MINI-REVIEW

DNA Methylation Biomarkers for Nasopharyngeal Carcinoma: Diagnostic and Prognostic Tools

Wei Jiang*, Rui Cai, Qiu-Qiu Chen

Abstract

Nasopharyngeal carcinoma (NPC) is a common tumor in southern China and south-eastern Asia. Effective strategies for the prevention or screening of NPC are limited. Exploring effective biomarkers for the early diagnosis and prognosis of NPC continues to be a rigorous challenge. Evidence is accumulating that DNA methylation alterations are involved in the initiation and progression of NPC. Over the past few decades, aberrant DNA methylation in single or multiple tumor suppressor genes (TSGs) in various biologic samples have been described in NPC, which potentially represents useful biomarkers. Recently, large-scale DNA methylation analysis by genome-wide methylation platform provides a new way to identify candidate DNA methylated markers of NPC. This review summarizes the published research on the diagnostic and prognostic potential biomarkers of DNA methylation for NPC and discusses the current knowledge on DNA methylation as a biomarker for the early detection and monitoring of progression of NPC.

Keywords: DNA methylation - nasopharyngeal carcinoma - biomarker - diagnosis - prognosis

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Introduction

Nasopharyngeal carcinoma (NPC) is one of the most prevalent malignancies in southern China and south-eastern Asia. In southern China, the incidence rate is about 25-50 per 100,000 person years (Jeannel et al., 1999; McDermott et al., 2001; Wei and Sham, 2005). Despite considerable advances in NPC treatment, local recurrence or distant metastasis is observed frequently. The main challenge of NPC management remains a lack of effective biomarkers for developing more precise diagnostic, prognostic, treatment and prevention approaches (Heng et al., 1999; Hong et al., 2000; Ayan et al., 2003).

To date, abundant evidence convincingly demonstrated that aberrant epigenetic silencing of many tumor suppressor genes (TSGs), cellular functional genes and micro-RNAs (miRNAs) affect the normal cell growth and development (Esteller, 2007; Lujambio et al., 2008), which leads to various human malignancies (Belinsky et al., 1998; Mittag et al., 2006), that has been recognized as a common and early event in human cancers(Jones, 1996; Baylin and Herman, 2000). Of particular interest, the patterns of DNA methylation of normal tissues are distinct from those of tumor tissues, DNA methylation as a potential biomarker for diagnosis, prognosis, personalized therapy and disease management is just beginning to emerge. Recently, investigators have employed specific sets of methylated genes served as biomarkers for clinical practice in several types of cancer, such as lung (Koga et al., 2011), melanoma (You et al., 2010), and breast (Ramos et al., 2010).

Like other types of cancers, NPC is associated with multiple genetic mutations and epigenetic aberrations (Lo and Huang, 2002; Lo et al., 2004). Studies have suggested that aberrant DNA methylation at the promoter CpG islands underlie the development and progression of NPC (Tao and Chan, 2007; Razak et al., 2010). In addition, growing evidence demonstrates that many genes are predominantly silenced by DNA methylation in NPC epithelial cells (Li et al., 2011a; Bruce et al., 2015). Identification of differential DNA methylation genes could contribute to the understanding of pathogenetic mechanisms and develop the available biomarkers to diagnose NPC early and optimize and personalize treatment for NPC.

Here, we detail the the current knowledge of DNA methylation biomarkers in terms of the diagnosis and prognosis of NPC.

