulator that inhibits the cell cycle progression from G1 to S phase. *p16* abnormalities are perhaps the most common mechanism for inactivating pRb/cyclin D1/cdk4/p16 cell cycle control pathway in NPC. Loss of functional p16 will lead to Rb phosphorylation and therefore the release of E2F transcription factor, which constitutively activates the S phase genes for DNA synthesis and results in uncontrolled cell proliferation.[41,42] Restoration of *p16* expression in NPC cells induced G0/G1 arrest and suppressed tumorigenicity in vivo.[43] ARF functions as a tumor suppressor by binding to and inactivating the MDM2 protein that negatively regulates p53. Loss of ARF enables MDM2 to counter-act p53 function more efficiently in response to aberrant growth or oncogenic stresses. Since p53 mutation is rare in NPC, inactivating ARF may be a common mechanism for disrupting the functional p53 in this cancer.[44-46] Interestingly, homozygous deletion of *INK4/ARF* locus was observed during the establishment of an immortalized nasopharyngeal epithelial cell line.[30] The findings suggested that genetic and epigenetic inactivation of *p16* and *ARF* are the critical steps for nasopharyngeal epithelial cells transformation.

On the short arm of chromosome 3, multiple tumor suppressor loci have been reported from a variety of human cancers. These regions include 3p12-13 (*ROBO1/DUTT1* gene region), 3p14.2 (*FHIT* gene region), 3p21.1-p21.2 (*DRR1*, *BAP1*, *ARP*), 3p21.3C (centromeric, LUCA), 3p21.3T (telomeric, A20) and 3p24-26 (*VHL* and *RARβ*).[47] However, NPC-associated tumor suppressor(s) is believed to be located on 3p21.3C (LUCA) in which high frequency of deletion was consistently found in LOH and CGH studies.[9-12,48,49] In our early study, we have demonstrated high frequencies of genetic and epigenetic alterations of the *RASSF1A* (*Ras Association Domain Family 1A*) tumor suppressor gene, which is located within a 120-kb common homozygous deletion region at 3p21.3, in EBV-positive

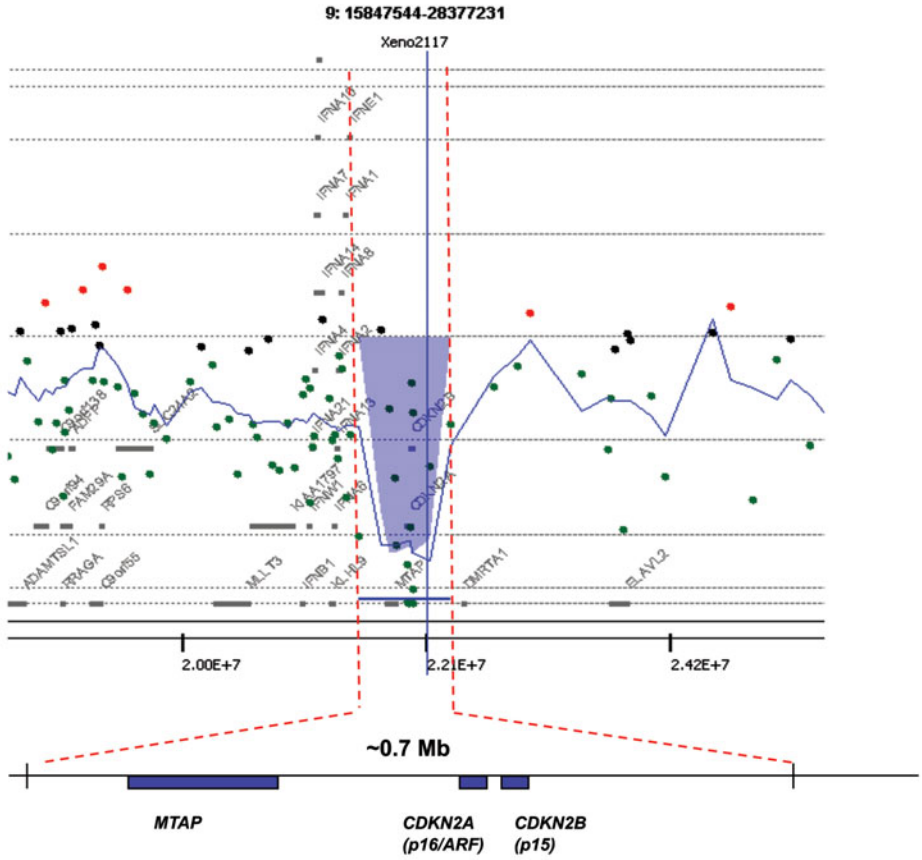

Figure 2. Mapping of homozygous deletion of *INK41ARF* locus at 9p21.3 in a NPC Xenograft (xeno-2117) by high-density array CGH.

