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Author(s)	**Dai, W; ZHENG, H; Cheung, AKL; Lung, ML**
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Genetic and epigenetic landscape of nasopharyngeal carcinoma

Wei Dai[1], Hong Zheng[1], Arthur Kwok Leung Cheung[1,2], Maria Li Lung[1,2,3]

[1]Department of Clinical Oncology, [2]Center for Cancer Research, [3]Center for Nasopharyngeal Carcinoma Research, University of Hong Kong, Hong Kong SAR, China

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Correspondence to: Maria Li Lung. Department of Clinical Oncology, University of Hong Kong, Pokfulam, Hong Kong SAR, China. Email: mlilung@hku.hk.

Abstract: Nasopharyngeal carcinoma (NPC) is a unique epithelial malignancy that shows a remarkable geographical and ethic distribution. Multiple factors including predisposing genetic factors, environmental carcinogens, and Epstein-Barr virus (EBV) infection contribute to the accumulation of genetic and epigenetic alterations leading to NPC development. Emerging technologies now allow us to detailedly characterize and understand cancer genomes. Genome-wide studies show that typically NPC tumors are characterized as having comparatively low mutation rates, widespread hypermethylation, and frequent copy number alterations and chromosome abnormalities. In this review, we provide an updated overview of the genetic and epigenetic aberrations that likely drive nasopharyngeal tumor development and progression. We integrate the previous knowledge and novel findings from whole-exome sequencing (WES) and methylome studies in NPC, and further discuss the potential use of these findings to identify biomarkers for NPC diagnosis and prognosis.

Keywords: Nasopharyngeal carcinoma (NPC); whole-exome sequencing (WES); epigenetics; biomarkers

Introduction

Nasopharyngeal carcinoma (NPC) is a unique malignancy that arises from the epithelium of the nasopharynx and has a restricted prevalence in certain regions of the world. NPC shows the highest incidence rate in the Southeast China, Southeast Asia, North Africa, Middle East, and the Arctic regions, but it is rare in most other parts of the world (1). NPC patients are relatively young at diagnosis, when compared to other cancer types that usually are diagnosed at 65 years of age or older, while NPC has a median age at diagnosis of around 50.

Previous studies suggested that there are several cofactors that are associated with NPC development. They are the dietary factors, infection with an oncovirus, Epstein-Barr virus (EBV), and genetic predisposition. NPC is associated with genetic alterations on particular chromosomal regions and genes, harboring of specific cancer-associating single nucleotide polymorphisms (SNPs), and familial aggregation. Interestingly, recent studies confirm that epigenetic alterations, including the promoter hypermethylation (2), are also one of the crucial factors that are highly associated with NPC.

