CpG methylation as epigenetic biomarkers for nasopharyngeal carcinoma diagnostics

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Abstract: CpG methylation at gene promoters or regulatory regions, as one of the well-studied epigenetic modifications, plays essential roles in normal physiology and the pathogenesis of nasopharyngeal carcinoma (NPC). Strongly associated with Epstein-Barr virus (EBV) infection, NPC manifests a unique epigenetic phenotype with high-grade genome-wide CpG methylation at CpG islands. Meanwhile, EBV acts as a strong epigenetic driver for NPC tumorigenesis. Through hijacking the cell epigenetic machinery (DNMTs, TETs, HDACs, etc.), EBV modulates the CpG methylation and histone modification profiles of both viral and cellular genes (especially tumor suppressor genes—TSG) to regulate their expression, even at the very early stage of NPC pathogenesis. It is believed that the high CpG methylation pressure and epigenetic dysregulation of gene expression induced by EBV infection create an ideal epigenetic environment in pre-malignant nasopharyngeal (NP) epithelial cells for further malignant transformation with subsequent genetic mutations, eventually potentiates NPC initiation and promotes its progression, together with genetic alterations. Clinically, tumor-specific methylation of TSG promoters can be used as epigenetic biomarkers. In this review, we summarized the recent development of TSG CpG methylation-based biomarkers for NPC diagnostics. We further discussed the advantages of DNA methylation biomarkers, the detection methods and sample sources (tissue biopsy, NP brushing, plasma). We believe that CpG methylation-based biomarkers using cell-free circulating DNA and NP brushing samples should have a great perspective for NPC diagnostics in future.

Keywords: CpG methylation; epigenetic biomarkers; nasopharyngeal carcinoma (NPC); diagnostics

Received: 15 June 2021; Accepted: 24 December 2021; Published: 30 June 2022.
doi: 10.21037/anpc-21-9
View this article at: https://dx.doi.org/10.21037/anpc-21-9

Introduction

Nasopharyngeal carcinoma (NPC) is a specific type of head and neck cancer, occurring at the epithelium surface of the posterior nasopharynx (1,2). NPC incidence is ~2–3 times more common in males than in females, with a peak at ages 45–59 years (3). NPC is highly prevalent in some regions of southern China, Southeast Asia, North Africa, and the Arctic/Alaska (4). Due to its limited geographic and ethnic distribution, genetic susceptibility and environmental factors have been considered to contribute to its development. These include Cantonese ancestry, eating habits like food preserved with salt, tobacco smoking, as well as herpesvirus Epstein-Barr virus (EBV) infection (5,6). All undifferentiated NPC cases are associated with EBV infection, especially the high-risk EBV variants (7). In terms of the distinct expression pattern of EBV latent

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genes, NPC is classified as type II viral latency. Although the current clinical treatment could achieve an overall 5-year survival rate of >80% for NPC patients especially at early stages, still ~20% of patients eventually present with local-regional relapse and distant metastasis (8). Thus, developing useful biomarkers for the early diagnosis of NPC is in good need.

A unique epigenetic etiology of NPC pathogenesis

Epigenetic modifications can occur at multiple levels, from direct DNA CpG modification to more complex histone modifications/chromatin conformational changes, as well as the newly discovered various RNA modifications (9-11). The dysregulation of these epigenetic modifications contributes critically to multi-tumorigeneses, including NPC.

