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Research Paper

Diagnostic Capacity of *RASSF1A* Promoter Methylation as a Biomarker in Tissue, Brushing, and Blood Samples of Nasopharyngeal Carcinoma

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ABSTRACT

Methylation of the RAS association domain family protein 1A (*RASSF1A*) promoter has been observed in nasopharyngeal carcinoma (NPC). This study investigated the correlation of *RASSF1A* promoter methylation with clinicopathological features and its utility as a diagnostic biomarker in NPC. A total of 926 patients with NPC and 495 non-tumor controls were analyzed in this study. *RASSF1A* promoter methylation was notably higher in NPC compared with non-tumor tissue, brushing and blood samples. *RASSF1A* promoter methylation was associated with clinical stage, lymph node status, distant metastasis, and T classification of patients with NPC, although it was not linked to age and sex. The pooled sensitivity, specificity, and AUC (area under the curve) of *RASSF1A* promoter methylation were determined in NPC samples vs. non-tumor samples (tissue: sensitivity = 0.72, specificity = 0.99, AUC = 0.98; brushing: sensitivity = 0.56, specificity = 1.00, AUC = 0.94; blood: sensitivity = 0.11, specificity = 0.98, AUC = 0.97). Our findings show that *RASSF1A* promoter methylation may be correlated with the development, progression and metastasis of NPC. *RASSF1A* promoter methylation is a promising noninvasive biomarker for the diagnosis of NPC from tissue and brushing samples.

1. Introduction

Nasopharyngeal carcinoma (NPC) is an uncommon malignancy with distinct geographic and ethnic characteristics. GLOBOCAN estimates that approximately 86,700 new cases of NPC have been reported, leading to an estimated 50,800 deaths in 2012 (Torre et al., 2015). NPC occurs frequently, with an incidence rate of 15 to 50 per 100,000 people annually in Southeast Asia. However, the incidence rate is not higher than 1 per 100,000 people in Western countries (Zhou et al., 2007; Yu and Yuan, 2002). Unfortunately, distant metastasis is a main cause of death for NPC patients, which often has an unfavorable prognosis (Chen et al., 2012; Chua et al., 2012; Liu et al., 2003). Although computed tomography (CT) and magnetic resonance imaging (MRI) are effective, they cannot accurately provide a prognosis for NPC or predict the effectiveness of biological therapeutic targets (Lin et al., 2013; Gong et al., 1991). Thus, a novel, noninvasive low-cost biomarker for early detection of NPC is of great importance to clinical practice.

DNA methylation, which is a common mechanism in epigenetic alterations, may be correlated with NPC (Jiang et al., 2015; Nawaz et al., 2015a). Promoter methylation of tumor suppressor genes (TSGs), such as calcium channel voltage-dependent alpha 2/delta subunit 3 (*CACNA2D3*) and cadherin 4 (*CDH4*), may play a crucial role in NPC development and progression (Wong et al., 2013; Du et al., 2011). Localized in human chromosomal region 3p21.3, the RAS association domain family protein 1A (*RASSF1A*) is an important TSG involved in multiple biological functions, including cell cycle regulation, microtubule stabilization, and apoptosis (Allen et al., 2007; Agathanggelou et al., 2005; Burbee et al., 2001). In NPC, *RASSF1A* gene expression is often blocked due to promoter methylation (Wang et al., 2009; Fendri et al., 2009; Lo et al., 2001). *RASSF1A* promoter methylation can be detected in tissue, brushing and blood samples of patients with NPC (Nawaz et al., 2015b; Yang et al., 2015; Hutajulu et al., 2011).

However, there are some inconsistencies in reports on the level of the *RASSF1A* promoter methylation in NPC. For example, Chang et al. reported that the rate of *RASSF1A* promoter methylation in NPC patients was different in tissue (66.7%), blood (3.3%), and brushing samples (33.3%) (Chang et al., 2003). Yang et al. reported that the *RASSF1A* promoter region was frequently methylated in 68.8% of brushing samples from NPC patients (Yang et al., 2015). Therefore, the aim of this study was to assess the relationship between *RASSF1A* promoter methylation and NPC risk in tissue, brushing, and blood samples. Moreover, we

Abbreviations: *RASSF1A*, RAS association domain family protein 1A; NPC, nasopharyngeal carcinoma; OR, odds ratio; 95% CI, 95% confidence interval; AUC, the summary receiver operator characteristic (SROC) curve; TSG, tumor suppressor gene.

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analyzed the correlation of *RASSF1A* promoter methylation with clinicopathological features of patients with NPC. Finally, we determined the diagnostic utility of *RASSF1A* promoter methylation as a noninvasive biomarker in samples of tissue, brushings, and blood.

2. Materials and Methods

2.1. Search Strategy

We conducted a systematic search of online electronic databases (PubMed, Embase, EBSCO, Web of Science, Scopus and the Cochrane Library) to identify eligible literature published prior to January 11, 2017. The following combination of key words and search terms were used to identify studies: 'nasopharyngeal cancer or nasopharyngeal neoplasm or nasopharyngeal carcinoma or nasopharyngeal tumor or NPC', 'RASSF1A or RAS association domain family protein 1A', 'methylation or methylated or epigene*'. We also carefully checked the references of eligible articles to identify other potential studies. This study was conducted based on the preferred reporting items for systematic reviews and meta-analyses (PRISMA) statement criteria (Moher et al., 2009) (Table S1).

2.2. Inclusion Criteria

Studies were included in this meta-analysis if they fulfilled the following selection criteria: 1) patients were diagnosed with primary NPC based on histopathological examination of samples, including tissue, brushing, and blood; 2) articles were published in English; 3) there was sufficient information on the level of *RASSF1A* promoter methylation in NPC and non-tumor samples; 4) there was sufficient data for estimating the relationship between *RASSF1A* promoter methylation and the clinicopathological characteristics of patients with NPC. If multiple papers were published using overlapping sample data, we only included the most appropriate article with the most detailed information.

2.3. Data Extraction

Two authors independently scanned and abstracted the following information from available studies: surname of first author, year of publication, country, population by race, sample types, number of cases and non-tumor controls, methodology for the detection of methylation, rate of *RASSF1A* promoter methylation, expression status of the *RASSF1A* gene, and clinicopathological parameters, such as age (>50 years vs. ≤ 50 years), sex (male vs. female), clinical stage (stage 3–4 vs. stage 1–2), lymph node status (positive vs. negative status), distant metastasis (yes vs. no), and T classification (T3–4 vs. T1–2). Any inconsistent data or information was resolved by a discussion including all authors.