NPC cell lines, xenografts and primary tumors.[50,51] Inactivation of *RASSF1A* by promoter hypermethylation is commonly found in a variety of human cancers, including lung cancer, breast cancer, renal cell carcinoma, liver cancer and medulloblastoma.[52-57] In NPC, aberrant methylation and transcription silencing of *RASSF1A* were detected in all EBV-positive xenografts and cell line (4/4, 100%).[50] Aberrant methylation and mutation of *RASSF1A* were also detected in 66.7-83% and 9.5%, respectively, of primary tumors.[50] The presence of missense and silence mutations in primary tumors strengthens the hypothesis that *RASSF1A* is the critical tumor suppressor of NPC. The tumor suppressor function of *RASSF1A* in NPC cells has been demonstrated by transfecting wild-type *RASSF1A* clone in a RASSF1A-deficient NPC cell line. Restoration of wild-type *RASSF1A* led to marked growth inhibition and dramatic reduction in tumorigenic potential of NPC cells.[58] These findings provide functional evidence that *RASSF1A* is a target tumor suppressor gene on 3p21.3 in NPC. RASSF1A is a member of the RASSF family of proteins characterized by a consensus RAS-association domain at the C-terminus. Studies have shown that it may function in the Ras-regulated pro-apoptotic pathway.[59-61] RASSF1A can also inhibit cell

cycle progression by blocking the c-Jun-NH2-kinase pathway and suppressing cyclin D1 accumulation.[62,63] In our recent study, we demonstrated that *RASSF1A* can transcriptionally regulate a number of target genes (*ATF5, TCRB, RGS1, activin betaE, HNRPH1, HNRPD, ID2* and *CKS2*) which are involved in multiple cellular pathways such as transcription, signal transduction, cell adhesion and RNA processing.[64] RASSF1A may function as a tumor suppressor in NPC by repressing ID2 (*inhibitor of DNA binding 2*) expression, whereby its loss leads to epithelial-mesenchymal transition and failure of differentiation. Recently, several groups have reported that RASSF1A is a microtubule-binding protein, which regulates microtubule stability, controls mitotic progression and maintains genomic stability.[61,65-72] As the guardian of mitosis, it regulates APC^{cdc20} activity and ensures the sequential progression of mitosis through direct interaction with Cdc20. Our recent finding suggested that the specific tumor suppressive function of *RASSF1A* in NPC is dependent on the unique N-terminus mediated APC^{cdc20} regulation in mitosis.[73] *RASSF1A*-knockout mice were prone to spontaneous tumorigenesis at advanced age.[74] $RASSF1A^{-/-}$ cells from the knockout mice were much more sensitive to nocodazole induced microtubule destruction than the wild-type cells. By using siRNA targeting RASSF1A, we also found that knockdown of *RASSF1A* in immortalized nasopharyngeal epithelial cells resulted in mitotic failures and enhanced tumorigenic potential. These findings implied that RASSF1A is a major tumor suppressor gene on 3p21.3 in NPC. Aside from RASSF1A, other family members of Ras association domain family (RASSF) are suspected to be candidate tumor suppressors. However, we have previously shown that abnormalities of *RASSF1C*, *NORE1* and *RASSF4* rarely occur in NPC.[58,75] Recently, Zhang et al[76] have reported that promoter hypermethylation of *RASSF2A* was found in 50% primary tumors and correlated with lymph node metastases. Loss of *RASSF2A* in NPC may be beneficial for tumor cell survival by reducing K-ras apoptotic signals.