As the vast majority of NPC is EBV-associated, EBV acts as a strong epigenetic driver during NPC pathogenesis (12,13). Previous methylome studies have shown a special epigenetic phenotype of NPC: a significantly higher grade of methylation at cellular CpG islands (CGIs) (14-16). EBV dysregulates host cell epigenetic mechanisms through abducting cellular epigenetic machinery (DNMTs, TETs) (17), then causes methylation aberrations (epi-mutation) of both viral and cellular genes, especially the promoter CpG methylation of multiple tumor suppressor genes (TSGs). Therefore, genetic and epigenetic alterations are complementary to each other during early NPC pathogenesis, and potentiate NPC initiation and progression altogether. For example, high frequency of chromosome 3p deletion is an early event in NPC development (18). Meanwhile, TSGs at this region like *RASSF1A* (19), *DLEC1* (20,21), *PLCD1*, *ZMYND10/BLU* are also frequently methylated in NPC primary tissues but not in normal nasopharyngeal (NP) epithelia. *p16* (*INK4A*) inactivation by promoter CpG methylation is one of the most common and earliest epigenetic events in human carcinogenesis including NPC (22). Recently, direct functional evidence using engineered mice model demonstrated that *p16* (*INK4A*) epi-mutation is an epigenetic driver for tumor formation and malignant progression (23). Thus, CpG methylation are ideal and valuable tumor biomarkers for NPC early diagnostics.

Advantages of detecting CpG methylation biomarkers for NPC

Epigenetic modifications can be accurately and repeatedly detected, in line with the definition of biomarkers. Epigenetic molecular markers include CpG methylation, 5hmC of DNA, histone protein modifications, and even various RNA modifications. Among these, CpG methylation of DNA has its unique advantages and feasibility as a good biomarker: (I) DNA molecules of clinical samples can exist stably for many years even after routine histopathology treatment, thus can be used in archival samples; (II) CpG methylation detection is a positive test with absolute indication and remains relatively stable, thus methylation detection does not need internal control design; (III) CpG methylation of TSG promoters is tumor-specific, thus its detection has great specificity for tumor cells; (IV) CpG methylation detection can use polymerase chain reaction (PCR) amplification-based detection systems, thus with great sensitivity; (V) different patients usually have different gene mutations while promoter CpG methylation is commonly present in most individuals, thus its detection is more convenient; (VI) most importantly, aberrant TSG promoter methylation occur at the early stage of tumorigenesis, thus makes it a valuable biomarker for early cancer diagnosis.

Methods of detecting CpG methylation and clinical sample sources

To develop CpG methylation biomarkers with high sensitivity and specificity for NPC diagnostics, proper selection of appropriate methods for methylation biomarker screening and detection is very important. Various techniques to detect DNA methylation levels have been established. Genome-wide screening using next-generation sequencing- or microarray-based platforms can screen candidate methylation biomarkers comprehensively. These approaches are based on affinity enrichment, methyl-sensitive restriction enzyme (RE) digestion, and bisulfite conversion. Affinity-based enrichment methods enrich methylated DNA fragments using antibodies specific to 5mC (as in MeDIP) or methyl-CpG-binding domain (MBD) proteins, followed by profiling with microarray (MeDIP-chip, MBD-chip) or sequencing (MeDIP-seq, MethylCap-seq). DNA bisulfite conversion coupled with sequencing, or methylation array or RE digestion can quantitatively measure genome-wide methylation, including whole-genome bisulfite sequencing (WGBS), Illumina Infinium Array (MethylationEPIC, Infinium Methylation850, 450 BeadChips), and reduced-representation bisulfite sequencing (RRBS, dRRBS,

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xRRBS). NPC methylomes have been carried out by methylated DNA immunoprecipitation (MeDIP) (14) and Illumina HumanMethylation450 BeadChip (15).

Selected single specific methylation biomarkers or a panel of limited methylation markers can be further examined in large cohort samples, using rapid and cost-effective gene-specific methylation detection assays. For this purpose, bisulfite DNA sequencing remains as a gold standard for DNA methylation analysis at high resolution. Other methods include methylation-specific PCR (MSP), quantitative MSP (qMSP) such as MethyLight assay, and pyrosequencing. All can detect the methylation status of single genes with high sensitivity and specificity, which meets the key requirements for biomarker validation and subsequent clinical usage. However, for each gene with epigenetic alterations, careful optimization of its methylation detection system for rapid detection is necessary and critical. We list some common methods of CpG methylation marker detection for NPC with different purposes in *Table 1*.

