RESEARCH ARTICLE

RASSF1A Gene Methylation is Associated with Nasopharyngeal Carcinoma Risk in Chinese

Kun Wu[1,2&], Xiao-Ning Xu[3&], Yu Chen[1&], Xiao-Lin Pu[1], Bo-Yuan Wang[2], Xiao-Dan Tang[1*]

Abstract

In order to explore the association between *RASSF1A* methylation and nasopharyngeal carcinoma (NPC) risk of Chinese, we carried out a meta-analysis with searches of PubMed, Web of Science, ProQest and Medline databases. Ultimately, 14 articles were identified and analysised using R Software (R version 3.1.2) including meta packages. Overall, we found a significant relationship between *RASSF1A* methylation and NPC risk (OR 30.7; 95 % CI, 16.71~56.23; z=11.0591; p<0.0001) in a fixed effects model and (OR 32.1; 95% CI, 14.27~72.01; z=8.3984; p<0.0001) in a random effects model pooled. In tissue and NP brushings groups , similar results were found. Hence, our study identified a strong association between *RASSF1A* methylation and NPC and highlighted a promising potential for *RASSF1A* methylation in NPC risk prediction of Chinese.

Keywords: *RASSF1A* - nasopharyngeal carcinoma - methylation

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Introduction

Nasopharyngeal cancer (NPC) is a prevalent human malignancy in Southern China and Southeast Asia (Bhattacharyya, 2004; Bernard W. Stewart, 2014). According to the data from the International Agency for Research on Cancer, the incidence rate of NPC is 20-50 per 100,000 in Southern China, while it is only 1 per 100,000 in most parts of the world. Nasopharyngeal carcinoma is a distinct type of head and neck cancer and is very different from other head and neck cancers because of its specific multifactorial etiology and its geographic distribution. The molecular basis of NPC pathogenesis is not well understood, but is believed to involve a multistep process including Epstein-Barr Virus (EBV) infection, environmental factors, as well as genetic and epigenetic alterations (Tao and Chan, 2007). Once tumor cells have spread, the long-term prognosis is poor since no curative treatments are available. Thus, the development of biomarkers for effective early diagnosis of NPC is clearly necessary.

DNA methylation is one of the best-studied epigenetic alterations in cancer. Aberrant methylation at the promoter regions lead to gene silencing. Hypermethylation of tumor suppressor genes (TSGs) has been found in most human cancers (Baylin, 2005; Hesson et al., 2007). The gene Ras association domain family 1A (RASSF1A) has been widely studied as a methylation biomarker and a TSG in NPC since its discovery in lung cancer in 2000 (Dammann et al., 2000; Lo et al., 2001). Taking into consideration the above findings we found that *RASSF1A* is an important tumor-suppressor gene and is likely to be involved in the genesis of NPC, and plays an important role in the progression of tumorigenesis.

However, even if lots of studies have been evaluated the association between promoter methylation of *RASSF1A* gene and the risk of NPC (Fendri et al., 2009; Challouf et al., 2012; Yang et al., 2014), the results from those studies remain conflicting rather than conclusive. Thus, we carried out a meta-analysis based on all eligible case-control literature to assess the association of *RASSF1A* gene promoter methylation with the risk of NPC.

Materials and Methods

Search strategy and selection criteria

We conducted a comprehensive search strategy towards electronic databases, including PubMed, Web of Science, ProQest and Medline, using the key words: 'nasopharyngeal cancer or nasopharyngeal neoplasm or nasopharyngeal carcinoma or NPC', '*RASSF1A* or Ras association domain family 1A or *Rassf1a*', 'methylation or hypermethylation or epigenetic'. The included articles meet the following criteria: *(i)* original study; *(ii)* the diagnosis of nasopharyngeal cancer was based on histopathology; *(iii)* the subjects in every study comprised of prostate cancer patients and non-cancer controls; *(iv)* When the same or overlapping data appeared in multiple publications, we used the most recent or largest population; *(v)* Data was included in the analysis only if the full text of the article was in English. Exclusion criteria were: *(i) RASSF1A* methylation conducted only in the cell

*[1]Medical Faculty, Kunming University of Science and Technology, [2]Digestive system department, [3]Clinical psychology department, the First People's Hospital of Yunnan Province, Panlong Campus, 157 Jinbi Road, Kunming, Yunnan, P.R. China [&]Equal contributors *For correspondence: txd06@163.com*

lines; *(ii)* no raw data available or cannot retrieve any raw data; *(iii)* review papers.