BLU/ZMYD10 (*zinc finger, MYND-type containing 10*), a candidate 3p21.3 tumor suppressor gene immediately upstream of *RASSF1A*, was also commonly inactivated in NPC. *BLU* is a stress-responsive gene regulated by *E2F* and contains a MYNF domain at its C-terminus. Hypermethylation and downregulation of *BLU* were demonstrated in 66-80% primary tumors.[77-79] Partial methylation of *BLU* was also shown in several EBV-positive xenografts and cell line. Several studies have shown that overexpression of *BLU* led to growth inhibition and tumor suppression in cancer cell lines.[77,80] Although the biological function of BLU is still not known, the MYNF domain at C-terminus is believed to be important for its tumor suppressor activity. It is likely that *BLU* transcription regulates several important target genes involved in cancer development. Further study of the BLU function and its associated pathways is important in understanding the roles of this protein in NPC tumorigenesis. Recently, we have shown that *DLEC1* (*Deleted in Lung and Esophageal Cancer 1*) located at A20 region (3p21.3-3p22.2) is another candidate tumor suppressor gene of NPC.[81] The gene was frequently inactivated in NPC and ovarian cancers by promoter methylation and histone deacetylation.[81,82] Overexpression of DLEC1 suppressed growth, reduced invasiveness, and inhibited tumorigenic potential of cancer cells although its biochemical function is still unclear.

In addition to chromosomes 3p and 9p, two candidate tumor suppressor genes of NPC, *TSLC1* and *THY1*, have been reported at 11q22-23, a region frequently deleted in NPC. *TSLC1* (*tumor suppressor in lung cancer 1*), also known as *IGSF4* (*Immunoglobulin superfamily 4*), encodes an immunoglobulin superfamily cell adhesion molecule (IgCAM), which is a membrane protein involved in cell-cell interactions.[83-85] The protein can directly interact with DAL-1/4.1B and MAGuk to form a ternary complex that participates in

epithelial-like cell structures associated with cell adhesion.[86,87] *TSLC1* may suppress tumor formation by inhibiting epithelial-mesenchymal transition. Loss of its function could lead to invasion or metastasis. The tumor suppression function of TSLC1 has been shown in a variety of cancer cell lines. In NPC, aberrant methylation of *TSLC1* was reported in 34.2% primary tumors.[88] Using tissue microarray and immunohistochemistry analysis, loss of TSLC1 expression were found in 12% of primary NPC and 35% of metastatic tumors.[89] The significantly higher frequency of loss of TSLC1 expression in metastatic tumors suggested that its inactivation might be involved in NPC progression. Apart from *TSLC1*, *THY1* (*Thy-1 cell surface antigen*)/*CD90* on 11q22-23, was also found to be a candidate tumor suppressor of NPC. The protein participates in multiple signaling cascades affecting cellular adhesion, proliferation, survival, and cytokine/growth factor responses.[90,91] A recent study also reported that *THY1* can upregulate thrombospondin-1 and fibronectin, which are associated with cell differentiation and angiogenesis inhibition.[92] Lung et al[93] reported that 40% of primary tumors and 74% metastatic NPCs showed downregulation or loss of THY1 expression in a tissue microarray study. Aberrant methylation may be a possible mechanism for transcriptional silencing of *THY1* in these tumors. These finding suggested that inactivation of *THY1* is involved in lymph node metastasis of NPC.

In addition to the regions mentioned above, inactivation of tumor suppressor gene(s) at chromosome 14q is also believed to be an important event in NPC tumorigenesis since LOH on 14q was detected in more than 85% of primary tumors.[10] However, few candidate tumor suppressor genes for NPC have been identified in this region yet. Searching for the target gene(s) at this chromosomal region may provide further insight in NPC tumorigenesis.

EPIGENETIC ALTERATIONS

For the past decades, epigenetic alterations, including promoter hypermethylation and histone modifications, have been recognized as an important mechanism for the inactivation of cancer-associated genes.[94-96] In NPC, promoter hypermethylation was found to be the major mechanism for inactivation of critical tumor suppressor genes, such as *p16* and *RASSF1A*. Recent studies have detected a widespread hypermethylation of CpG islands of cancer genes over the NPC genome while the contribution of histone modifications in this cancer was rarely reported. The epigenetic changes influenced multiple cellular mechanisms involved in initiation and progression of NPC.