DNA samples could be acquired from clinical tissues or body fluids. NPC, due to its unique anatomical location, traditional diagnosis of primary tumor is usually the pathological assessment of tissue biopsy obtained by invasive nasal endoscopy, which is not easy for large population screening and disease monitoring. Moreover, tissue biopsy of early-stage NPC is difficult to obtain due to the absence of clinical symptoms. Thus, NP brushing (14) and circulating cell-free nucleic acids (ccfNAs) (30) from body fluids (plasma, serum) are ideal samples for NPC methylation marker detection. Previous studies have shown that these clinical samples are well suitable for EBV viral copy number quantitative assessment as a biomarker for NPC non-invasive diagnosis (47). The detection of CpG methylation as non-invasive biomarkers for NPC, in parallel with EBV-DNA copy number analysis, would provide a highly specific diagnostic tool for NPC risk assessment and early diagnosis.

TSG methylated DNA as biomarkers for NPC diagnostics

Aberrant promoter CpG methylation of TSGs occurs at the early stage of cancer development, and has become an attractive biomarker for cancer screening and early detection. We summarize the reported CpG methylation biomarkers for NPC screening and diagnosis in *Tables 1,2*. Multiple methylation biomarkers in a variety of sample sources, tested as a single gene (*Table 1*) or panels of genes (*Table 2*), appeared to be effective in discriminating NPC from non-cancer controls. Biomarkers mostly investigated in both NPC tumor biopsy and liquid biopsy (NP brushing, plasma) include *RASSF1A*, *p16*, *CDH1*, *DLEC1*, *UCHL1*, which achieves good sensitivity and specificity.

The sensitivity and specificity of methylation detection using NPC tissue or liquid samples (plasma, brushing, swabs) are often similar: for examples, methylation panel of *DAPK*, *E-cadherin*, *RASSF1A*, *p15*, *p16* by MSP (methylation: tissue: 97%, swabs: 80%); methylation panel of *RASSF1A*, *WIF1*, *DAPK1*, *RARβ2* by methylation-sensitive high resolution melting (MS-HRM) [plasma: sensitivity 96%/specificity 64.6%, area under the curve (AUC) =0.87; brushing: sensitivity 95.8%/specificity 67.4%, AUC =0.82], although some variations among different sample types have also been observed. Gene methylation panels are always satisfactory based on sensitivity, while the specificity could be improved by combining with EBV DNA or antibodies markers. The majority of methylation biomarker data mentioned above are based on comparing primary NPC tissue with healthy control samples. For screening and early diagnosis purposes, future NPC methylation marker studies should be more focused on high-risk populations and suspected patients at early stages.

Conclusion and future perspective

The special epigenetic feature of high-grade CpG methylation of NPC highlights the importance of its epigenetic etiology, and also the good perspective of developing methylation biomarkers for its early detection and diagnostics. Methylation biomarker is actually complementary to other non-invasive markers such as EBV-DNA for NPC diagnosis. The studies reviewed here exemplify recent progress of CpG methylation biomarkers in NPC diagnostics, including both single methylation biomarker and panel of multiple methylation biomarkers with diagnostic power. Although methylation biomarkers are very promising, there is still no reliable NPC methylation diagnostic kit available for clinical use so far. Further in-depth technical investigations are still needed to facilitate the realistic usage in-clinic diagnostics of NPC, such as optimized sample storage, standardized DNA extraction, and selection of methylation detection systems. With the recent technical advance of CpG methylation detection, the future of methylation biomarkers for NPC early detection and diagnostics is indeed bright.

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Table 1 DNA methylation biomarkers for NPC diagnostics