2.4. Statistical Analysis

Pooled data in this meta-analysis were analyzed using Stata software, version 12.0 (STATA Corp., College Station, TX, USA). The strength of the correlation between *RASSF1A* promoter methylation and NPC was estimated by the combined odds ratios (ORs) with 95% confidence intervals (95% CIs). The pooled ORs and corresponding 95% CIs were also used to analyze the relationship between *RASSF1A* promoter methylation and the clinicopathological features of NPC patients, including age, sex, clinical stage, lymph node status, distant metastasis, and T classification. Potential heterogeneity among studies was detected using Cochran's Q test (Coory, 2010). The random-effects model was applied when Q-test P values were <0.1, indicating obvious heterogeneity. A fixed-effect model was applied to the data when the P values were >0.1, indicating no evidence of heterogeneity (Higgins et al., 2003; DerSimonian, 1996). Meta-regression analyses were performed to assess the sources of heterogeneity. Sensitivity analyses were conducted to determine whether removing individual studies with substantial heterogeneity changed the overall OR (Lau et al., 1997). Egger's test was used to evaluate potential publication bias for results with more than nine studies (Egger et al., 1997). Based on the bivariate analysis, we generated the combined sensitivity, specificity, and the summary receiver operator characteristic (SROC) curve (AUC) to evaluate the diagnostic capacity of *RASSF1A* promoter methylation in tissue, blood, and brushing samples from NPC patients in the meta-analysis (Reitsma et al., 2005; Jones and Athanasiou, 2005).

3. Results

3.1. Study Characteristics

Fig. 1 lists a detailed procedure for our literature search in a range of online electronic databases. After a careful screen based on the inclusion criteria described above, we identified 16 studies, including 926 patients with NPC and 495 non-tumor controls, with sufficient data in the final meta-analysis (Nawaz et al., 2015b; Yang et al., 2015; Tian et al., 2013; Challouf et al., 2012; Hutajulu et al., 2011; Wang et al., 2009; Fendri et al., 2009; Zhou et al., 2005; Pan et al., 2005; Qiu et al., 2004; Wong et al., 2004; Chang et al., 2003; Wong et al., 2003; Tong et al., 2002; Kwong et al., 2002; Lo et al., 2001). Of the 16 eligible studies, 11 investigated the correlation between *RASSF1A* promoter methylation and NPC in tumor versus non-tumor tissues (Nawaz et al., 2015b; Challouf et al., 2012; Wang et al., 2009; Fendri et al., 2009; Zhou et al., 2005; Qiu et al., 2004; Chang et al., 2003; Wong et al., 2003; Tong et al., 2002; Kwong et al., 2002; Lo et al., 2001). Four studies determined the relationship between *RASSF1A* promoter methylation and NPC in tumor versus non-tumor blood samples (Yang et al., 2015; Tian et al., 2013; Wong et al., 2004; Chang et al., 2003). Four studies analyzed the association between *RASSF1A* promoter methylation and NPC in tumor versus non-tumor brushing samples (Yang et al., 2015; Hutajulu et al., 2011; Chang et al., 2003; Tong et al., 2002). Eight studies involving 502 NPC patients assessed the relationship between *RASSF1A* promoter methylation and the clinicopathological characteristics of patients with NPC (Yang et al., 2015; Tian et al., 2013; Wang et al., 2009; Fendri et al., 2009; Zhou et al., 2005; Pan et al., 2005; Wong et al., 2004; Tong et al., 2002). Table 1 and Table S2 present the general characteristics of the studies included in the meta-analysis.

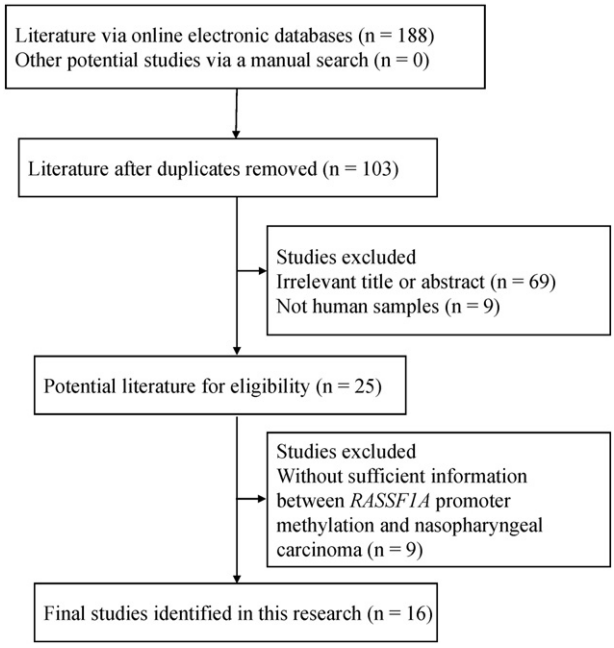

Fig. 1. PRISMA flow chart of the procedure for selecting literature.

Table 1
Baseline characteristics of the eligible studies considered in this report.

First author	Country	Ethnicity	Age	Method	Stage	Sample	Control sample	Cancer		Control		Clinical features	Expression
								M+	N (M+ %)	M+	N (M+ %)		
(Lo et al., 2001)	China	Asians	NA	MSP	NA	Tissue	Normal	14	21 (66.7)	0	6 (0.0)	NA	Loss
(Kwong et al., 2002)	China	Asians	NA	MSP	NA	Tissue	Normal	24	29 (82.8)	0	6 (0.0)	NA	NA
(Tong et al., 2002)	China	Asians	52.3	MSP	1–4	Brushing	Non-tumor	11	28 (39.3)	0	12 (0.0)	Yes	NA
(Tong et al., 2002)	China	Asians	NA	MSP	1–4	Tissue	Non-tumor	8	16 (50.0)	0	12 (0.0)	Yes	NA
(Wong et al., 2003)	China	Asians	NA	MSP	NA	Tissue	Normal	13	28 (46.4)	0	5 (0.0)	NA	NA
(Chang et al., 2003)	China	Asians	49	MSP	1–4	Tissue	Normal	20	30 (66.7)	0	6 (0.0)	NA	NA
(Chang et al., 2003)	China	Asians	49	MSP	1–4	Brushing	Normal	10	30 (33.3)	0	37 (0.0)	NA	NA
(Chang et al., 2003)	China	Asians	49	MSP	1–4	Blood	Normal	1	30 (3.3)	1	43 (2.3)	NA	NA
(Wong et al., 2004)	China	Asians	46	MethyLight	1–4	Blood	Normal	2	41 (4.9)	0	43 (0.0)	Yes	NA
(Qiu et al., 2004)	Singapore	Asians	NA	MSP	NA	Tissue	Normal	20	27 (74.1)	0	20 (0.0)	NA	NA
(Pan et al., 2005)	China	Asians	NA	MSP	1–4	Tissue	NA	17	23 (73.9)	NA	NA	Yes	NA
(Zhou et al., 2005)	China	Asians	NA	MSP	1–4	Tissue	Adjacent	23	28 (82.1)	34	56 (60.7)	Yes	NA
(Zhou et al., 2005)	China	Asians	NA	MSP	1–4	Tissue	Non-tumor	23	28 (82.1)	0	8 (0.0)	NA	NA
(Fendri et al., 2009)	Tunisia	Caucasians	42	MSP	1–4	Tissue	Normal	62	68 (91.2)	0	9 (0.0)	Yes	Loss
(Wang et al., 2009)	China	Asians	NA	MSP	1–4	Tissue	Normal	27	38 (71.1)	0	14 (0.0)	Yes	Loss
(Hutajulu et al., 2011)	The Netherlands	Caucasians	NA	MSP	1–4	Brushing	Non-tumor	40	53 (75.5)	1	47 (2.1)	NA	NA
(Challouf et al., 2012)	Tunisia	Caucasians	45	MSP	1–4	Tissue	Non-tumor	27	36 (75.0)	0	19 (0.0)	NA	NA
(Tian et al., 2013)	China	Asians	50.2	MSP	2–4	Blood	Normal	7	40 (17.5)	2	41 (4.9)	Yes	NA
(Yang et al., 2015)	China	Asians	NA	MS-HRM	NA	Brushing	Non-tumor	66	96 (68.8)	0	43 (0.0)	NA	NA
(Yang et al., 2015)	China	Asians	NA	MS-HRM	1–4	Blood	Non-tumor	53	220 (24.1)	0	50 (0.0)	Yes	NA
(Nawaz et al., 2015b)	Sweden	Caucasians	NA	MSP	NA	Tissue	Non-tumor	29	44 (65.9)	1	18 (5.6)	NA	NA

MSP: methylation-specific polymerase chain reaction; MS-HRM: methylation-sensitive high resolution melting; M: methylation; N: number of participants; NA: not applicable.