Previous reviews suggested the multi-step progression model for NPC. Lo *et al.* proposed that the loss of chromosomes 3p and 9p regions is an early event for the transformation of the normal nasopharyngeal epithelium (3). During the transformation process, the inactivation of the *p16* gene on chromosome 9 and *RASSF1* on chromosome 3, and/or the over-expression of the cyclin D1 or amplification of particular regions on chromosome 11 are followed by the EBV latent infection and multiple genetic and epigenetic changes. The subsequent loss of chromosomes 11q, 13q, 14q, and 16q regions and promoter hypermethylation of numerous tumor suppressor genes (TSGs), including E-cadherin, finally result in NPC (3). This model proposes
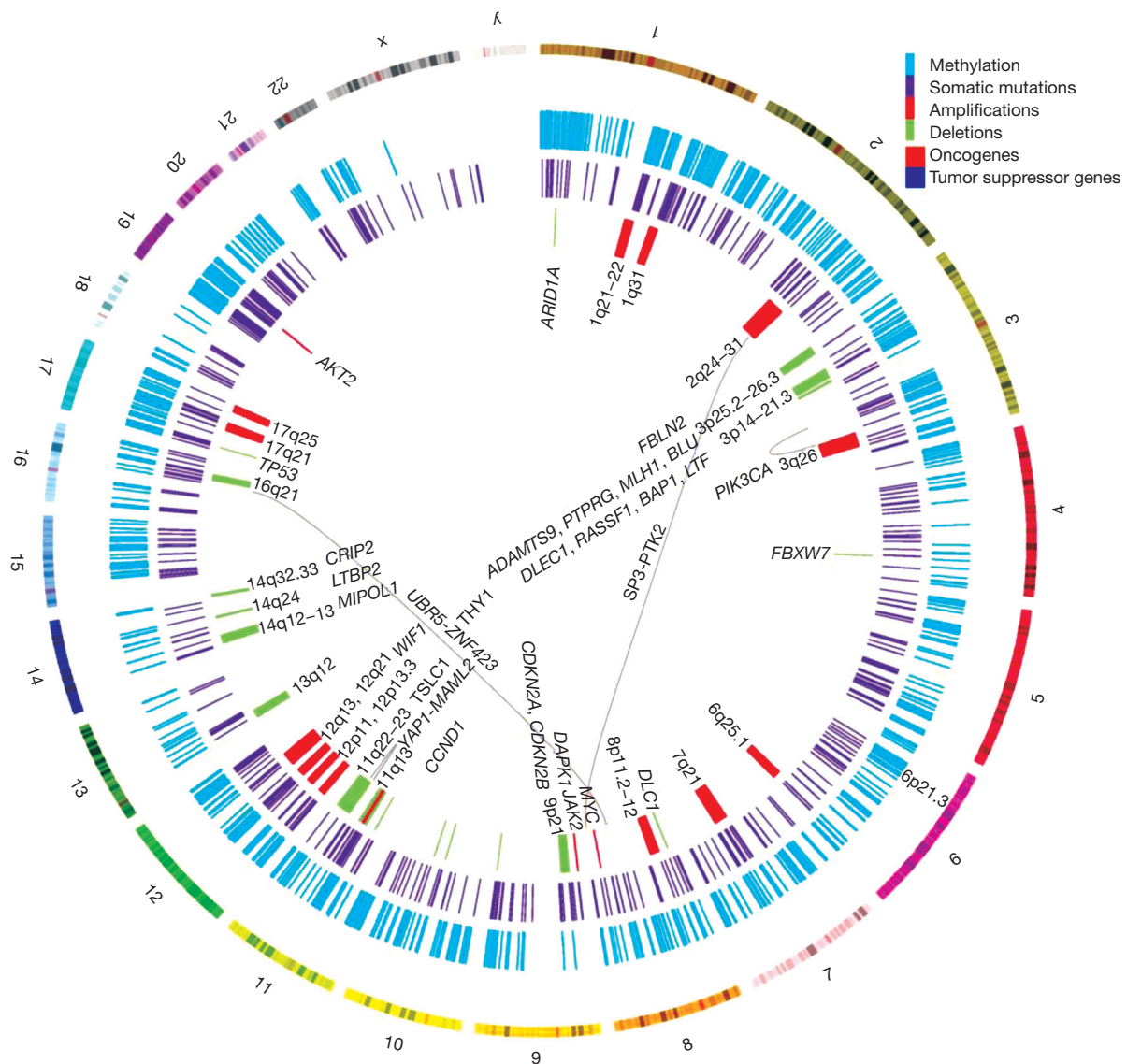
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Figure 1 Summary of genetic and epigenetic changes in NPC by Circos plot. Circles from outer to inner direction show loci with hypermethylation, somatic mutations, amplifications and deletions in NPC tumors. Rearrangements between two genes are linked by grey lines. Important regions and genes are labeled according to their genomic locations. The large genomic regions with copy number gains reported previously, such as 4q12–q21, 6q14–q22, and 8q11.2-q23, are not presented on the graph. NPC, nasopharyngeal carcinoma.

a critical role of the genetic and epigenetic factors in the pathogenesis of NPC. In this review, we summarize the genetic and epigenetic changes in NPC, as seen in *Figure 1*, and focus discussions on these critical events that contribute to the initiation and progression of this EBV-associated tumor, as well as the potential use of these findings as biomarkers for NPC diagnosis and prognosis.