The retinoid signaling pathway in almost all NPC tumors were disrupted by epigenetic inactivation of multiple components including nuclear retinoic acid receptor (*RARB2*), cellular retinoic acid-binding proteins (*CRBP1*, *CRBPV*) and/or retinoid response gene *TIG1*.[40,97,98] The transcriptional silencing of these genes by promoter hypermethylation may result in the loss of cellular retinoic acid homeostasis, inability to uptake natural retinol, and synthesis of retinoic acid. These findings suggested NPC may resist retinoic acid treatment.

Several members of cadherin superfamily, which participate in intercellular and cell-extracellular matrix interactions of cancer, were reported to be the targets for epigenetic inactivation in NPC. E-cadherin (*CDH1*), H-cadherin (*CDH13*), and Protocadherin 10 (*PCDH10*) are methylated in 52%, 89.7% and 82% of primary tumors, respectively.[99-101] Transcription silencing of H-cadherin by promoter methylation was also consistently shown in three EBV-positive tumor lines. Loss of these cell adhesion molecules may

contribute to the progression of NPC by promoting tumor cell invasion and metastasis. Furthermore, inactivation of these genes may be involved in interruption of various cellular functions, including signal transduction, cell growth and differentiation.

High frequencies of promoter hypermethylation in cancer-related genes (*IRF8, GADD45*, *DAPK1, ENDRB, HIN-1, WIF1, RASAL, DAB2, UCHL1*) that are involved in interferon-γ stimulation and DNA damages responses, cell death-signaling network, endothelin-1, AKT, Wnt, Ras GTPase and p53 signaling pathways were reported in several studies.[40,102-109] In addition to *RASSF1A*, the *CHFR* (*checkpoint with forkhead-associated domain (FHA) and RING finger domain*) gene that participates in mitotsis checkpoint regulation is also inactivated by hypermethylation in most of NPC tumor lines and primary tumors.[110] Loss of both *RASSF1A* and *CHFR* may lead to genomic instability. Furthermore, transcriptional silencing of *RIZ1* by promoter hypermethylation may influence the chromatin-mediated gene expression.[111] In comparison with other EBV-negative head and neck cancers, much higher frequencies of promoter hypermethylation in cancer genes were found in EBV-positive NPC. The widespread hypermethylation in the NPC genome implies a methylator phenotype of this EBV-associated cancer. Interestingly, EBV-positive gastric cancer has been reported to show a higher frequency of aberrant methylation than EBV-negative gastric cancer.[112] The observation suggests a relationship between latent EBV infection and epigenetic changes in these EBV-associated epithelial cancers. In NPC, DNA methylation not only contributes to inactivation of cancer genes, it also modifies the Wp and Cp promoters leading to silencing of several EBV latent genes (nuclear antigens EBNA2, 3A, 3B and 3C) and establishment of cell specific type II latency.[113,114] It is likely that epigenetic modification of both viral and cellular genes is crucial in transforming nasopharyngeal epithelial cells. Interestingly, EBV oncoprotein LMP1 was shown to participate in DNA methylation. LMP1 is able to activate cellular DNA methyltransferases via c-jun NH_2-terminal kinase signaling and subsequently induce hypermethylation of several cellular genes, such as E-cadherin.[115,116] On the other hand, LMP1 can upregulate the polycomb group (PcG) protein Bmi-1 which may in turn be responsible for promoter hypermethylation of tumor suppressor genes.[117] The PcG-mediated histone modifications may render certain cancer genes vulnerable to DNA hypermethylation.[118] Thus, latent LMP1 protein expressed in EBV-infected nasopharyngeal cells would induce promoter methylation of several EBV and cellular cancer genes through the upregulation of methyltransferase and PcG protein, and thereby participate in both tumor initiation and progression.

ABERRANT SIGNAL TRANSDUCTION PATHWAYS

In EBV-associated malignancies, the viral latent proteins have been shown to activate multiple signaling pathways and contribute to disease progression. For examples, the LMP1 and LMP2A, which are often expressed in NPC tumors and are known to be able to activate NF-κB, mitogen-activated protein kinase (MAPK), and Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathways.[119-122] On the other hand, there are also evidences suggesting that genetic and epigenetic alterations are involved in those activating cellular pathways via EBV-independent mechanisms in NPC.