3.2. Association Between RASSF1A Promoter Methylation and NPC in Cancer vs. Control Samples

Fig. 2 shows the significant relationship between *RASSF1A* promoter methylation and NPC risk in cancerous samples compared with control samples (tissue: OR = 29.81, 95% CI = 11.27–78.86, P < 0.001; brushing: OR = 75.74, 95% CI = 20.70–277.10, P < 0.001; blood: OR = 5.21, 95% CI = 1.50–18.04, P = 0.009). This comparison included 365 NPC and 179 non-tumor tissue samples, 207 NPC and 139 non-tumor brushing samples, and 331 NPC and 177 non-tumor blood samples.

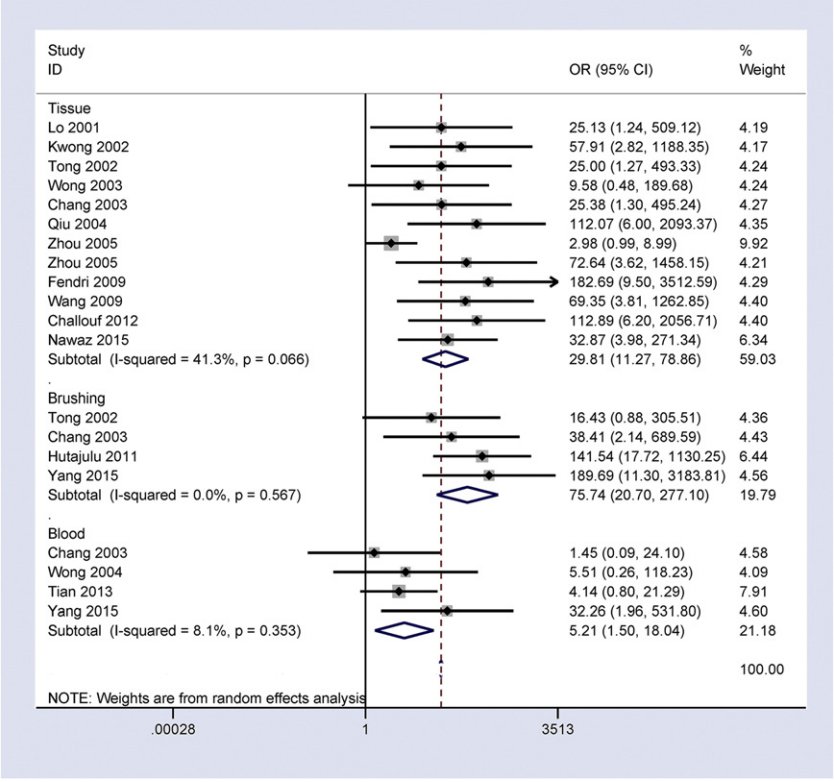

Fig. 2. Forest plot of the association between *RASSF1A* promoter methylation and NPC risk in cancer vs. non-tumor tissue, brushing and blood samples.

3.3. Subgroup, Sensitivity and Meta-Regression Analyses in Tumor Versus Non-tumor Tissues

Subgroup analysis was conducted by ethnicity (Asian and Caucasian populations), and the results showed that *RASSF1A* promoter methylation was closely correlated with NPC risk in both Asian and Caucasian populations (OR = 14.73, 95% CI = 7.33–29.60, P < 0.001 and OR = 67.89, 95% CI = 15.41–299.10, P < 0.001, respectively) (Fig. 3).

There was a slight heterogeneity in measurements comparing NPC and non-tumor tissues (P = 0.066 < 0.1). A sensitivity analysis was carried out to estimate the influence of deleting an individual study on the overall result. When we removed the study by Zhou et al. (2005) (control: adjacent tissue samples) and recalculated, the combined OR was 47.35 (95% CI = 20.08–111.66, P < 0.001) and there was no significant heterogeneity (P = 0.971).

To explore sources of heterogeneity, we performed meta-regression analyses using race (Asian and Caucasian populations) and multiple control types (normal, non-tumor, and adjacent tissue samples) (Table 2). The results demonstrated that ethnicity was not the source of the heterogeneity we observed (P > 0.1). However, control type analysis revealed that the heterogeneity was from adjacent tissue samples (P = 0.012), which is consistent with the sensitivity analysis.

Table 2
Meta-regression analysis in tumor versus non-tumor tissues.

Subgroup	Coefficient (95% CI)	t	P value
Race	1.308 (−1.113, 3.728)	1.2	0.257
Control types			0.012
Normal	0.009 (−1.998, 2.015)	0.01	0.993
Adjacent	−2.761 (−4.756, −0.767)	−3.13	0.012

95% CI: 95% confidence interval.

3.4. Association Between RASSF1A Promoter Methylation and Age or Sex of NPC

The results showed that the status of *RASSF1A* promoter methylation was not associated with age (133 NPC patients) and sex (471 NPC patients) in NPC (OR = 0.77, 95% CI = 0.36–1.64, P = 0.496 and OR = 1.42, 95% CI = 0.86–2.34, P = 0.168, respectively) (Fig. 4).

3.5. Association Between RASSF1A Promoter Methylation and Clinical Stage or Lymph Node Status of NPC

The analysis included data on the clinical stage of 403 patients with NPC and the lymph node status of 214 patients with NPC. The results showed that *RASSF1A* promoter methylation was associated with clinical stage and lymph node status (OR = 2.16, 95% CI = 1.26–3.70, P = 0.005 and OR = 3.96, 95% CI = 1.17–13.48, P = 0.027, respectively) (Fig. 5).

3.6. Association Between RASSF1A Promoter Methylation and Distant Metastasis or T Classification of NPC

The analysis included data on of distant metastasis in 359 NPC patients and T classification in 252 NPC patients. The results showed that methylation of the *RASSF1A* promoter was associated with distant metastasis and T classification in NPC (OR = 6.16, 95% CI = 2.85–13.31, P < 0.001 and OR = 2.04, 95% CI = 1.16–3.59, P = 0.014, respectively) (Fig. 6).

3.7. Publication Bias

The analysis of publication bias was measured in tumor versus non-tumor tissue samples (Fig. S1) and revealed obvious evidence of publication bias (P < 0.001). After one study was removed (Zhou et al., 2005), (control: adjacent tissue samples), the recalculated publication bias was significantly decreased (P = 0.674).