Genetic alterations in NPC

In 2014, the first genomic landscape study of NPC was published using the whole-exome sequencing (WES), targeted deep sequencing, and SNP array analysis approaches to characterize NPC genetic alterations (4). This integrated study revealed the enrichment of genetic lesions affecting several important cellular processes and
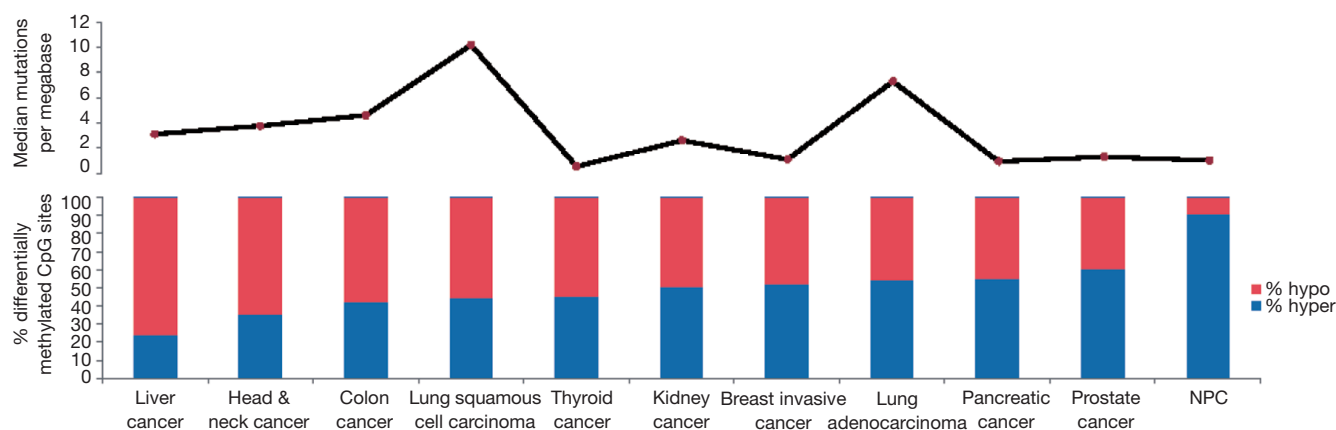
Figure 2 The somatic mutation prevalence and the percentage of differential methylation across human cancer types. Top: somatic mutation rates in NPC were calculated based on two WES studies of NPC by Lin *et al.* (4) and our study (manuscript in revision). Somatic mutation rates of other cancers were adapted from Alexandrov *et al.* (5). Somatic mutations rates in exomes were calculated based on the identified mutations in protein-coding genes (including synonymous mutations) and assuming that an average exome has 30 Mb in protein-coding genes with sufficient coverage. Somatic mutations rates in whole genomes were calculated based on all identified mutations and assuming that an average whole genome has 2.8 gigabases with sufficient coverage; Bottom: percentage of hypermethylated and hypomethylated CpG sites identified by LIMMA analysis with FDR <0.001 in NPC and other types of solid tumours. hypo, hypomethylated CpG sites; hyper, hypermethylated CpG sites. The graph is adapted from previous methylome study by Dai *et al.* (2). NPC, nasopharyngeal carcinoma; WES, whole-exome sequencing; LIMMA, linear models for microarray and RNA-Seq data; FDR, false discovery rate.

pathways including chromatin modification, ERBB-PI3K signaling, and autophagy machinery.

Somatic mutations

The somatic mutation rate in NPC is relatively low compared to other types of cancers, with a median of one somatic mutation per megabase (*Figure 2*). Interestingly, *TP53* is the most frequently mutated gene in NPC. Most of the earlier studies using the single-strand conformation polymorphism (SSCP) method to screen for mutations reported infrequent *TP53* mutations in NPC biopsies (6,7). Using a sensitive yeast screening assay developed by Flaman *et al.* (8), that covers both the exons and introns of p53, Lung *et al.* (9) were able to detect up to 27% mutated p53 in a small number of NPC biopsies, indicating that the SSCP methods were inadequate to detect many of the p53 mutations harbored in NPC. Now with sensitive WES approaches, higher *TP53* mutation frequencies of 8.5% were observed in a Singapore study (4), as well as in 7.3% of our NPC biopsies in a Hong Kong study (manuscript in revision).

Most well-known oncogenes are rarely mutated in NPC. Mutation hotspots in *EGFR*, *PIK3CA*, *KRAS*, *HRAS*, *NRAS*, *BRAF*, *KIT*, *ABL1*, *AKT1*, *AKT2*, *CDK4*, *ERBB2*, *FGFR1*, *FGFR3*, *FLT3*, *JAK2*, *MET*, *RET*, and *PDGFRA* were screened using SNaPshot or OncoCarta panel assays in 70 and 160 NPC patients, respectively. In each study, most genes were mutated in less than three NPC samples at the mutation hotspots in each study, except for *KIT*, which was reported to be mutated in 10% (7/70) and 2% (3/160) of NPC samples (10,11). WES identified mutations in multiple members of the ERBB-PI3K pathway including *PIK3CA* (1.7%), *ERBB2* (1.7%), *ERBB3* (1.7%), *FGFR2* (2.6%), *KRAS* (1.7%), and *NRAS* (0.9%) (4). In another study *PIK3CA* was shown to be mutated in 9.6% (7/73) of NPC cases using polymerase chain reaction (PCR)-sequencing methods (12).

Both the published WES study (4), as well as our WES data, show that epigenetic regulators are frequently mutated in NPC, including *ARID1A*, *BAP1*, *KMT2B*, *KMT2C*, *KMT2D*, *TSHZ3*, *HDAC4*, *PAXIP1*, and others. Other mutated genes identified by WES include cadherin members (*FAT1*, *FAT2*, *FAT3*), Notch family members (*NOTCH1*, *NOTCH2*, *NOTCH3*), and autophagy-related genes (*ATG2A*, *ATG7*, *ATG13*) (4).