Activating NF-κB signaling pathway is implicated in the development of NPC. Constitutive NF-κB nuclear activity has been consistently demonstrated in EBV-positive NPC cell lines, xenografts and primary tumor.[19-22] There are multiple functions by which

NF-κB can promote transformation and progression of this cancer. Target genes induced by NF-κB are important for controlling cell survival, proliferation, invasion, angiogenesis, innate and acquired immunity. By gene expression profiling, Shi et al[123] have revealed that *NFκB2* (*p100/p52*) and its transcriptional cofactors *RELB* and *BCL3* were significantly upregulated in NPC primary tumors, together with a number of NF-κB target genes, such as *MMP9*, *Bcl-2*, *BFL1*, *BIRC3* and *BIRC5*. Upregulation of other NF-κB target genes (e.g., *VCAM1*, *ICAM1*, *EGFR*, *A20*, *CXCR4*) in NPC has also been reported in several studies.[124-128] It is also possible that abnormal activation of NF-κB is involved in the initial step of transformation. Although NF-κB activity in precancerous lesions is unknown, we have demonstrated that activation of NF-κB was observed in immortalized nasopharyngeal epithelial cell line at later population doublings.[30] These finding suggests the possible role of constitutive NF-κB activation in supporting the growth and survival of immortalized cells. Moreover, activation of NF-κB can inhibit lytic replication of EBV and may therefore contribute to the maintenance of viral latency in nasopharyngeal epithelial cells.[129]

In tumors expressing LMP1, the viral latent protein, NF-κB dimers (e.g., p50/p65, p52/RelB) may be activated through canonical or noncanonical pathways. Interestingly, Thornburg et al[21] has clearly shown that p50/p50 homodimers were specifically activated in NPC while other NF-κB dimers were not detected in the nuclear extracts of both LMP1 expressing and non-expressing NPC cells. The p50/p50 homodimers transcriptional activate downstream targets by binding with Bcl-3 that is overexpressed in most of NPC tumors. Genetic alterations in NF-κB pathways, such as loss of IκB-α and overexpression of Bcl-3, are likely contributing to the abnormal regulation of NF-κB in NPC. Recently, we have found a crosstalk between NF-κB and NOTCH3 signaling pathways. The transcription of *p50/p105* (*NFKB1*) is directly regulated by NICD3 signal which are constitutively activated in NPC cells.[130] Overexpression of NOTCH3 receptor and ligands (DLL4 and JAG1) were detected in almost all EBV-positive tumor lines and primary tumors. We also showed that the activated NICD3 signal is important in maintaining the cancer stem-like cells features, chemoresistance and survival of NPC cells.[130] Dysregulation of NOTCH3 and NF-κB pathways play crucial roles in the development of this EBV-positive epithelial cancer.

By gene expression profiling studies, multiple deregulated signal transduction pathways have been revealed in primary NPCs. The expression microarray showed the differentiated expression of multiple components of WNT/beta-catenin signaling pathway, including two major inhibitors of Wnt/beta-catenin pathway, WIF1 (Wnt inhibitory factor 1, WIF1) and FRZB (secreted Frizzle-related protein 3), which are commonly inactivated in human cancers by promoter methylation.[123] Loss of these inhibitors may disrupt the regulation and enhance the LMP1 and LMP2A mediated activation of Wnt/beta-catenin pathway. In NPC, these viral latent proteins may induce beta-catenin activity through activation of PI3K/AKT pathways.[119,120,122] Activated AKT, phosphorylated GSK-3 and nuclear beta-catenin accumulation were found in NPC.[131] Interestingly, *PIK3CA*, a gene coding for the catalytic subunit p110alpha of PI3K, is amplified and may also cause deregulation of AKT pathway in a subset of NPCs. The findings support that both EBV infection and genetic/epigenetic changes contribute to the constitutively activation of PI3K/AKT and WNT/beta-catenin signaling in NPC. Thus, further comprehensive elucidation of both EBV-related and EBV-independent mechanisms involved in deregulation of critical signal transduction pathways (e.g., STAT, MARK, NOTCH and TGFbeta) from representative EBV-positive NPC tumor lines and primary tumors is important for deciphering the molecular basis of this cancer.