3.8. Diagnostic Utility of RASSF1A Promoter Methylation in Cancer vs. Controls

To evaluate the diagnostic capacity of *RASSF1A* promoter methylation, we compared sample types (tissue, brushing, and blood) from NPC and control. Based on their identification as a source of

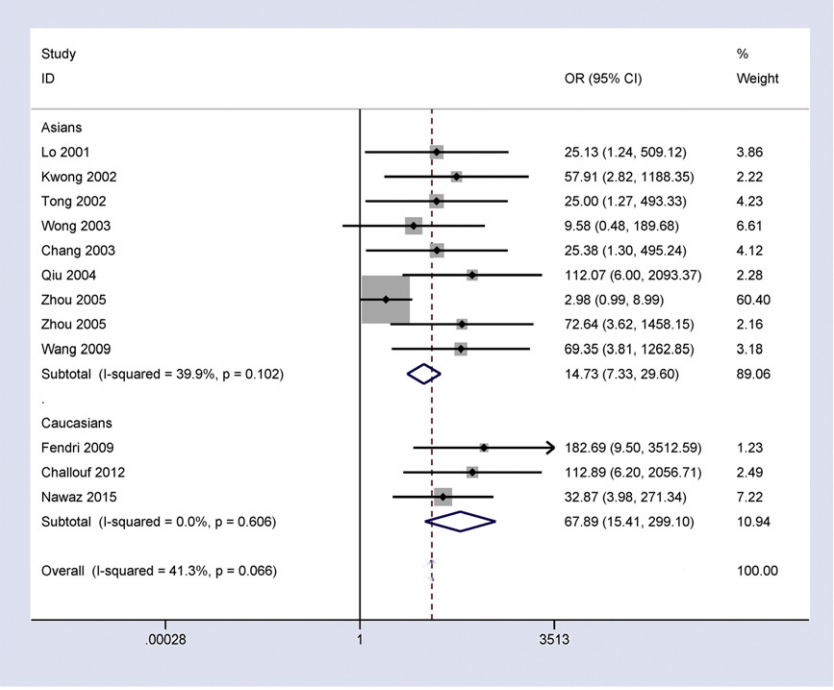

Fig. 3. Forest plot of subgroup analyses by ethnicity in NPC vs. non-tumor tissue samples.

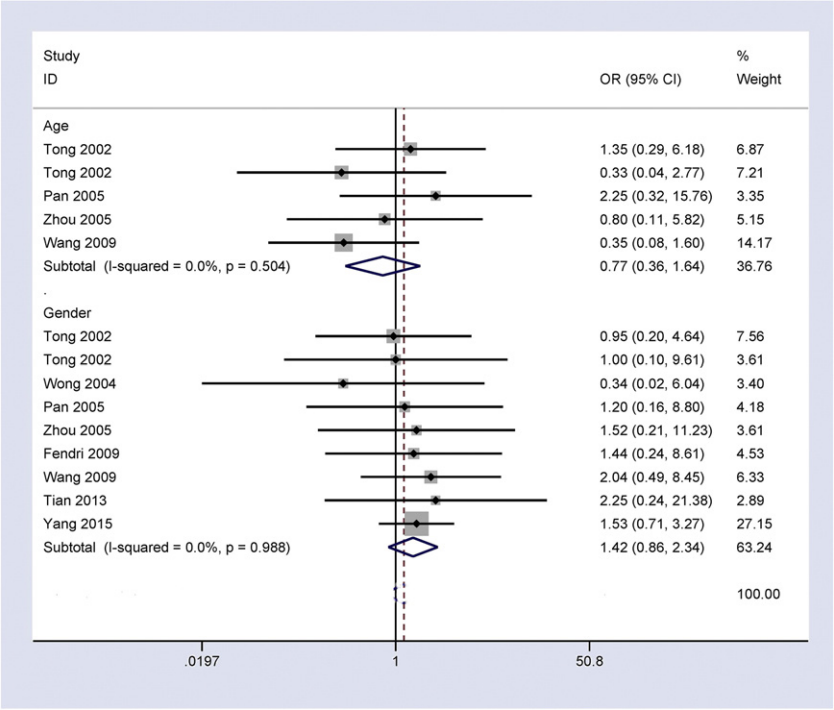

Fig. 4. Forest plot of the association between *RASSF1A* promoter methylation, age, and sex of patients with NPC.

heterogeneity, adjacent tissue samples were excluded from the analysis. The pooled sensitivity, specificity and AUC of *RASSF1A* promoter methylation in tissue samples were 0.72 (95% CI = 0.64–0.80), 0.99 (95% CI = 0.92–1.00), and 0.98 (95% CI = 0.96–0.99), respectively (Fig. 7). The overall sensitivity, specificity and AUC of the brushing samples were 0.56 (95% CI = 0.37–0.73), 1.00 (95% CI = 0.63–1.00), and 0.94 (95% CI = 0.91–0.95), respectively (Fig. 8). The combined sensitivity, specificity and AUC of the blood samples were 0.11 (95% CI = 0.05–0.25), 0.98 (95% CI = 0.93–1.00), and 0.97 (95% CI = 0.95–0.98), respectively (Fig. 9). The sensitivity of the tissue and brushing groups (tissue: 0.72 and brushing: 0.56) was higher compared with the blood group (a weak sensitivity = 0.11). These results suggest that testing for *RASSF1A* promoter methylation may provide a non-invasive method for diagnosing NPC in tissue and brushing samples.

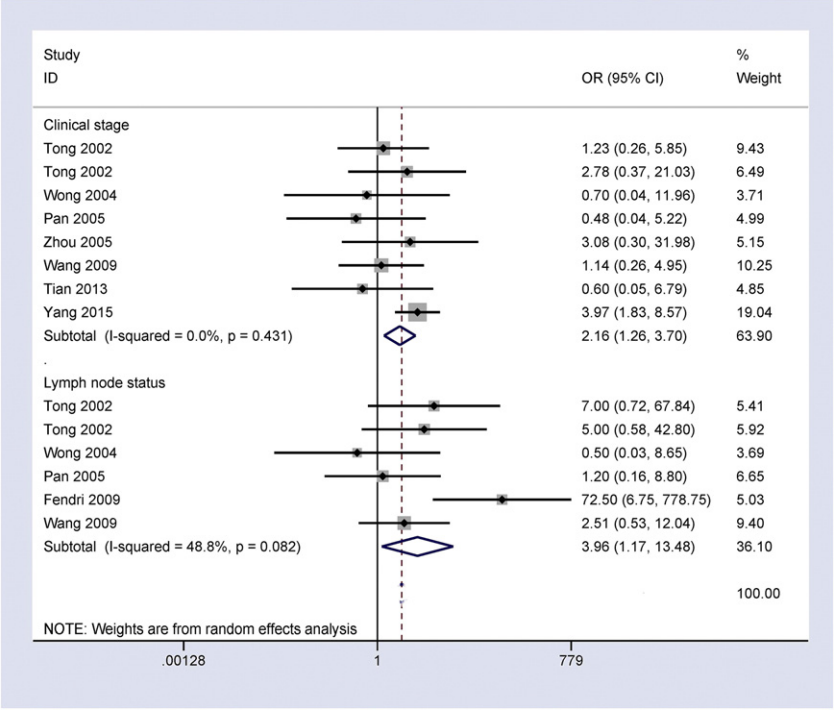

Fig. 5. Forest plot of the correlation between *RASSF1A* promoter methylation, clinical stage, and lymph node status of NPC patients.