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Copy number gains

Multiple chromosomal abnormalities and gene copy number alterations have been identified in NPC by various approaches including comparative genomic hybridization (CGH), microsatellite marker analysis, karyotyping, array-based CGH (aCGH), and SNP array. Chromosomes 1q, 2q, 3q, 4q, 6q, 7q, 8p, 8q, 11q, 12p, 12q, and 17q frequently have copy number gains in NPC (13-17). Cyclin D1 (*CCND1*), located at 11q13, has the highest frequency of copy number changes in NPC, with a reported frequency ranging from 9.6% (5/52 using SNP array) to 61.9% (13/21 using aCGH) (4,18). Another oncogene related to G1/S transition, *MYC*, located in 8q24, was also amplified in 5.8% (3/52 by SNP array) to 62% (21/34 by FISH) of NPC tumors (4,19).

Multiple members of the ERBB-PI3K pathway also have copy number gains apart from somatic mutations. SNP array and aCGH analysis revealed amplifications in PIK3CA (75%, 24/32 with aCGH and FISH), AKT2 (5.8%, 3/52 with SNP array), and JAK2 (3.8%, 2/52 with SNP array) (4,20). Mutations and amplifications were frequently observed in the ERBB-PI3K pathway regulators, indicating the importance of this pathway in NPC.

Other frequent amplifications identified by CGH include *LTBR* at 12p13, *TERC* at 3q26.3, and *ESR* at 6q25.1 (21,22).

Copy number losses and loss of heterozygosity (LOH)

Previous studies suggested the loss of chromosomes 1p, 3p, 9p, 9q, 11q, 13q, 14q, and 16q regions are common events in NPC (13-17,23-25). Functional complementation studies were performed by utilizing the microcell-mediated chromosome transfer (MMCT) approach to transfer intact or truncated chromosomes to identify the critical regions (CRs) that are important for tumor suppression in NPC cells, including 3p21.3, 14q12, 14q13.2–13.3, 14q24.1, 14q32.33, 11q13, and 11q22–23 (26-32). The 3p21.3 region contains several important TSGs. *BLU/ZMYND10*, *PTPRG*, *RASSF1A*, and *FBLN2*, mapped to or next to this CR, were identified by MMCT, cDNA microarray assay, tissue microarray staining, and NotI chromosome 3 genomic microarray analysis (33-36). Other candidate TSGs located in 3p include *ADAMTS9*, *CACNA2D3*, *GNAT1*, *LTF*, *LARS2*, *RBMS3*, and *CHL1* (13,37). Several TSGs on chromosome 14, including the *MIPOL1*, *CRIP2*, and *LTBP2*, showed significant *in vitro* and *in vivo* tumor-suppressive effects (26,38-40). CRs on chromosome 11,

11q13, and 11q22–23, harbored TSGs including *TSLC1/CADM1* (41), *THY1* (42), and *CRYAB* (27).

Frequent chromosome 9p losses were found in histologically normal nasopharyngeal epithelia and, thus, were considered as a critical early event in NPC tumorigenesis (23). Homozygous deletions of the 9p21.3 region containing *CDKN2A* (*p16*) and *CDKN2B* (*p15*) were found in NPC xenografts and primary tumors (43). Consistently, the genomic landscape study showed the most frequent deletion occurs in the 9p21 region spanning *CDKN2A* and *CDKN2B* (4).

Apart from somatic mutations, SNP array analysis revealed several chromatin modification regulators that also harbor frequent number losses in NPC, including *ARID1A* (15.4%), *BAP1* (1.9%), and *TET1* (3.8 %), which further emphasized the importance of this pathway. *TP53*, which is the most frequently mutated gene in NPC, was also reported to be deleted in 15.4% of NPC cases (8/52) (4).

Gene translocations and rearrangements

Only a few structural variants were reported in NPC. A fusion of ubiquitin protein ligase E3 component n-recognin 5 (UBR5) on 8q22.3 and zinc finger protein 423 (ZNF423) on 16q12.1 was first identified by whole-transcriptome sequencing from the C666 cell line and later was recurrently detected in 8.3% (12/144) of primary tumors (44). Functional analysis demonstrated that the fusion protein affected cell proliferation and colony-forming ability *in vitro* and promoted tumor formation *in vivo* (44). Another study developed a new algorithm to detect structural variants from high-throughput sequencing from long-insert paired-end DNA libraries of NPC, and identified gene fusions at *YAP1-MAML2*, *PTPLB-RSRC1*, and *SP3-PTK2*. FISH analysis of an additional 196 cases identified more rearrangements at *MAML2* (two cases), *PTK2* (six cases), and *SP3* (three cases) (45).