Data extraction

Data were extracted from each study by two reviewers (K Wu and X Xu) independently using pre-specified selection standards. Decisions were made and disagreements about study selection were resolved by consensus or by involving a third reviewer (X Pu). The following information was extracted from the studies: the first author's last name, publication year, original country of patients in the subjects, and the number of *RASSF1A* methylation of cases and controls in individuals, etc.

Statistical analysis

The strength of the association between the *RASSF1A* promoter methylation and NPC risk was measured by pooled odds ratio (OR) with its 95 % confidence interval (CI).The significance of the pooled OR was determined by the Z test and $p<0.05$ was considered as statistically significant. Subgroup analysis was performed stratified by the study character of tissue sample and NP brushings sample. Heterogeneity was tested using the I^2 statistic with values over 50% and Chi-squared test with $p\leq0.1$ indicating strong heterogeneity between the studies (Higgins et al., 2003). Tau-squared ($\tau2$) was used to determine how much heterogeneity was explained by subgroup differences. The data were pooled using the DerSimonian and Laird random effects model ($I^2>50\%$, $p\leq0.1$) or fixed effects model ($I^2<50\%$) according to heterogeneity statistic I^2 (DerSimonian and Laird, 1986). With a lack of heterogeneity among included studies, the pooled odds ratio estimates were calculated using the fixed-effects model (Mantele-Haenszel) (Mantel and Haenszel, 1959). Otherwise, the random-effects model (DerSimonian and Laird method) was used (DerSimonian and Laird, 1986). Sensitivity analyses were performed to assess the contributions of single studies to the final results. Begg's test were used to examine whether the results of a meta-analysis may have been affected by publication bias. Egger's test was implemented to testing for funnel plot asymmetry (Egger, 1997). All statistical analyses were performed using R Software (R version 3.1.2) including meta packages.

Results

After being selected in accordance with the inclusive criteria, our final eligible studies included 14 studies, as shown in Figure 1 (Lo et al., 2001; Kwong et al., 2002; Tong et al., 2002; Chang et al., 2003; Wong et al., 2003; Qiu et al., 2004; Wong et al., 2004; Zhou et al., 2005; Tan et al., 2006; Fendri et al., 2009; Challouf et al., 2012; Yang et al., 2014).

Study characteristics

The characteristics of retained 14 studies are listed in Table 1. The subjects were conducted in 3 countries (Tunisia, China and Netherlands) and published between 2001 and 2014, twelve of the 14 studies focus on tissue sample and 2 of the 14 studies about the brushing sample. There was 52.53% of NPC patients had the methylated *RASSF1A* allele with a frequency ranging from 4.88% to 89.71% in individual trials, however, the *RASSF1A* methylation was only found 4.28% in normal sample.