Overview of research on DNA methylation and NPC

Over the past decade, many studies have specifically explored DNA methylation in NPC, and a large variety of genes with aberrant methylation (including the different pathways involved in carcinogenesis) have been reported

Earlier studies specifically assessed DNA methylation as either "present" or "absent" in a single gene or multiple genes. For instance, Lo and colleagues (Lo et al., 2002) reported that hypermethylation of a single gene *EDNRB* was detected in 19/21 (90.5%) primary tumors, whereas no methylation was found in normal nasopharyngeal

*Department of Radiation Oncology, the Affiliated Hospital of Guilin Medical University, Guilin, China *For correspondence: weijiang@glmc.edu.cn*

epithelia. Liu and colleagues (Liu et al., 2003) revealed that the BLU promoter region occured hypermethyaltion in 74% of primary NPC tumors, whereas non-neoplastic nasopharyngeal tissue exhibited low rmethylation. From multiple-gene studies, Kwong and colleagues (Kwong et al., 2002) detected the prevalence of several genes methylation in NPC tumors including RARbeta2 (80%), DAP-kinase (76%), p16 (46%), p15 (17%), p14 (20%), and MGMT (20%), respectively. More recently, Yanatatsaneejit *P* and colleagues (Yanatatsaneejit et al., 2008) examined the methylation status of eight genes and higher frequencies of CCNA1 (48%), RARRES1 (51%), and HRASLS (17%), respectively, were found in NPC tumors. Significant differences among numerous the studied DNA methyaltion were scored both in the NPC and control tissues.

Detailed mechanistic studies that further elucidated biologic roles in NPC suggested that transcriptional inactivation of different TSGs by promoter hypermethylation is associated with many important cellular processes involved in tumorigenesis. Several reports have provided convincing evidence that UCHL1, WIF1, RASSF1A, FEZF2, LOX, Kank1 and RRAD are frequently inactivated by promoter methylation in NPC (Chow et al., 2004; Lin et al., 2006; Li et al., 2010; Mo et al., 2012; Shu et al., 2013; Sung et al., 2014; Luo et al., 2015). Restoration of the expression of these genes after demethylation always suppressed NPC cell growth, colony formation and apoptosis of NPC cells, as well as inhibiting their migration and invasion. Similarly to TSGs, silencing of miRNA by hypermethyaltion in NPC has shown its important involvement in various factors during carcinogenesis. For instance, restorations of miR-148a, miR31, miR34c and miR24 expressions inhibit cell growth and migration in NPC cells by targeting different downstream genes (Cheung et al., 2014; Li et al., 2014a; Wang et al., 2014; Li et al., 2015b).

In addition, DNA methyaltion of TSGs were found to involved in multiple biological pathways during carcinogenesis and progression. It was recently reported that hypermethylated gene ADAMTS8 plays a promoting role in NPC progression by triggering EGFR-MEK-ERK signaling (Choi et al., 2014) Similarly, highly methylated gene ROR2 participated in the negative regulation of cell functions through suppressing β-catenin and AKT pathway (Li et al., 2014c). Tao and colleagues also revealed that gene methylation disrupts Wnt signaling, MAPK signaling, regulation of the actin cytoskeleton, Hedgehog signaling and TGF-β signaling pathways in NPC using microarray screening methods (Li et al., 2015a).

Analyses of DNA methylation not only provides the opportunity for understanding the molecular pathogenesis of the disease but can also be used to develop new potential new markers for diagnosis, prognosis and prediction of NPC.

DNA methylation as a potential diagnostic and prognostic markers of NPC

Due to the early occurrence and stability of DNA methylation, analysis of DNA methylation status has been suggested as a useful markers for the early detection and for prediction of outcome of multiple types of cancer (Delpu et al., 2013). DNA methylation is strongly associated with NPC, and hypermethylated DNA has great potential to become a biomarker for the early detection and prognosis of NPC. (Figure 1)

Marks for NPC Diagnosis

In 1996, Lo et al.(Lo et al., 1996) firstly reported high methylation level of p16 in NPC xenograft, cell line