Epigenetic alterations in NPC

Multiple factors including host genetics, viral infection, and environmental factors contribute to NPC tumorigenesis, which all play integral roles in modulating the epigenetic changes, ultimately leading to the critical gene expression alterations. Aberrant epigenetic alterations, particularly DNA methylation, have been frequently reported in NPC. In our recent comparative methylome study, we found NPC had the highest hypermethylation frequency, as compared

to nine other cancer types including liver, head and neck, colon, lung, thyroid, kidney, breast, pancreatic, and prostate cancers (2) (*Figure 2*). A number of tumor suppressors were found to be downregulated by promoter methylation. Aberrant methylation of tumor suppressors *RASSF1* and *CDKN2A* was considered as important early events in NPC tumorigenesis. Promoter methylation of *RASSF1* and *CDKN2A* was found in 49–83% and 16–66% of NPC patients, respectively. Moreover, we identified a number of novel tumor suppressors including *ADAMTS9* (37), *PTPRG* (34), *ZMYND10* (33), *FBLN2* (35), *CRYAB* (27), *CADM1* (41), *THY1* (42), *MMP19* (46), *DUSP6* (47), *MIPOL1* (26), and *LTBP2* (39) by MMCT studies in NPC; the majority of these tumor suppressors were downregulated in NPC by promoter methylation (48). Important DNA methylation changes are frequently reported in several CRs such as 3p21.3, 9p21, and 6p21.3 regions as summarized in *Table 1*.

3p21.3 region

Promoter methylation at multiple 3p21.3 genes has been reported in NPC. The most important epigenetic event was *RASSF1* promoter methylation, which was originally reported in NPC xenografts, cell lines, and in primary tumors, but not in the normal nasopharyngeal epithelia (55). These results were further confirmed by several studies carried out at the single gene or genome-wide levels (2,49,50,52,55-63), while somatic mutations at this gene were detected at only low frequencies (9.5%) in primary tumors and were not observed in recent somatic landscape studies with the Singapore cohort (4) and in our Hong Kong study (manuscript in revision). Besides *RASSF1*, promoter methylation was also detected in *MLH1* (50), *DLEC1* (53,64), *LARS2* (65), *SLC6A20* (2), *LTF* (2), and *ZMYND10* (*BLU*) (52-54) from the 3p21.3 region in NPC patients, suggesting DNA methylation plays an important role in regulating the expression of the genes in this region.

6p22.1–6p21.3 region

The human major histocompatibility complex (MHC) locus is located at the 6p22.1–6p21.3 region. In NPC, the association between human leukocyte antigen (HLA) polymorphisms with NPC genetic susceptibility was confirmed by genome-wide association study (GWAS). Interestingly, the methylome study using the Illumina Infinium assay on HumanMethylation450 BeadChip reveals

significant methylation changes in this region in NPC (2). A number of genes including *B3GALT4*, *FLOT1*, *PXT1*, *TNXB*, *TRIM31*, *LY6G5C*, *PPP1R18*, *GNL1*, *IER3*, and *NKAPL* in this region were hypermethylated in tumors compared to non-tumor tissues, indicating the importance of DNA methylation in regulating gene expression. Although none of the MHC class I HLA genes was found significantly methylated in NPC, previous study using gene expression profiling microarrays showed the inhibition of the expression of the MHC class I genes in NPC by EBV (78). It is likely that epigenetic changes have a role in regulating the transcription of HLA genes in NPC. Further investigation of aberrant methylation in this region in a larger patient cohort is necessary. Although next-generation sequencing (NGS)-based bisulfite sequencing has emerged as a powerful method to characterize the methylation changes in high resolution, MHC is a highly polymorphic region, which limits the utility of NGS-based methods, since the complexity of the DNA sequence is greatly reduced after bisulfite conversion. An alignment and methylation analysis pipeline specifically designed for the MHC region having adequate sequencing depth is required to improve the accuracy of methylation assessment in this region.

9p21 region

Homozygous deletion of the 9p21.3 region containing *CDKN2A* (*p16*) and *CDKN2B* (*p15*) was found in NPC. In addition to genomic alterations, inactivation of both genes by promoter methylation was reported in 16–66% and 21–50% of the tumors, respectively (50,53,58,60,62). These results emphasize the importance of integrating the genetic and epigenetic changes to provide a comprehensive view of molecular changes in NPC.