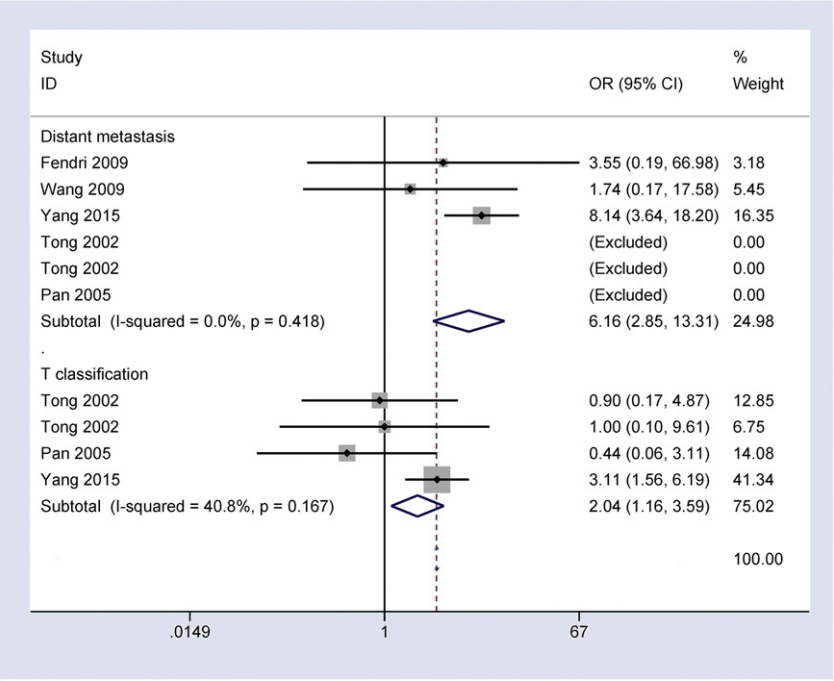

Fig. 6. Forest plot of the relationship between *RASSF1A* promoter methylation, distant metastasis, and T classification of NPC patients.

4. Discussion

Multiple factors are involved in NPC pathogenesis, including the Epstein-Barr virus, environmental, genetics and epigenetic components (Tsao et al., 2014; Lo et al., 2004). *RASSF1A* is a key TSG in various human cancers (Donninger et al., 2007). DNA methylation of TSG promoters leads to dysfunction or loss of gene expression, including *RASSF1A*, which may play a key role in the development of NPC (Fendri et al., 2009; Kong et al., 2006; Lo et al., 1996). Numerous studies with small populations have indicated that the frequency of *RASSF1A* promoter methylation is significantly increased in NPC tissue samples compared with non-tumor tissue samples (Nawaz et al., 2015b; Challouf et al., 2012; Wang et al., 2009; Fendri et al., 2009). Our results, comprised of 11 studies forming a large population, confirm that *RASSF1A* promoter methylation was notably more common in NPC compared with non-tumor tissues, which indicates that methylation of the *RASSF1A* promoter is closely linked to NPC tumorigenesis.

Subgroup analysis by ethnicity (Asian and Caucasian populations) on *RASSF1A* promoter methylation in NPC compared with non-tumor tissues showed that methylation was associated with an increased risk of NPC in both Asian and Caucasian populations. These results suggest that *RASSF1A*, with promoter methylation, may be a susceptibility

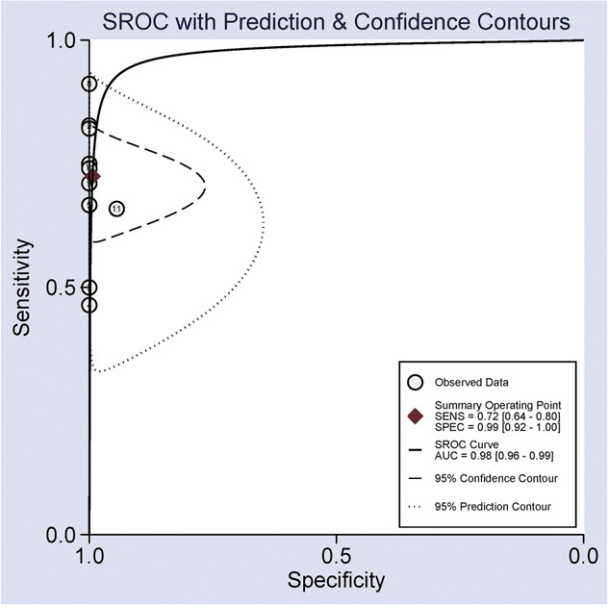

Fig. 7. Summary receiver operating characteristics (SROC) evaluation of *RASSF1A* promoter methylation in the tissue of NPC patients compared with non-tumor tissue samples, sensitivity = 0.72 (95% CI = 0.64–0.80), specificity = 0.99 (95% CI = 0.92–1.00), and AUC = 0.98 (95% CI = 0.96–0.99).

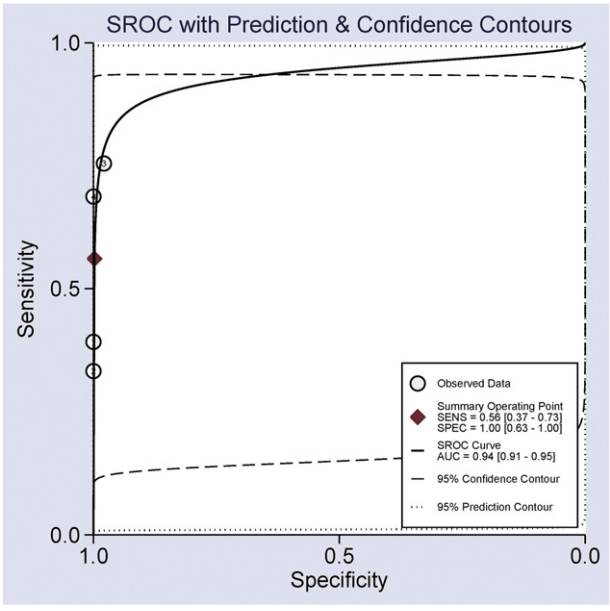

Fig. 8. Summary receiver operating characteristics (SROC) assessment of *RASSF1A* promoter methylation in the brushing of NPC patients compared with non-tumor brushing samples, sensitivity = 0.56 (95% CI = 0.37–0.73), specificity = 1.00 (95% CI = 0.63–1.00), and AUC = 0.94 (95% CI = 0.91–0.95).