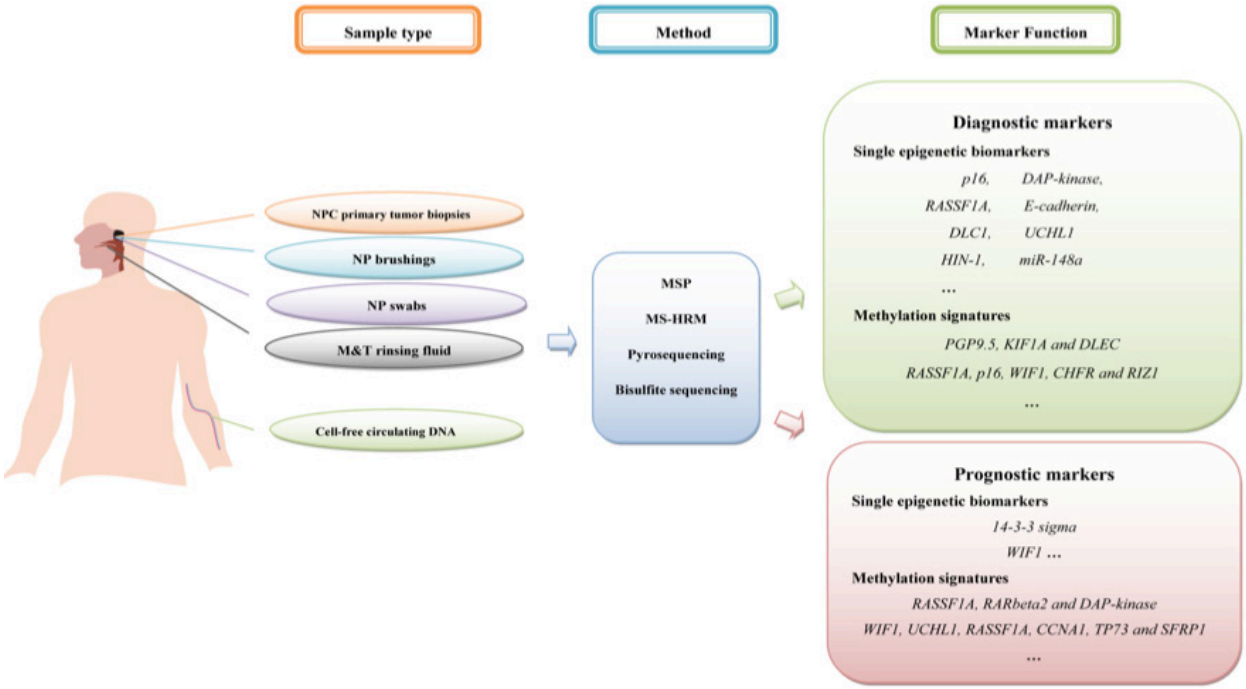

Figure 1. DNA Methylation Can Potentially Serve as a Bomarker for the Early Detection and Prognosis of Nasopharyngeal Carcinoma (NPC). NP: nasopharyngeal; M&T: mouth and throat; MSP: methylation-specific polymerase chain reaction; MS-HRM: methylation-sensitive high-resolution melting.

Table 1. Summary of genes shown to be hypermethylated in nasopharyngeal carcinoma