DNA methylation marker or gene	Sample	Method	Methylation frequency	Sensitivity/specificity	Ref.	Comments
RASSF1A	Tissue, brushing, blood	MSP, MethyLight, MS-HRM	–	Tissue: 72%/99%, AUC =0.98 Brushing: 56%/100%, AUC =0.94 Blood: 11%/98%, AUC =0.97	(19,24-30)	Meta-analysis enrolled 16 eligible studies
RERG	Tissue	methyl qPCR	–	Tissue: 78.3%/100%, AUC =0.897	(31,32)	
	ccfDNA	qAMP		ccfDNA: 60%/100%, AUC =0.855	(33)	All cases are non-keratinizing carcinoma
ITGA4	Tissue, ccfDNA	qAMP	–	Tissue: 69.1%/94.4%, AUC =0.871 ccfDNA: 75%/60%, AUC =0.683		
ZNF671				Tissue: 91.5%/89.5%, AUC =0.946 ccfDNA: 64.7%/80%, AUC =0.724		
SHISA3				Tissue: 74.1%/84.2%, AUC =0.809 ccfDNA: 42.9%/90%, AUC =0.600		
SLIT2	Tissue, plasma	Bisulfite pyrosequencing	–	Tissue: 77%/81.6%, AUC =0.846 Plasma: 70.5%/94.7%, AUC =0.866	(34)	Patients were treatment-naïve; the diagnostic value of *SLIT2* methylation in plasma is better than that from tissue samples
EBNA1	Tissue	MSP	–	82%/94%	(26)	Samples from Morocco
LMP1				59%/94%		
ITGA9				50%/100%		
P16				45%/100%		
WNT7A				69%/80%		
CHFR				40%/67%		
CYB5R2				47%/75%		
WIF1				100%/25%		
FSTL1				57%/87%		
SEPT9	Tissue, swabs	qMSP	Tissue: 92% (23/25) Swabs: 72.7% (16/22)	AUC =0.882	(35)	NPC biopsies and paired swabs had a good correlation
CDH13	Tissue, swabs	MSP	Tissue: 89.7% (52/58) Swabs: 81% (34/42)	Swabs: 81%/100%	(36)	Methylation was not detected in swab samples whose corresponding biopsies were unmethylated
DAPK	Tissue, plasma, buffy coat	MSP	Tissue: 75% (24/32) Plasma: 50% (6/12) Buffy coat: 25% (3/12)	–	(26-30,37)	12 patients with blood samples available are all *DAPK*-methylated in tissue samples

Table 1 *(continued)*

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Table 1 *(continued)*

DNA methylation marker or gene	Sample	Method	Methylation frequency	Sensitivity/specificity	Ref.	Comments
RARβ2	Tissue, brushing, plasma	MS-HRM	Brushing: 53.1% (51/96)	Brushing: 50.0%/83.7%, AUC =0.68	(27)	
			Plasma: 15.9% (35/220)	Plasma: AUC =0.58		
E-cadherin	Swabs, M&T rinsing fluid, plasma, buffy coat	MSP	Tissue: 53%	Swabs: 50%/100%	(28,38)	
			Swabs: 27%	M&T: 81%/100%		
			M&T: 43%	Plasma: 13%/100%		
			Plasma: 7%	Buffy coat: 38%/100%		
			Buffy coat: 20%			
p15			Tissue: 80%	Swabs: 67%/100%	(28,30)	
			Swabs: 53%	M&T: 46%/98%		
			M&T: 40%	Plasma: 0/98%		
			Plasma: 0	Buffy coat: 8%/100%		
			Buffy coat: 7%			
p16			Tissue: 33%	Swabs: 50%/100%		
			Swabs: 17%	M&T: 50%/100%		
			M&T: 17%	Plasma: 0/100%		
			Plasma: 0	Buffy coat: 0/100%		
			Buffy coat: 0			
CDKN2A	Serum	MSP	22.5% (9/40)	22.5%/97.6%, AUC =0.6	(29)	Number of early-stage NPC patients was limited (stage I/II: 4/40)
DLEC1			25.0% (10/40)	25%/92.7%, AUC =0.59		
UCHL1			64.9% (24/37)	64.9%/80.5%, AUC =0.7		
AIM1	Tissue	qMSP	30% (15/50)	30%/92%, AUC =0.61	(39)	All tumor tissues enrolled were type II non-keratinizing carcinomas and EBV+. This is the first study utilizing qMSP for the examination of NPC
APC			34% (17/50)	34%/96%, AUC =0.65		
CALCA			44% (22/50)	40%/92%, AUC =0.68		
DCC			50% (23/46)	50%/96%, AUC =0.77		
DLEC			60.4% (29/48)	60%/96%, AUC =0.73		
DLC1			43.8% (21/48)	43%/100%, AUC =0.71		
ESR			26% (13/50)	26%/96%, AUC =0.61		
FHIT			44% (22/50)	44%/21%, AUC =0.38		
KIF1A			56% (28/50)	56%/96%, AUC =0.76		
UCHL1/ PGP9.5			64% (32/50)	66%/96%, AUC =0.78		
TIG1			30% (15/50)	26%/92%, AUC =0.43		