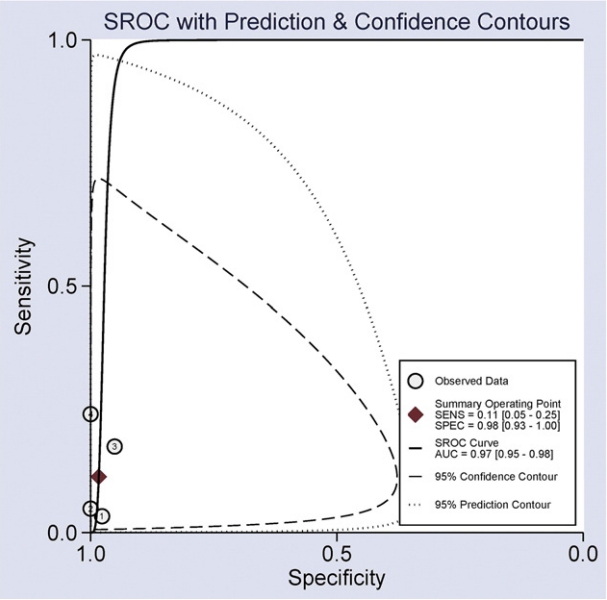

Fig. 9. Summary receiver operating characteristics (SROC) estimation of *RASSF1A* promoter methylation in the blood of NPC patients compared with non-tumor blood samples, sensitivity = 0.11 (95% CI = 0.05–0.25), specificity = 0.98 (95% CI = 0.93–1.00), and AUC = 0.97 (95% CI = 0.95–0.98).

gene for Asians and Caucasians with NPC. We found a slight heterogeneity (P = 0.066) and performed a sensitivity analysis to determine the stability of the pooled OR by omitting an individual study (Zhou et al., 2005), control: adjacent tissue samples). The combined OR from the remaining studies was also significant and heterogeneity was dramatically reduced (P = 0.971). The main reason for bias in the current result may be contamination of tissue samples adjacent to the nasopharynx by NPC cells. Furthermore, the result of meta-regression analysis was consistent with the sensitivity analysis, suggesting that our analyses are stable and credible.

RASSF1A promoter methylation was not correlated with age in the four studies that analyzed it (Wang et al., 2009; Zhou et al., 2005; Pan et al., 2005; Tong et al., 2002). *RASSF1A* promoter methylation was not associated with sex in the eight studies that analyzed it (Yang et al., 2015; Tian et al., 2013; Wang et al., 2009; Fendri et al., 2009; Zhou et al., 2005; Pan et al., 2005; Wong et al., 2004; Tong et al., 2002). Our results were consistent, showing no relationship between *RASSF1A* promoter methylation, age and sex of NPC patients. In a large population (189 NPC patients), Yang et al. (Yang et al., 2015) reported a significant relationship between *RASSF1A* promoter methylation and clinical stage, distant metastasis, and T classification. The remaining articles, which had small populations, reported no correlation between *RASSF1A* promoter methylation clinical stage (Tian et al., 2013; Wang et al., 2009; Zhou et al., 2005; Pan et al., 2005; Wong et al., 2004; Tong et al., 2002), distant metastasis (Wang et al., 2009; Fendri et al., 2009; Pan et al., 2005; Tong et al., 2002), and T classification (Pan et al., 2005; Tong et al., 2002). There was a significant relationship between *RASSF1A* promoter methylation and lymph node status in 68 patients with NPC (Fendri et al., 2009). However, the remaining papers (<42 NPC patients per study) showed no association between *RASSF1A* promoter methylation and lymph node status (Wang et al., 2009; Pan et al., 2005; Wong et al., 2004; Tong et al., 2002). The current findings, based on multiple studies, reveal that *RASSF1A* promoter methylation was correlated with clinical stage, lymph node status, distant metastasis, and T classification. Furthermore, it was notably higher in advanced stage compared with early stage NPC patients, higher in lymph node positive- compared with lymph node negative patients, higher in patients with distant metastasis compared with patients without distant metastasis, and higher in patients with T3–4 classification compared with patients with T1–2 classification. These results suggest that *RASSF1A* promoter methylation plays an important role in the progression and metastasis of NPC. Thus, *RASSF1A* promoter methylation may be associated with a poor prognosis for patients with NPC and serve as a potential therapeutic drug target.

This study reveals a significant relationship between *RASSF1A* promoter methylation and NPC in tissue, brushing and blood samples, indicating that *RASSF1A* promoter methylation may be a noninvasive biomarker for NPC. Several studies have suggested that aberrant DNA methylation of cancer-specific genes (e.g., TSGs) in various types of human samples could be used for noninvasive cancer screening and diagnosis (Ye et al., 2016; Yang et al., 2016; Ma et al., 2015; Renard et al., 2010). Therefore, we investigated whether *RASSF1A* promoter methylation can serve as a diagnostic biomarker for NPC. The pooled specificity and AUC of *RASSF1A* promoter methylation were very good in tissue, brushing and blood samples of patients with NPC vs. corresponding non-tumor samples (tissue: specificity = 0.99, AUC: 0.98; brushing: specificity = 1.00, AUC: 0.94; blood: specificity = 0.98, AUC: 0.97 > 0.9). The combined sensitivity of *RASSF1A* promoter methylation was higher in the tissue and brushing groups (0.72, 95% CI = 0.64–0.80 and 0.56, 95% CI = 0.37–0.73, respectively) compared with the blood group, which had a bad value (0.11, 95% CI = 0.05–0.25). We also found that the sensitivity and specificity of *RASSF1A* promoter methylation were better in the tissue (91.2% and 100%, respectively) and brushing samples (75.5% and 97.9%, respectively). These findings suggest that *RASSF1A* promoter methylation is an effective noninvasive biomarker for the diagnosis of NPC in tissue and brushing samples. In the future, additional well-designed clinical studies with large populations will be necessary to validate the diagnostic potential of *RASSF1A* promoter methylation for NPC patients, particularly in brushing samples.

This study has several limitations. First, only papers published in English were included. Publications in languages other than English were excluded due to insufficient information, which may lead to selection bias. Second, this study involved largely Asians and Caucasians; other ethnic subgroups (e.g., Africans) were lacking. In addition, several of the studies were based on a small Caucasian population. In the future, additional studies with large Caucasian and African populations are necessary. Third, further prospective studies using quantitative detection methodologies (i.e., pyrosequencing, MethyLight, methylation-sensitive high resolution melting, etc.) are needed to confirm the role of *RASSF1A* promoter methylation as a biomarker for the diagnosis of NPC. Finally, the correlation between *RASSF1A* promoter methylation and the clinicopathological characteristics of NPC patients requires further validation because of the limited sample size.

5. Conclusions

The findings from this study suggest that *RASSF1A* promoter methylation is more common in NPC than in non-tumor tissue, brushing, and blood samples. Furthermore, *RASSF1A* promoter methylation was higher in later stage than in early stage patients, higher in patients with lymph node metastasis than without, higher in patients with distant metastasis than those without, and higher in patients with T3–4 classification than in patients with T1–2 classification. In addition, *RASSF1A* promoter methylation may be a diagnostic biomarker in tissue and brushing samples that could be used for the clinical diagnosis of NPC. In the future, well-matched prospective studies are essential for determining the prognostic and diagnostic significance of *RASSF1A* promoter methylation in patients with NPC.

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Conflicts of Interest

The authors declare that they have no competing financial interests.

Authors' Contributions

MY and TH contributed to the conception and design of this research. CN and SC contributed to the retrieval of articles and the extraction of data. PY, CN and SC contributed to the data analysis and design of the figures and tables. All authors approved the final manuscript.

Ethical Review from Patients

Although the present study was not primary research involving human samples, our study was a secondary analysis of human subject data published in the public domain.

References

Agathanggelou, A., Cooper, W.N., Latif, F., 2005. Role of the Ras-association domain family 1 tumor suppressor gene in human cancers. Cancer Res. 65, 3497–3508.