Biomarker	Type of Sample	Method	Range of Methylation frequency	Marker Function	Reference
p16	NPC primary tumor biopsies	MSP	23-66%	diagnosis	(Ayadi et al., 2008; Challouf et al., 2012; Tian et al., 2013)
	NP brushing		46.40%		(Tong et al., 2002)
	NP swabs		17%		(Chang et al., 2003b)
	M&T rinsing fluid		17%		(Chang et al., 2003b)
	Plasma		42%		(Wong et al., 2004)
RASSF1A	NPC primary tumor biopsies	MSP	46-67%	diagnosis	(Chang et al., 2003b; Wong et al., 2003b)
	NP brushing		39.30%		(Tong et al., 2002)
	NP swabs		33%		
	M&T rinsing fluid		37%		
DAP-kinase	NPC primary tumor biopsies	MSP	75%-77%	diagnosis	(Wong et al., 2002; Chang et al., 2003b)
	NPC cell lines		80%		(Tong et al., 2002)
	NP brushing		50.00%		
	NP swabs		63%		
	M&T rinsing fluid		50%		
	Plasma		20%		(Wong et al., 2004)
CDH1	NPC primary tumor biopsies	MSP	50%	diagnosis	(Wong et al., 2003b)
	Plasma		46%		(Wong et al., 2004)
E-cadherin	NPC primary tumor biopsies	MSP	52% -65%	diagnosis	(Chang et al., 2003b; Tsao et al., 2003; Ran et al., 2011)
	NPC cell lines		100%		(Ran et al., 2011)
	NP swabs		27%		(Chang et al., 2003b)
	M&T rinsing fluid		43%		(Chang et al., 2003b)
HIN-1	NPC primary tumor biopsies	MSP	77%	diagnosis	(Wong et al., 2003a)
	NPC cell lines		100%		
	nasopharyngeal swabs		46%		
	throat-rinsing fluids		19%		
	Plasma		18%		
	buffy coats of peripheral blood		46%		
MGMT	NPC primary tumor biopsies	MSP	28%	diagnosis	(Wong et al., 2003b)
MLH1	NPC primary tumor biopsies	MSP	40%	diagnosis	(Wong et al., 2003b)
p15	NPC primary tumor biopsies	MSP	50-80%	diagnosis	(Chang et al., 2003b; Wong et al., 2003b)
	NP swabs		53%		
	M&T rinsing fluid		40%		
	Plasma		20%		
RIZ1	NPC paraffin and/or brushing	MSP	56.60%	diagnosis	(Chang et al., 2003a; Hutajulu et al., 2011)
THBS1	NPC primary tumor biopsies	MSP	50%	diagnosis	(Wong et al., 2003b)
TP73	NPC primary tumor biopsies	MSP	20%	diagnosis	(Wong et al., 2003b)
CHFR	NPC primary tumor biopsies	MSP	61.1% (22/36)	diagnosis	(Cheung et al., 2005)
	NPC cell lines		100%		
GADD45G	NPC primary tumor biopsies	MSP	73% (8/11)	diagnosis	(Ying et al., 2005)
TIG1	NPC primary tumor biopsies	MSP	90.7% (39/43)	diagnosis	(Kwong et al., 2005a)
CRBPs	NPC primary tumor biopsies	MSP	54.2%- 87.8%	diagnosis	(Kwong et al., 2005b)
DLC1	NPC primary tumor biopsies	MSP	79% (31/39)	diagnosis	(Peng et al., 2006)
	NPC paraffin and/or brushing	MSP	77%		(Hutajulu et al., 2011)
	NPC (endemic and sporadic types)		89% (64/72)		(Seng et al., 2007)
LTF	NPC primary tumor biopsies		63.6% (21/33)	diagnosis	(Yi et al., 2006)
PCDH10	NPC primary tumor biopsies	MSP	82%	diagnosis	(Ying et al., 2006)
CDH13	NPC primary tumor biopsies	MSP	89.7% (52/58)	diagnosis	(Sun et al., 2007)
	NPC cell lines		20% (1/5)		
	NPC xenografts		100% (2/2)		
BRD7	NPC primary tumor biopsies	MSP	100%	diagnosis	(Liu et al., 2008)