Table 1 *(continued)*

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Table 1 *(continued)*

DNA methylation marker or gene	Sample	Method	Methylation frequency	Sensitivity/specificity	Ref.	Comments
CDK10	Tissue, PB	MSP	Tissue: 52.5% (21/40) PB: 37.5% (15/40)	PB: 37.5%/100%	(40)	Methylation was observed only in blood samples derived from patients with tissues exhibiting methylation
RIZ1	Tissue, swabs, M&T rinsing fluid, plasma, buffy coat	MSP	Tissue: 60% (18/30) Swabs: 37% (11/30) M&T: 30% (9/30) Plasma: 23% (7/30) Buffy coat: 10% (3/30)	Tissue: 0/100%	(26,41)	Methylation in tissues was necessary but not sufficient for methylation detection in PB
HIN-1	Tissue, swabs, throat rinsing fluid, plasma, buffy coat	MSP	Tissue: 77% (36/47) Swabs: 46% (12/26) Throat rinsing fluid: 19% (5/26) Plasma: 18% (2/11) Buffy coat: 45% (5/11)	Swabs: 67%/100% Throat rinsing fluid: 28%/100% Plasma: 18%/100% Buffy coat: 46%/100%	(42)	High specificity and moderate sensitivity; might be used in combination with EBV antibody markers
CDH1	Plasma	qMSP	46% (19/41)	–	(30)	
ECRG4	Tissue, PB	MSP, BGS	Ttissue: 72.5% (29/40) PB: 57.5% (23/40)	–	(43)	The first study to detect *ECRG4* methylation in PB samples of NPC patients
LOX	Tissue, swabs	MSP, BGS	Tissue: 85.7% (42/49) Swabs: 18.75% (3/16)	–	(44)	
FEZF2	Tissue, swabs	MSP, BGS	Tissue: 75.5% (37/49) Swabs: 75% (12/16)	–	(45)	All NPC tissues were EBV+
BRD7	Tissue, PB	MSP	Tissue: 100% (18/18) PB: 100% (18/18)	–	(46)	A provocative observation based on limited samples

NPC, nasopharyngeal carcinoma; MSP, methylation-specific polymerase chain reaction; MS-HRM, methylation-sensitive high resolution melting; AUC, area under the curve; qPCR, quantitative polymerase chain reaction; ccfDNA, circulating cell-free DNA; qAMP, quantitative analysis of DNA methylation using real-time polymerase chain reaction; qMSP, quantitative methylation-sensitive polymerase chain reaction; M&T, mouth and throat; EBV, Epstein-Barr virus; PB, peripheral blood; BGS, bisulfite genomic sequencing.

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Table 2 Panels of DNA methylation biomarkers for NPC diagnostics