Signaling pathways deregulated by DNA methylation in NPC

Two genome-wide methylome studies consistently identified a few important signaling pathways and functions often deregulated by DNA methylation in NPC, including the Wnt, MAPK, Hedgehog, and TGF-β signaling pathways and focal adhesion (2,49). In the Wnt signaling pathway, a number of Wnt inhibitors including *DKK1*, *WIF1*, *SFRP1*, *SFRP2*, *SFRP4*, and *SFRP5* are silenced by promoter methylation in NPC. Inactivation of these inhibitors may lead to the aberrant activation of Wnt

Table 1 Tumor suppressor genes frequently altered by epigenetic and genetic mechanisms in NPC

Gene	Location	Full name	Mechanisms of silencing	Function	References
TP73	1p36.3	Tumor protein p73	Hypermethylation	Cell cycle, DNA damage	(49,50)
CASP8	2q33–q34	Caspase 8, apoptosis-related cysteine peptidase	Hypermethylation	Apoptosis	(50)
ADAMTS9	3p14.1	ADAM metallopeptidase with thrombospondin type 1 motif, 9	Hypermethylation	Angiogenesis	(37)
FHIT	3p14.2	Fragile histidine triad	Hypermethylation/ allelic deletion	Cell cycle, DNA damage response	(51)
ZMYND10	3p21.3	Zinc finger, MYND-type containing 10	Hypermethylation	Angiogenesis	(33,52-54)
RASSF1	3p21.3	Ras association (RalGDS/AF-6) domain family member 1	Hypermethylation	Cell growth, proliferation	(2,49,50, 52,55-63)
DLEC1	3p21.3	Deleted in lung and esophageal cancer 1	Hypermethylation	Cell communication, signaling transduction	(53,64)
MLH1	3p21.3	MutL homolog 1	Hypermethylation	Mismatch repair	(50)
LARS2	3p21.3	Leucyl-tRNA synthetase 2, mitochondrial	Hypermethylation/ allelic deletion	Aminoacyl-tRNA synthetase, mitochondrial leucyl-tRNA synthetase	(65)
PTPRG	3p21–p14	Protein tyrosine phosphatase, receptor type, G	Hypermethylation	Cell cycle	(34)
RARB	3p24.2	Retinoic acid receptor, beta	Hypermethylation	Hormone receptor, transcriptional regulator, retinoic acid signalling	(60,66)
FBLN2	3p25.1	Fibulin 2	Hypermethylation/ allelic deletion	Cell growth, angiogenesis, migration	(35)
UCHL1	4p14	Ubiquitin carboxyl-terminal esterase L1 (ubiquitin thiolesterase)	Hypermethylation	Deubiquitination	(58,67)
PCDH10	4q28.3	Protocadherin 10	Hypermethylation	Protocadherin, cell-cell adhesion, apoptosis, cell signalling	(68)
IER3	6p21.3	Immediate early response 3	Hypermethylation	DNA damage/repair, apoptosis	(2)
SFRP1	8p11.21	Secreted frizzled-related protein 1	Hypermethylation	Inhibitor of Wnt/β-catenin signaling pathway	(49)
DLC1	8p22	DLC1 Rho GTPase activating protein	Hypermethylation/ allelic deletion	Cell cytoskeleton organization, GTPase activator, signal transduction, cell adhesion, invasion	(64,69)
CDKN2A	9p21	Cyclin-dependent kinase inhibitor 2A	Hypermethylation/ allelic deletion	Cell cycle	(31,53,58,60, 62,70-74)
CDKN2B	9p21	Cyclin-dependent kinase inhibitor 2B (p15, inhibits CDK4)	Hypermethylation/ allelic deletion	Cell cycle	(60,70,72)
DAPK1	9q21.33	Death-associated protein kinase 1	Hypermethylation	Cell cycle	(57,58,62,72)
MGMT	10q26	O-6-methylguanine-DNA methyltransferase	Hypermethylation	DNA repair, cell cycle, genomic stability	(50,60)

Table 1 (*continued*)

Table 1 (*continued*)

Gene	Location	Full name	Mechanisms of silencing	Function	References
CRYAB	11q22.3–q23.1	Crystallin, alpha B	Hypermethylation/ allelic deletion	Epithelial-mesenchymal transition (EMT)	(27)
CADM1	11q23.2	Cell adhesion molecule 1	Hypermethylation	Cell growth, apoptosis	(41)
THY1	11q23.3	Thy-1 cell surface antigen	Hypermethylation	Cell invasion, cell growth	(42)
MMP19	12q14	Matrix metallopeptidase 19	Hypermethylation/ allelic deletion	Angiogenesis	(46)
WIF1	12q14.3	WNT inhibitory factor 1	Hypermethylation	Inhibitor of Wnt signaling pathway	(57,62,75)
DUSP6	12q22–q23	Dual specificity phosphatase 6	Hypermethylation/ allelic deletion	Migration, invasion, cell growth, EMT	(47)
CHFR	12q24.33	Checkpoint with fork head and ring finger domains, E3 ubiquitin protein ligase	Hypermethylation	Cell cycle	(76,77)
MIPOL1	14q13.3	Mirror-image polydactyly 1	Hypermethylation/ allelic deletion	Cell cycle	(26)
LTBP2	14q24	Latent transforming growth factor beta binding protein 2	Hypermethylation/ histone deacetylation	Cell mobility, invasiveness	(39)

NPC, nasopharyngeal carcinoma.

signaling and transcription of its downstream targets. Gene expression profiling of primary tumors shows the differential expression of several components of the Wnt canonical signaling pathway (79). These data suggested the important involvement of aberrant Wnt signaling in NPC tumorigenesis.