	Matched blood samples		100%		
IRF8	NPC primary tumor biopsies	MSP	78%	diagnosis	(Lee et al., 2008)
	NPC cell lines		100%		
14-3-3 sigma	NPC primary tumor biopsies	MSP	84%	prognosis	(Yi et al., 2009)
	NPC cell lines		100%		
LARS2	NPC primary tumor biopsies	MSP	64% (23/36)	diagnosis	(Zhou et al., 2009)
DAB2	NPC primary tumor biopsies	MSP	72% (33/46)	diagnosis	(Tong et al., 2010)
TFPI-2	NPC primary tumor biopsies	MSP	88.6% (62/70)	diagnosis	(Wang et al., 2010)
	NPC cell lines		66.7% (4/6)		
CADM1	NPC paraffin and/or brushing	MSP	69.80%	diagnosis	(Hutajulu et al., 2011)
CDH4	NPC primary tumor biopsies	MSP	94.30%	diagnosis	(Du et al., 2011)
	NPC cell lines		100%		
CHFR	NPC paraffin and/or brushing	MSP	58.50%	diagnosis	(Hutajulu et al., 2011)
LTF	NPC cell lines		100% (7/7)	diagnosis	(Zhang et al., 2011)
Myocardin	NPC primary tumor biopsies	MSP	73.8% (48/65)	diagnosis	(Chen et al., 2011)
	NPC cell lines		4 of 5 (80%)		
NOR1	NPC primary tumor biopsies	MSP	61.9% (13/21)	diagnosis	(Li et al., 2011b)
	NPC cell lines		100% (4/4)		
WIF1	NPC paraffin and/or brushing	MSP	61.20%	diagnosis	(Hutajulu et al., 2011)
PCDH8	NPC primary tumor biopsies	MSP	85.3% (35/41)	diagnosis	(He et al., 2012)
	NPC cell lines		100% (5/5)		
RRAD	NPC primary tumor biopsies	MSP	74.3% (26/35)	diagnosis	(Mo et al., 2012)
CACNA2D3	NPC primary tumor biopsies	MSP	100% (5/5)	diagnosis	(Wong et al., 2013)
	NPC cell lines		100% (3/3)		
CDK10	NPC primary tumor biopsies	MSP	52.50%	diagnosis	(You et al., 2013)
DLEC1	Cell-free circulating DNA	MSP	25.00%	diagnosis	(Tian et al., 2013)
FEZF2	NPC primary tumor biopsies	MSP	75.5%(37/49)	diagnosis	(Shu et al., 2013)
	nasal swab		75% (12/16)		
SOX11	NPC primary tumor biopsies	MSP	67.4% (29/43)	diagnosis,prognosis	(Zhang et al., 2013)
UCHL1	Cell-free circulating DNA	MSP	64.90%	diagnosis	(Tian et al., 2013)
TTC40	NPC primary tumor biopsies	MSP	71.12%(32/45)	diagnosis	(Ayadi et al., 2014)
LOX	NPC primary tumor biopsies	MSP	85.7% (42/49)	diagnosis	(Sung et al., 2014)
	Nose swab		18.75% (3/16)		
miR-148a	NPC primary tumor biopsies	Bisulfite sequencing	53-97%	diagnosis	(Li et al., 2014a)
PTEN	NPC primary tumor biopsies	MSP	82.2%(37/45)	diagnosis	(Li et al., 2014b)
	NPC cell lines		80% (4/5)		
WWOX	NPC primary tumor biopsies	MSP	56.9%(37/65)	diagnosis	(Yang et al., 2014)
CYB5R2	NPC primary tumor biopsies	MSP	84%(42/50)	diagnosis	(Xiao et al., 2014)
	NPC cell lines		100%(6/6)		
ECRG4	NPC primary tumor biopsies	MSP	72.5% (29/40)	diagnosis	(You et al., 2015)
	Peripheral blood samples from the NPC patients		57.5 % (23/40)		
PCDH20	NPC primary tumor biopsies	MSP	78.4% (40/51)	diagnosis	(Chen et al., 2015)
	NPC cell lines		80% (4/5)		
ITGA9	NPC primary tumor biopsies	MSP	56%	diagnosis	(Nawaz et al., 2015a)