Allen, N.P., Donninger, H., Vos, M.D., Eckfeld, K., Hesson, L., Gordon, L., Birrer, M.J., Latif, F., Clark, G.J., 2007. RASSF6 is a novel member of the RASSF family of tumor suppressors. Oncogene 26, 6203–6211.

Burbee, D.G., Forgacs, E., Zochbauer-Muller, S., Shivakumar, L., Fong, K., Gao, B., Randle, D., Kondo, M., Virmani, A., Bader, S., Sekido, Y., Latif, F., Milchgrub, S., Toyooka, S., Gazdar, A.F., Lerman, M.I., Zabarovsky, E., White, M., Minna, J.D., 2001. Epigenetic inactivation of RASSF1A in lung and breast cancers and malignant phenotype suppression. J. Natl. Cancer Inst. 93, 691–699.

Challouf, S., Ziadi, S., Zaghdoudi, R., Ksiaa, F., Ben Gacem, R., Trimeche, M., 2012. Patterns of aberrant DNA hypermethylation in nasopharyngeal carcinoma in Tunisian patients. Clin. Chim. Acta 413, 795–802.

Chang, H.W., Chan, A., Kwong, D.L., Wei, W.I., Sham, J.S., Yuen, A.P., 2003. Evaluation of hypermethylated tumor suppressor genes as tumor markers in mouth and throat rinsing fluid, nasopharyngeal swab and peripheral blood of nasopharygeal carcinoma patient. Int. J. Cancer 105, 851–855.

Chen, L., Hu, C.S., Chen, X.Z., Hu, G.Q., Cheng, Z.B., Sun, Y., Li, W.X., Chen, Y.Y., Xie, F.Y., Liang, S.B., Chen, Y., Xu, T.T., Li, B., Long, G.X., Wang, S.Y., Zheng, B.M., Guo, Y., Sun, Y., Mao, Y.P., Tang, L.L., Chen, Y.M., Liu, M.Z., Ma, J., 2012. Concurrent chemoradiotherapy plus adjuvant chemotherapy versus concurrent chemoradiotherapy alone in patients with locoregionally advanced nasopharyngeal carcinoma: a phase 3 multicentre randomised controlled trial. Lancet Oncol. 13, 163–171.

Chua, D.T., Yiu, H.H., Seetalarom, K., Ng, A.W., Kurnianda, J., Shotelersuk, K., Krishnan, G., Hong, R.L., Yang, M.H., Wang, C.H., Sze, W.K., Ng, W.T., 2012. Phase II trial of capecitabine plus cisplatin as first-line therapy in patients with metastatic nasopharyngeal cancer. Head Neck 34, 1225–1230.

Coory, M.D., 2010. Comment on: heterogeneity in meta-analysis should be expected and appropriately quantified. Int. J. Epidemiol. 39, 932 (author reply 933).

Dersimonian, R., 1996. Meta-analysis in the design and monitoring of clinical trials. Stat. Med. 15, 1237–1248 (discussion 1249-52).

Donninger, H., Vos, M.D., Clark, G.J., 2007. The RASSF1A tumor suppressor. J. Cell Sci. 120, 3163–3172.

Du, C., Huang, T., Sun, D., Mo, Y., Feng, H., Zhou, X., Xiao, X., Yu, N., Hou, B., Huang, G., Ernberg, I., Zhang, Z., 2011. CDH4 as a novel putative tumor suppressor gene epigenetically silenced by promoter hypermethylation in nasopharyngeal carcinoma. Cancer Lett. 309, 54–61.

Egger, M., Davey Smith, G., Schneider, M., Minder, C., 1997. Bias in meta-analysis detected by a simple, graphical test. BMJ 315, 629–634.

Fendri, A., Masmoudi, A., Khabir, A., Sellami-Boudawara, T., Daoud, J., Frikha, M., Ghorbel, A., Gargouri, A., Mokdad-Gargouri, R., 2009. Inactivation of RASSF1A, RARbeta2 and DAP-kinase by promoter methylation correlates with lymph node metastasis in nasopharyngeal carcinoma. Cancer Biol. Ther. 8, 444–451.

Gong, Q.Y., Zheng, G.L., Zhu, H.Y., 1991. MRI differentiation of recurrent nasopharyngeal carcinoma from postradiation fibrosis. Comput. Med. Imaging Graph. 15, 423–429.

Higgins, J.P., Thompson, S.G., Deeks, J.J., Altman, D.G., 2003. Measuring inconsistency in meta-analyses. BMJ 327, 557–560.

Hutajulu, S.H., Indrasari, S.R., Indrawati, L.P., Harijadi, A., Duin, S., Haryana, S.M., Steenbergen, R.D., Greijer, A.E., Middeldorp, J.M., 2011. Epigenetic markers for early detection of nasopharyngeal carcinoma in a high risk population. Mol. Cancer 10, 48.

Jiang, W., Liu, N., Chen, X.Z., Sun, Y., Li, B., Ren, X.Y., Qin, W.F., Jiang, N., Xu, Y.F., Li, Y.Q., Ren, J., Cho, W.C., Yun, J.P., Zeng, J., Liu, L.Z., Li, L., Guo, Y., Mai, H.Q., Zeng, M.S., Kang, T.B., Jia, W.H., Shao, J.Y., Ma, J., 2015. Genome-wide identification of a methylation gene panel as a prognostic biomarker in nasopharyngeal carcinoma. Mol. Cancer Ther. 14, 2864–2873.

Jones, C.M., Athanasiou, T., 2005. Summary receiver operating characteristic curve analysis techniques in the evaluation of diagnostic tests. Ann. Thorac. Surg. 79, 16–20.

Kong, W.J., Zhang, S., Guo, C.K., Wang, Y.J., Chen, X., Zhang, S.L., Zhang, D., Liu, Z., Kong, W., 2006. Effect of methylation-associated silencing of the death-associated protein kinase gene on nasopharyngeal carcinoma. Anti-Cancer Drugs 17, 251–259.

Kwong, J., Lo, K.W., To, K.F., Teo, P.M., Johnson, P.J., Huang, D.P., 2002. Promoter hypermethylation of multiple genes in nasopharyngeal carcinoma. Clin. Cancer Res. 8, 131–137.

Lau, J., Ioannidis, J.P., Schmid, C.H., 1997. Quantitative synthesis in systematic reviews. Ann. Intern. Med. 127, 820–826.

Lin, G.W., Wang, L.X., Ji, M., Qian, H.Z., 2013. The use of MR imaging to detect residual versus recurrent nasopharyngeal carcinoma following treatment with radiation therapy. Eur. J. Radiol. 82, 2240–2246.

Liu, M.T., Hsieh, C.Y., Chang, T.H., Lin, J.P., Huang, C.C., Wang, A.Y., 2003. Prognostic factors affecting the outcome of nasopharyngeal carcinoma. Jpn. J. Clin. Oncol. 33, 501–508.

Lo, K.W., Cheung, S.T., Leung, S.F., Van Hasselt, A., Tsang, Y.S., Mak, K.F., Chung, Y.F., Woo, J.K., Lee, J.C., Huang, D.P., 1996. Hypermethylation of the p16 gene in nasopharyngeal carcinoma. Cancer Res. 56, 2721–2725.

Lo, K.W., Kwong, J., Hui, A.B., Chan, S.Y., To, K.F., Chan, A.S., Chow, L.S., Teo, P.M., Johnson, P.J., Huang, D.P., 2001. High frequency of promoter hypermethylation of RASSF1A in nasopharyngeal carcinoma. Cancer Res. 61, 3877–3881.