MOLECULAR GENETIC CHANGES IN PRE-INVASIVE LESIONS

Despite of the high frequency of NPC, pre-invasive lesions of nasopharynx were encountered only rarely (~0.6%) during routine examination of nasopharyngeal biopsies in endemic area. To date, very little is known about the molecular changes in these pre-invasive lesions. Activation of telomerase and overexpression of BCL2 were consistently found in the dysplastic lesions.[132-134] These events may contribute to maintaining telomere length and enhancing survival of the pre-invasive epithelial cells. Earlier report has demonstrated clonal EBV genome and latent transcripts including LMP1 in high grade dysplasia and carcinoma in situ.[5] We have also detected EBV latent infection in the high grade dysplasia, but not in the low grade dysplasia and normal nasopharyngeal epithelia.[16] However, in vitro study has proven that EBV infection alone is not sufficient to transform immortalized nasopharyngeal epithelial cells although the viral latent products can induce the invasive property and modulate multiple signaling cascades.[19] Accumulation of other genetic changes might be necessary for malignant transformation of the EBV-infected cells. LOH analysis of microdissected nasopharyngeal epithelia has revealed high frequencies of chromosomes 3p and 9p deletions in the dysplastic lesions and histologically normal epithelia.[16,17] Interestingly, allelic loss at 3p and 9p in the normal nasopharyngeal epithelia is significantly higher in the population from endemic area than non-endemic region. These findings suggested the field cancerization may be common in nasopharyngeal epithelia of Southern Chinese. The occurrence of multiple genetic instable lesions in this population may associate with the exposure to specific environmental carcinogens that increase the susceptibility to further genetic damages. The specific clonal genetic changes disrupting cellular mechanisms (e.g., cell cycle regulation, genetic stability) and signaling pathways (e.g., NF-κB pathway) may predispose for EBV infection, maintenance of permanent viral latency, and tumor initiation. Our recent work has shown the aberrant methylation of *RASSF1A*, the critical tumor suppressor on 3p21.3, in pre-invasive lesions. In this study, multiple dysplasia lesions of the nasopharynx from two Chinese patients were microdissected for investigation.[8,10] *RASSF1A* methylation was detected in some of the microdissected nasopharyngeal epithelia, either with or without EBV infection (Fig. 3). Thus, inactivation of *RASSF1A* may be already involved in the early development of NPC. Interestingly, homozygous deletion of *INK4/ARF* locus, downregulation of *RASSF1A* and activation of NF-κB pathway were also noted in the telomerase-immortalized nasopharyngeal epithelial cells. In vitro selection of the clonal immortalized cells with *INK4/ARF* and *RASSF1A* abnormalities suggests that those changes may be important for in vivo formation of immortalized nasopharyngeal cells.

TUMORIGENESIS MODEL OF NPC

Although there is limited information on pre-invasive lesions, studies suggested that genetic and epigenetic changes collaborate with EBV latent infection in disrupting major cellular mechanisms that contribute to the initiation and progression of NPC. Based on these exciting findings, a collaborative model for NPC tumorigenesis driven by specific genetic and environmental factors is proposed. In individuals from endemic regions, the NPC-associated genotypes for various alleles (such as HLA and the polymorphic