MSP : methylation-specific polymerase chain reaction; M&T: mouth and throat; NP: nasopharyngeal

and primary tumors. Several subsequent studies using methylation-specific polymerase chain reaction (MSP) approaches to investigate the promoter methylation profile of p16 found methylation frequencies to be 23-66% in primary undifferentiated NPC(Ayadi et al., 2008; Challouf et al., 2012; Tian et al., 2013), 46.4% in nasopharyngeal (NP) brushings(Tong et al., 2002), and 42% in plasma DNA(Wong et al., 2004). There was a perfect concordance in methylation among corresponding samples. The results demonstrated that the methylation level in p16 was a potential diagnostic tool for the differential diagnosis between benign NP tissue and malignant NP tumors.

Another example of the potential use of hypermethylated DNA as a biomarker was the involvement of the methylated gene RASSF1A in the early detection of NPC. Lo et al. (Lo et al., 2001) used MSP analyses to investigate hypermethylation of promoter regions of RASSF1A in nasopharyngeal primary tumors, xenografts, and cell lines for the first time. Other reports also presented the methylation frequency of RASSF1A promoters to be as high as 39.3% in nasopharyngeal brushings, 46%-67% in primary undifferentiated NPC, 33% in nasopharyngeal swabs, 37% in mouth and throat (M&T) rinsing fluid, respectively (Chang et al., 2003b; Wong et al., 2003b). From these efforts, it is clear that this molecular event is an early and important marker of NPC.

In the past decade, a multitude of studies demonstrated that other classical TSGs undergo hypermethylation in various biologic samples of NPC, including RRAD, DAP-kinase, CDH13, E-cad, TIG1, CHFR, DAB2, Myocardin, TFPI-2 and CDH4 and so on (Chang et al., 2003b; Tsao et al., 2003; Cheung et al., 2005; Kwong et al., 2005a; Sun et al., 2007; Tong et al., 2010; Wang et al., 2010; Chen et al., 2011; Du et al., 2011; Mo et al., 2012), thereby rendering thier potential as alternative surrogate markers for the early diagnosis. We have summarized some examples of putative biomarkers in Table 1.

However, single epigenetic biomarkers are not sufficiently sensitive to detect early NPC accurately in tissue or body fluids, specific gene-methylation signatures have been suggested to improve sensitivity(Nawaz et al., 2015b). Hutajulu et al. applied quantitative profiling of DNA methylation in 10 TSGs in nasopharyngeal brushings and corresponding NPC paraffin-embedded tissue. The study found that combined analyses of five methylation markers (RASSF1A, p16, WIF1, CHFR and RIZ1) provided good discrimination between NPC and non-NPC with detection rate of 98% (Hutajulu et al., 2011). Myriam et al. used quantitative MSP to investigate promoter hypermethylation of 18 TSGs in NPC cell lines and NPC tumors biopsies. Authors suggested that combinatorial analyses of methylation of three genes (PGP9.5, KIF1A and DLEC) would detected NPC early with 84% sensitivity and 92% specificity (Loyo et al., 2011). In other methylation signature reports, the methyaltion level of four-gene marker (CDKN2A, DLEC1, DAPK1 and UCHL1) could early predict NPC with the highest sensitivity and specificity (Tian et al., 2013). A panel of four methylated genes (RASSF1A, WIF1, DAPK1 and RARβ2) in combination with an EBV DNA marker significantly increased the prevalence of detection at an early stage and local recurrence in NPC (Yang et al., 2015).

With recent developments in methods of high-throughput screening, several studies have evaluated genome-wide methylation profiling in NPC. Tao and colleagues using a whole-genome methylation platform newly identified hypermethylated genes SFRP1, 2 and 5, DACT1, DACT2 and DKK3 in NPC cell lines and primary tumors and suggested their potential value as biomarkers for NPC detection (Li et al., 2015a). Another genome-wide study in NPC demonstrated that the top 500 hypermethylated regions were frequently located at 6p21.3 in NPC. This region contains several important genes which could be used as biomarkers for NPC detection (Dai et al., 2015). Recently, our group also took a global methylation approach (the Illumina HumanMethylation450 BeadChip) to reveal both hyper- and hypomethylation alterations are common events in NPC tumor tissues. As a result, 2173 CpG sites with methylation level change ≥0.2 (1880 hypermethylated, 293 hypomethylated) were identified (P<0.05), as well as use of potential markers for early diagnosis in NPC (Jiang et al., 2015).