Lo, K.W., To, K.F., Huang, D.P., 2004. Focus on nasopharyngeal carcinoma. Cancer Cell 5, 423–428.

Ma, Y., Zhang, H., Liu, F., Wu, Z., Lu, S., Jin, Q., Zhao, J., Zhong, X., Mao, H., 2015. Highly sensitive detection of DNA methylation levels by using a quantum dot-based FRET method. Nano 7, 17547–17555.

Moher, D., Liberati, A., Tetzlaff, J., Altman, D.G., Group, P, 2009. Preferred reporting items for systematic reviews and meta-analyses: the PRISMA statement. BMJ 339, b2535.

Nawaz, I., Hu, L.F., Du, Z.M., Moumad, K., Ignatyev, I., Pavlova, T.V., Kashuba, V., Almgren, M., Zabarovsky, E.R., Ernberg, I., 2015a. Integrin alpha9 gene promoter is hypermethylated and downregulated in nasopharyngeal carcinoma. Oncotarget 6, 31493–31507.

Nawaz, I., Moumad, K., Martorelli, D., Ennaji, M.M., Zhou, X., Zhang, Z., Dolcetti, R., Khyatti, M., Ernberg, I., Hu, L. F., 2015b. Detection of nasopharyngeal carcinoma in Morocco (North Africa) using a multiplex methylation-specific PCR biomarker assay. Clin. Epigenetics 7, 89.

Pan, Z.G., Kashuba, V.I., Liu, X.Q., Shao, J.Y., Zhang, R.H., Jiang, J.H., Guo, C., Zabarovsky, E., Ernberg, I., Zeng, Y.X., 2005. High frequency somatic mutations in RASSF1A in nasopharyngeal carcinoma. Cancer Biol. Ther. 4, 1116–1122.

Qiu, G.H., Tan, L.K., Loh, K.S., Lim, C.Y., Srivastava, G., Tsai, S.T., Tsao, S.W., Tao, Q., 2004. The candidate tumor suppressor gene BLU, located at the commonly deleted region 3p21.3, is an E2F-regulated, stress-responsive gene and inactivated by both epigenetic and genetic mechanisms in nasopharyngeal carcinoma. Oncogene 23, 4793–4806.

Reitsma, J.B., Glas, A.S., Rutjes, A.W., Scholten, R.J., Bossuyt, P.M., Zwinderman, A.H., 2005. Bivariate analysis of sensitivity and specificity produces informative summary measures in diagnostic reviews. J. Clin. Epidemiol. 58, 982–990.

Renard, I., Joniau, S., Van Cleynenbreugel, B., Collette, C., NAOME, C., Vlassenbroeck, I., Nicolas, H., De Leval, J., Straub, J., Van Criekinge, W., Hamida, W., Hellel, M., Thomas, A., De Leval, L., Bierau, K., Waltregny, D., 2010. Identification and validation of the methylated TWIST1 and NID2 genes through real-time methylation-specific polymerase chain reaction assays for the noninvasive detection of primary bladder cancer in urine samples. Eur. Urol. 58, 96–104.

Tian, F., Yip, S.P., Kwong, D.L., Lin, Z., Yang, Z., Wu, V.W., 2013. Promoter hypermethylation of tumor suppressor genes in serum as potential biomarker for the diagnosis of nasopharyngeal carcinoma. Cancer Epidemiol. 37, 708–713.

Tong, J.H., Tsang, R.K., Lo, K.W., Woo, J.K., Kwong, J., Chan, M.W., Chang, A.R., Van Hasselt, C.A., Huang, D.P., To, K.F., 2002. Quantitative Epstein-Barr virus DNA analysis and detection of gene promoter hypermethylation in nasopharyngeal (NP) brushing samples from patients with NP carcinoma. Clin. Cancer Res. 8, 2612–2619.

Torre, L.A., Bray, F., Siegel, R.L., Ferlay, J., Lortet-Tieulent, J., Jemal, A., 2015. Global cancer statistics, 2012. CA Cancer J. Clin. 65, 87–108.

Tsao, S.W., Yip, Y.L., Tsang, C.M., Pang, P.S., Lau, V.M., Zhang, G., Lo, K. W., 2014. Etiological factors of nasopharyngeal carcinoma. Oral Oncol. 50, 330–338.

Wang, T., Liu, H., Chen, Y., Liu, W., Yu, J., Wu, G., 2009. Methylation associated inactivation of RASSF1A and its synergistic effect with activated K-Ras in nasopharyngeal carcinoma. J. Exp. Clin. Cancer Res. 28, 160.

Wong, A.M., Kong, K.L., Chen, L., Liu, M., Wong, A.M., Zhu, C., Tsang, J.W., Guan, X.Y., 2013. Characterization of CACNA2D3 as a putative tumor suppressor gene in the development and progression of nasopharyngeal carcinoma. Int. J. Cancer 133, 2284–2295.

Wong, T.S., Kwong, D.L., Sham, J.S., Wei, W.I., Kwong, Y.L., Yuen, A.P., 2004. Quantitative plasma hypermethylated DNA markers of undifferentiated nasopharyngeal carcinoma. Clin. Cancer Res. 10, 2401–2406.

Wong, T.S., Tang, K.C., Kwong, D.L., Sham, J.S., Wei, W.I., Kwong, Y.L., Yuen, A.P., 2003. Differential gene methylation in undifferentiated nasopharyngeal carcinoma. Int. J. Oncol. 22, 869–874.

Yang, Q., Huang, T., Ye, G., Wang, B., Zhang, X., 2016. Methylation of SFRP2 gene as a promising noninvasive biomarker using feces in colorectal cancer diagnosis: a systematic meta-analysis. Sci. Rep. 6, 33339.

Yang, X., Dai, W., Kwong, D.L., Szeto, C.Y., Wong, E.H., Ng, W.T., Lee, A.W., Ngan, R.K., Yau, C.C., Tung, S.Y., Lung, M.L., 2015. Epigenetic markers for noninvasive early detection of nasopharyngeal carcinoma by methylation-sensitive high resolution melting. Int. J. Cancer 136, E127–E135.

Ye, M., Huang, T., Ying, Y., Li, J., Yang, P., Ni, C., Zhou, C., Chen, S., 2017. Detection of 14-3-3 sigma (sigma) promoter methylation as a noninvasive biomarker using blood samples for breast cancer diagnosis. Oncotarget 8, 9230–9242.

Yu, M.C., Yuan, J.M., 2002. Epidemiology of nasopharyngeal carcinoma. Semin. Cancer Biol. 12, 421–429.

Zhou, L., Jiang, W., Ren, C., Yin, Z., Feng, X., Liu, W., Tao, Q., Yao, K., 2005. Frequent hypermethylation of RASSF1A and TSLC1, and high viral load of Epstein-Barr Virus DNA in nasopharyngeal carcinoma and matched tumor-adjacent tissues. Neoplasia 7, 809–815.

Zhou, X., Cui, J., Macias, V., Kajdacsy-Balla, A.A., Ye, H., Wang, J., Rao, P.N., 2007. The progress on genetic analysis of nasopharyngeal carcinoma. Comput. Funct. Genomics 57513.