Panels of markers or genes	Sample	Method	Methylation frequency	Sensitivity/specificity	Ref.	Comments
LMP1, *ITGA9*, *RASSF1A*, *P16*	Tissue	MMSP	–	97%/94%	(26)	EBNA1+ NPC cases and normal samples; MMSP assay allows analyses of multiple marker methylation in a single reaction
RASSF1A, *WIF1*, *DAPK1*, *RARβ2*	Tissue, brushing, plasma	MS-HRM	Tissue: 100% (52/52)	Brushing: 95.8%/67.4%, AUC =0.82	(27)	The sensitivity of methylation panel was higher than EBV DNA at the early stage of NPC
			Brushing: 95.8% (92/96)	Plasma: 96%/64.6%, AUC =0.87		
			Plasma: 72.7% (160/220)			
DAPK, *E-cadherin*, *RASSF1A*, *p15*, *p16*	Tissue, swabs, M&T rinsing fluid, plasma, buffy coat	MSP	Tissue: 97%	Swabs: 83%/100%	(28)	The sensitivity was higher in NP swab and M&T rinsing fluid samples, compared to plasma and buffy coat samples
			Swabs: 80%	M&T: 90%/98%		
			M&T: 87%	Plasma: 10%/95%		
			Plasma: 10%	Buffy coat: 41%/93%		
			Buffy coat: 40%			
CDKN2A, *DLEC1*, *DAPK1*, *UCHL1*	Serum	MSP	–	85%/65.9%, AUC =0.82	(29)	The combination of these 4 markers is the best in considering both sensitivity and specificity
RERG, *ZNF671*	ccfDNA	qAMP	–	93.8%/80%, AUC =0.90	(33)	Its diagnostic accuracy is better than that of single marker in tissue DNA
AIM1, *APC*, *CALCA*, *DCC*, *DLEC*, *DLC1*, *ESR*, *FHIT*, *KIF1A*, *UCHL1*, *TIG1*	Tissue	qMSP	100% (50/50)	100%/57%, AUC =0.78	(39)	
CDH1, *DAPK1*, *p15*, *p16*, *RASSF1A*	Plasma	qMSP	71% (29/41)	71%/91%	(30)	Diagnostic accuracy is improved when combined with serological EBV antibodies
RASSF1A, *RARβ2*, *DAPK*, *p16*, *p15*, *p14*, *MGMT*, *GSTP1*	Tissue	MSP	–	100%/100%	(22)	
DAPK, *RASSF1A*, *p16*	Brushing	MSP	–	78.6%/100%	(24)	The sensitivity is increased to 100% when combined with quantitative EBV-DNA analysis
RASSF1A, *DAPK*, *EBNA1*, *LMP1*	Tissue, swabs	MMSP	–	98%/100%	(48)	Results of NPC swabs were similar to those of corresponding biopsies
CHFR, *RIZ1*, *WIF1*, *p16*, *RASSF1A*	Brushing or tissue	MSP	–	98%/96%	(49)	In Indonesian populations; NPC paraffin and brushing DNA samples showed 81.8% concordance in methylation by MSP. The panel is proposed as a complementary test for early NPC detection with EBV-DNA-based assay

NPC, nasopharyngeal carcinoma; MMSP, multiplex methylation-specific polymerase chain reaction; MS-HRM, methylation-sensitive high resolution melting; AUC, area under the curve; EBV, Epstein-Barr virus; MSP, methylation-specific polymerase chain reaction; M&T, mouth and throat; ccfDNA, circulating cell-free DNA; qAMP, quantitative analysis of DNA methylation using real-time polymerase chain reaction; qMSP, quantitative methylation-sensitive polymerase chain reaction.

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Acknowledgments

Funding: This work was supported by grants from Hong Kong RGC (GRF #14115920, #14118118), China National Natural Science Foundation (#81772869) and Health and Medical Research Fund (HMRF) of Hong Kong (#20190152).

Footnote

Provenance and Peer Review: This article was commissioned by the Guest Editors (Maria Li. Lung and Lawrence S. Young) for the series "NPC Biomarkers" published in *Annals of Nasopharynx Cancer*. The article has undergone external peer review.

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://anpc.amegroups.com/article/view/10.21037/anpc-21-9/coif). The series "NPC Biomarkers" was commissioned by the editorial office without any funding or sponsorship. LL receives Health and Medical Research Fund (HMRF) of Hong Kong (#20190152) and China National Natural Science Foundation (#81772869). QT receives Hong Kong RGC (GRF #14115920, #14118118). The authors have no other conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriate.

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doi: 10.21037/anpc-21-9
Cite this article as: Li L, Xie J, Ma B, Chan ATC, Tao Q. CpG methylation as epigenetic biomarkers for nasopharyngeal carcinoma diagnostics. Ann Nasopharynx Cancer 2022;6:5.

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