The studies mentioned above provide strong evidence that tumor promoter-specific hypermethylation is closely related with the development of NPC, and suggest DNA methylation biomarker that combines high sensitivity and specificity could be used for the early detection of NPC.

Marks for NPC Prognosis

DNA methylation profiles was shown with abilities to better defined the prognoses of numerous cancers(Ramos et al., 2010; You et al., 2010; Koga et al., 2011). Emerging research supports the notion that detection of aberrantly methylated genes in NPC can serve as biomarkers for the prognosis. In comparison, methylation of 14-3-3 sigma correlates with metastasis to lymph node and distant metastasis (Yi et al., 2009). WIF-1 methylation has been found to be associated with the tumor, node, and metastasis (TNM) classification (p= 0.003) and age (p=0.014) (Fendri et al., 2010). In primary NPC tumors, clinical studies have revealed that aberrant promoter methylation of the three genes (RASSF1A, RARβ2 and DAPK) are significantly associated with the lymph-node involvement (p<0.0001). In addition, hypermethylation of RASSF1A was found to be correlated with age at the diagnosis (p= 0.047) and T stage (p= 0.037), whereas the RARβ2 hypermethylation was associated with histological type (p = 0.011) (Fendri et al., 2009).

Latterly, our group examined the methylation level of paraffin-embedded specimens with NPC and provide reasonable assurance that the 6-hypermethylated gene panel (WIF1,UCHL1, RASSF1A, CCNA1, TP73 and SFRP1) was an independent prognostic factor in large sample size. The study revealed that NPC with high methylation level is associated with poorer survival and may increase the therapeutic options for patients diagnosed with NPC (Jiang et al., 2015)..

Clearly, the identification of new effective prognosis biomarkers for NPC will likely contribute to predict clinical outcomes and improved patient-tailored treatment. However, panels of candidate methylated-genes remain

to be validated in the prospective study.

Targeting DNA methylation for epigenetic therapy in NPC

Epigenetic changes are reversible, making DNA methylation a potential target for anticancer therapies. During the past decade, a growing number of drugs targeting DNA methylation have been developed, for example, azacytidine (5-azacytidine, 5-Aza-CR), and decitabine (5-aza-2'-deoxycytidine, 5-Aza-CdR). These agents have been used as single agents or combined with other anticancer therapies and validated in multiple clinical trials to reduce global DNA methylation in vivo. In particular, trials on hematologic malignancies have shown higher response rates. Among preclinical studies for NPC, decitabine decreased survival of NPC cell lines (Li et al., 2011b; Zhang et al., 2013; Luo et al., 2015) and azacytidine enhanced the radiosensitivity of NPC cells by promoting cell apoptosis (Jiang et al., 2014). Moreover, decitabine treatment reactivated the methylated gene ECRG4 and enhanced chemosensitivity to cisplatin in NPC cells (You et al., 2015). However, until recently, no clinical trials have been the process to shown an association between the level of induced demethylation and clinical response in patients with NPC.

Conclusion

NPC continues to be a major public-health problem in the China and and south-eastern Asia. However, a lack of effective biomarkers for early detection and monitoring of NPC progression contributes to its adverse outcomes. After more than a decade of studies, DNA methyaltion, with characteristics of high stability and easy evaluation, has been shown to be a high-potential tool with great sensitivity and specificity in the diagnosis and prognosis of NPC, which will extend our ability to improve NPC management. Further high-powered studies with perspective clinical data are required to establish the role of DNA methylation for the diagnosis and prognosis of NPC.

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