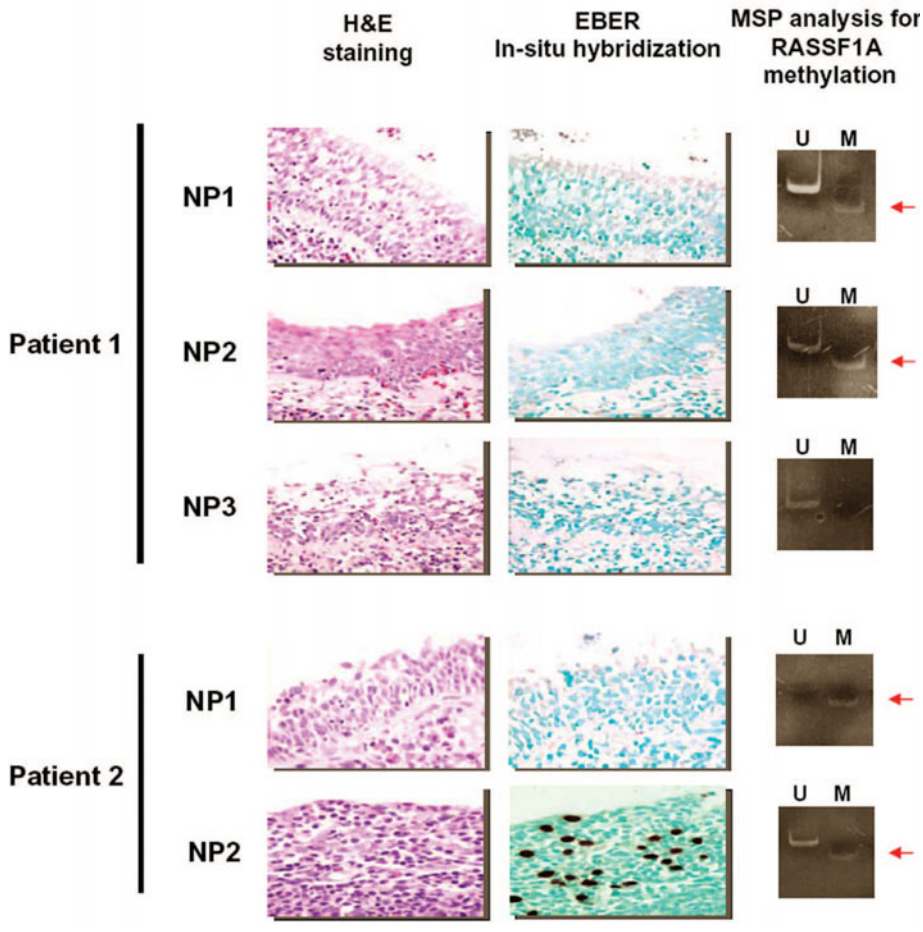

Figure 3. RASSF1A methylation in the precancerous lesions of nasopharynx. The methylation status of RASSF1A promoter in nasopharyngeal dysplastic lesions from five Chinese patients were examined by methylation specific PCR (MSP) analysis. Aberrant methylation (red arrows) was detected in multiple microdissected dysplasic lesions in 2/5 cases (NP1-2 of Patient 1 and NP1-2 of Patient 2). In-situ hybridization for EBER shows that only one lesion (NP2 of Patient 2) is positive for EBV latent infection. H & E: Haematoxylin and Eosin staining; U: unmethylated allele; M: Methylated allele.

genes for carcinogen metabolism, detoxification and DNA repair) may predispose the nasopharyngeal epithelial cells to DNA damage. As a consequence of chronic exposure to specific carcinogens (e.g., nitrosomine), increased DNA damage may lead to the formation of multiple lesions with clonal genetic changes in nasopharynx. The high frequencies of 3p and 9p loss in these lesions are likely due to the growth advantage achieved by p14, p16 and RASSF1A repression. Inactivation of Rb and p53 pathways through loss of *INK4/ARF* locus is critical for immortalization and resistance to

apoptosis of these clones. Suppression of *RASSF1A* in nasopharyngeal epithelial cells may inhibit differentiation and induce genetic instability. Phenotypic and morphological changes occur in these low-grade dysplastic lesions. Chronic inflammation induced by virus infection and chemical carcinogens may lead to persistent NF-κB activation and predispose the pre-invasive lesions to EBV latent infection. The virus may infect these epithelial cells through cell-cell contact with the EBV-bearing B lymphocytes or through polymeric IgA medicated mechanism. EBV latency is stably maintained in one of these progenitor cells. The latent viral gene products will drive the progenitor cell to rapid clonal expansion and invasion. EBV latent proteins, such as LMP1 and LMP2A, may modulate multiple signaling cascades, enhance genetic instability and induce epigenetic alterations. Through activating DNA methyltransferase and polycomb proteins by EBV oncoprotein LMP1, a number of NPC-associated genes will be transcriptional silenced by promoter methylation during the tumor initiation. Multiple cellular mechanisms (e.g., cell proliferation, apoptosis, genomic stability and cell adhesion) and signaling pathways (e.g., NF-κB, AKT, Wnt pathways), including those originally modulated by LMP1, may be permanently disrupted by both epigenetic and genetic changes under the continual selection process. LMP1 expression is then downregulated in a majority of invasive tumors to avoid its cytotoxic effect on epithelial cells. Furthermore, the genetic alterations on 11q, 13q, 14q, 16q may be involved in later steps during development of NPC. Inactivation of *TSLC1*, *THY1*, and other unknown genes may contribute to the late progression and metastasis of NPC (Fig. 4).

