Research Paper

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

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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., 2005). 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 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...
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 epigen*’. 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.](image-url)
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.](image-url)
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.

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 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 = 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. 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.](image-url)
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.
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
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 97.8%, 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.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ebiom.2017.03.038.

Funding

This research was supported by grants from the Natural Science Foundation of Zhejiang Province (LY16H160005), the Ningbo Natural
Agathanggelou, A., Cooper, W.N., Latif, F., 2005. Role of the Ras-association domain family science foundation (2014A610235), and the project of the scientific and technological project of the report.

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