Methylation status as biomarkers for NPC diagnosis and prognosis

Given the widespread aberrant methylation often observed in NPC, we and others have evaluated the usefulness of methylation biomarker panels for NPC early detection, using tumor biopsies and non-invasive biological specimens as summarized in *Table 2* (57-60,62,64,70-72). For example, Wong and his colleagues examined the methylation of *CDH1*, *DAPK1*, *CDKN2B*, *RASSF1*, and *CDKN2A* in plasma samples from 41 NPC patients before treatment and 43 normal individuals by real-time quantitative PCR after bisulfite conversion; they found aberrant methylation in at least one of the five genes was detectable in 71% plasma of NPC patients with a specificity of 91% (72). Methylation of five tumor suppressors (*RASSF1*, *CDKN2A*, *DLEC1*, *DAPK1*, *UCHL1*) in serum samples was investigated in 40

NPC patients and 41 age- and gender-matched healthy subjects using the methylation-specific PCR approach (58). Combination of four markers including *CDKN2A*, *DLEC1*, *DAPK1*, and *UCHL1* has the highest sensitivity and specificity for detecting NPC. Circulating EBV copy number changes have emerged as a promising biomarker to aid NPC diagnosis (80). We examined a methylation panel including *RASSF1*, *WIF1*, *DAPK1*, and *RARB2* using high-resolution melting curve analysis and EBV copy number changes in 220 plasma samples from NPC patients and found this methylation panel can be used as a supplementary test to the EBV copy number test for increased detection of early-stage NPC (57). Combinations of both the four-gene methylation panel and EBV DNA test can detect 63.6%, 86.7%, 88%, and 96.5% of the patients with stages I, II, III, and IV tumors, respectively, with a specificity of 88%. In addition to hypermethylation mainly at promoter CpG islands, global hypomethylation predominantly in the repetitive elements and gene body, is another feature of cancer. Chan *et al.* explored the usage of genome-wide hypomethylation and copy number aberrations in plasma DNA by bisulfite sequencing in multiple types of cancers including NPC with a sensitivity of 74% and a specificity of 94% (81). However, this NGS-based method is costly

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Table 2 Summary of the studies using a panel of genes as methylation biomarkers for detecting NPC

Methylation panel	Sample type	Method	# patients	# controls	Specificity (%)	Sensitivity (%)	References
RASSF1, *RARB2*, *DAPK1*, *CDKN2A*, *CDKN2B*, *MGMT*, *GSTP1*	Tumor tissue	MSP	39	6	100	100	(60)
RASSF1, *DAPK1*, *CDKN2A*	NP brushing	MSP	28	26	79	100	(71)
DAPK1, *CDH1*, *RASSF1*, *CDKN2B*	NP brushing	MSP	30	37	80	100	(70)
	Rinsing fluid	MSP	30	43	90	98	
	Plasma	MSP	30	43	10	95	
	Buffy coat	MSP	30	43	41	93	
CDH1, *CDKN2A*, *DPAK1*, *CDKN2B*, *RASSF1*	Plasma	Real-time quantitative PCR after bisulfite conversion	41	43	71	91	(72)
UCHL1, *KIF1A*, *DLEC*	Tumor biopsy	Quantitative MSP	50	28	84	92	(64)
CHFR, *RIZ1*, *WIF1*, *CDKN2A*, *RASSF1*	NP brushing	MSP	53	25	98	96	(62)
RASSF1, *DAPK1*, two *EBV* markers	NP brushing	MMSP	49	20	98	100	(59)
CDKN2A, *DLEC1*, *DAPK1*, *UCHL1*	Serum	MSP	40	41	85	66	(58)
RASSF1, *WIF1*, *DAPK1*, *RARB2*	Plasma	MS-HRM	220	50	73	96	(57)
RASSF1, *WIF1*, *DAPK1*, *RARB2* and *EBV DNA* test	Plasma	MS-HRM	220	50	89	88	
RASSF1, *WIF1*, *DAPK1*, *RARB2*	NP brushing	MS-HRM	96	43	96	67	

NPC, nasopharyngeal carcinoma; MSP, methylation-specific PCR; MMSP, multiplex methylation-specific PCR; MS-HRM, methylation-sensitive high resolution melting.

and further evaluation in a larger patient cohort is necessary prior to clinical use.