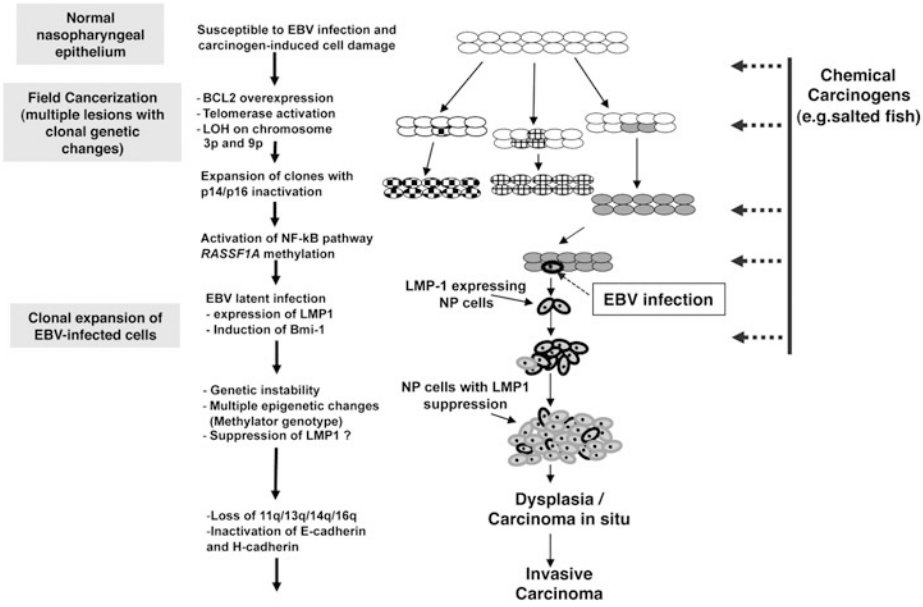

Figure 4. Tumorigenesis model for EBV-associated nasopharyngeal carcinoma.

CONCLUSION AND FUTURE DIRECTIONS

NPC represents an exciting model for the understanding of complex interactions among genetic, environmental and viral factors in human tumorigenesis. The availability of only small biopsies and the rich infiltrate of lymphocytes and plasma cells among cancer cells remain as the challenges of NPC research. Since NPC is strongly associated with EBV, one should be cautious in interpreting and extrapolating the laboratory findings of EBV negative cell line models. However, limited numbers of EBV positive NPC lines are established so far. Studies in the NPC pre-invasive lesion would certainly enhance our insights in the early genetic and epigenetic events and interactions with EBV infection. Nevertheless, investigations are limited by the rarity of pre-invasive lesions encountered in routine biopsies. The knowledge gap may be partially overcome by the establishment and in-depth studies of immortalized nasopharyngeal epithelial cell lines.

Knowledge of critical genetic and epigenetic events in NPC has been rapidly accumulated in the past two decades. Although our insight of this fascinating cancer is greatly enhanced, there are areas still require for more in-depth and active research. Genetic loses and amplification region in NPC genome have been delineated. However, potential important tumor suppressor genes and oncogenes located in some of theses region are not yet discovered. Recently, the next generation massive sequencing technology is rapidly transforming basic cancer biology and biomedicine by decoding DNA sequence of entire cancer genome. We believe that these advanced massive parallel genome sequencing approaches will help us to unveil the unknown driver events for NPC development via establishment of comprehensive catalogs of somatic alterations from NPC genome. Methylation of multiple cancer related genes is common in NPC. Although expression of viral oncoprotein, like LMP1, is implicated, the precise mechanism of this wide-spread methylation is unclear. Further studies are needed to dissect the complex interactions among the various down-stream targets and signaling pathways altered by both host and EBV gene expression. In particular, genetic or epigenetic changes leading to alterations in the inflammatory and immune responses are the exciting field to explore.

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