NPC local recurrence and distant metastasis contribute to treatment failure and NPC mortality. Therefore, identification of biomarkers for detecting the recurrence and metastasis and predicting the patient prognosis is extremely important for improving the treatment success in NPC. In our methylation biomarker study carried out in plasma samples, a combination of the four-gene methylation panel and EBV DNA tests has a sensitivity as high as 94.3% and 93.5% for detection of the metastasis and local recurrence, respectively, suggesting the combination testing has great potential for monitoring disease progression. Recently, a genome-wide methylation study identified a panel of prognostic methylation biomarkers comprising six hypermethylated genes *WIF1*, *CCNA1*, *UCHL1*, *RASSF1*, *TP73*, and *SFRP1* (56). The study was carried out in two stages including the training and validation cohorts. In the training cohort (n=150), the patients with high methylation levels had poorer disease-free survival (DFS) [HR, 2.26; 95% confidence interval (CI), 1.28–4.01; P=0.005] and overall survival (OS) [HR, 2.47; 95% CI, 1.30–4.71; P=0.006] than those with low methylation. The association of the methylation panel with survival was independent of stage. The prognostic value was further validated in two independent cohorts including 150 and 153 NPC patients, respectively. The patients with low methylation had a favorable response to concurrent chemotherapy with an improved DFS and OS, whereas patients with high methylation did not benefited a present chemotherapy. This study demonstrated the potential usefulness of a methylation panel as a prognostic and predictive tool in NPC.

Aberrant histone modifications in NPC

In the genomic landscape study in NPC, somatic mutations were identified in a number of histone modifiers, suggesting the importance of aberrant histone modification in NPC tumorigenesis. Consistently, our methylome study reveals that the aberrantly methylated genes often have bivalent markers, H3K4me3 mark for active transcription and H3K27me3 for repressive transcription, indicating DNA methylation functions together with histone modification for regulating gene expression in NPC tumorigenesis (2). The enrichment of the genes with homeobox domain in the aberrantly methylated genes in NPC indicates these aberrantly methylated genes are often polycomb complex targets. Many studies have demonstrated that the polycomb repressive complex genes encoding epigenetic gene-silencing proteins contribute to the oncogenesis of various cancers. Over-expression of the polycomb complex genes BIM1 and *EZH2* was demonstrated in NPC tumors (82,83). Importantly, ectopic expression of BMI1 is sufficient to immortalize the normal nasopharyngeal epithelial (NPE) cells (83). EZH2 encodes a histone-lysine N-methyltransferase, responsible for H3K27 methylation. The important roles of EZH2 in NPC cell migration/invasion, cell survival, and angiogenesis have been demonstrated *in vitro* in several studies (84-86). Cai *et al.* examined the H3K27me3 level in the NPC tumors and normal NPE tissues by Western blotting and immunohistochemistry (IHC). They found 60.8% of NPC and only 16% of normal NPE tissues have high level of H3K27me3 marks and the H3K27me3 level is associated with patient prognosis and chemoradiotherapy response (87).

EBV and epigenetic alterations

In NPC, EBV is harbored in a latent stage with restricted viral gene expression of *LMP1/2*, *EBNA1*, *EBER1/2*, and *BARTs*. LMP1 can activate cellular DNA methyltransferase via c-Jun NH2-terminal kinase signaling (88) and upregulate BMI1 expression (89), which is associated with epigenetic changes in NPC. On the other hand, latent episomal EBV genomes are subject to host cell-dependent epigenetic modifications including DNA methylation and histone modifications, which are important for maintenance of EBV latent infection (90).

In addition to NPC, extensive methylation changes are also often observed in the EBV-associated gastric cancer (91).

Previous studies suggested that the EBV-infected cells acquire extensive methylation to silence multiple TSGs and to transform cells into cancer cells (92). Although the initial step of virus infection in the epithelial cell and the methylation mechanism induced by EBV infection has not been fully elucidated, it was proposed that EBV infection is an epigenetic driver in tumorigenesis (92).

Conclusions and future directions

NPC is a complex disease that involves host genetics, viral infection, and environmental factors. It is characterized by a comparatively low mutation rate, extensive hypermethylation, as well as frequent chromosomal abnormalities and copy number alterations. Although the high-throughput characterization methods, such as massive parallel sequencing, SNP, and methylation assays, has resulted in a greater understanding of NPC biology, yet much remains to be uncovered. Moreover, integration of the whole-exome and whole-genome sequencing data, transcriptome, proteomics, and methylome data in more NPC patients, together with well-documented clinical outcome information, will aid us to further understand the importance of molecular alterations and their clinical relevance. Study of additional NPC patients will enhance our ability to identify the less frequent, but critical, genetic and epigenetic events in the pathogenesis of NPC. These insights will lead to the identification of powerful biomarkers for NPC diagnosis and prognosis and evaluation of novel and existing targeted therapies to improve the treatment success.

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Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

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