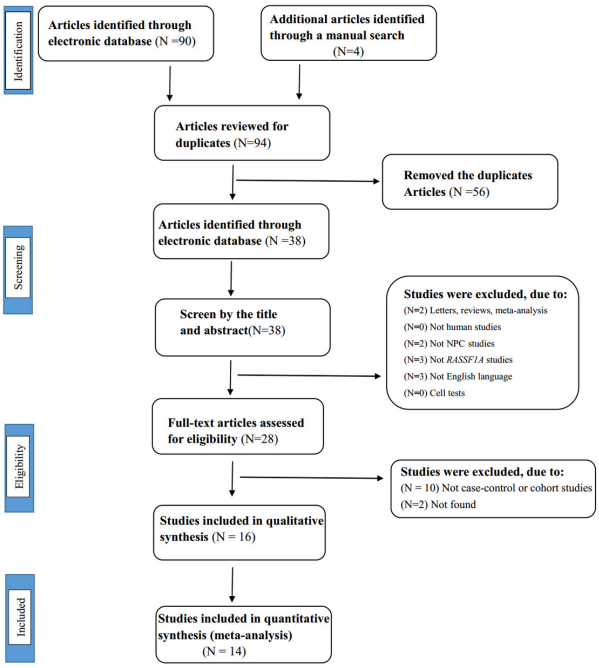

Figure 1. Flow Chart of Study Identification

Table 1. Main Characteristics of the Studies Included in the Meta-analysis

Authod	Year	Country	Sample	Method	Control			Case			Total
					M	T	%	M	T	%	
Yang et al.	2014	China	Tissue	MS-HRM	0	50	0.00%	53	220	24.09%	270
Challouf et al.	2012	Tunisia	Tissue	MSP	0	19	0.00%	27	36	75.00%	55
Hutajulu et al.	2011	Netherlands	Tissue	MSP	0	22	0.00%	40	53	75.47%	75
Wang et al.	2009	China	Tissue	MSP	0	14	0.00%	27	38	71.05%	52
Fendri et al.	2009	Tunisia	Tissue	MSP	0	9	0.00%	61	68	89.71%	77
Tan et al.	2006	China	Tissue	MSP	1	4	25.00%	13	19	68.42%	23
Zhou et al.	2005	China	Tissue	MSP	13	28	46.43%	23	28	82.14%	56
Wong et al.	2004	China	Tissue	QMSP	0	43	0.00%	2	41	4.88%	84
Qiu et al.	2004	China	Tissue	MSP	0	29	0.00%	20	27	74.07%	56
Chang et al.	2003	China	Brushing	MSP	2	129	1.55%	20	30	66.67%	159
Wong et al.	2003	China	Tissue	MSP	1	12	8.33%	14	30	46.67%	42
Tong et al.	2002	China	Brushing	MSP	0	26	0.00%	11	28	39.29%	54
Kwong et al.	2002	China	Tissue	MSP	0	6	0.00%	28	33	84.85%	39
Lo et al.	2001	China	Tissue	MSP	0	6	0.00%	14	21	66.67%	27

Table 2. Sensitivity Analysis of the Summary Odds Ratio Coefficients on the Relationships between *RASSF1A* Aberrant Promoter Methylation and the Pathogenesis of NPC

Study	OR	95%CI
Yang et al. (2014 China)	30.4880	16.7275; 55.5682
Challouf et al. (2012 Tunisia)	28.4649	15.2913; 52.9878
Hutajulu et al. (2011 Netherlands)	27.7268	14.8719; 51.6931
Wang et al. (2009 China)	29.3298	15.7732; 54.5378
Fendri et al. (2009 Tunisia)	28.7786	15.4901; 53.4668
Tan et al. (2006 China)	32.7963	17.4306; 61.7076
Zhou et al. (2005 China)	45.0297	21.2412; 95.4596
Wong et al. (2004 China)	32.5973	17.5067; 60.6960
Qiu et al. (2004 China)	27.9717	15.0121; 52.1187
Chang et al. (2003 China)	26.7264	14.0673; 50.7775
Wong et al. (2003 China)	33.4922	17.7328; 63.2570
Tong et al. (2002 China)	30.4445	16.3786; 56.5901
Kwong et al. (2002 China)	29.8742	16.1091; 55.4015
Lo et al. (2001 China)	30.8901	16.6245; 57.3971
Pooled estimate	30.6580	16.7143; 56.2341

Quantitative analysis

The main results of our meta-analysis and the heterogeneity test are shown in Figure 2 and Figure 3. No statistically significant heterogeneity was observed in overall and subgroup stratified analyses; all the pooled odds ratios for risk were calculated by a fixed-effects model and Random effects model.

The combined results based on all studies showed the relationship between the *RASSF1A* promoter methylation was significantly associated with increased risk of NPC (OR, 30.66; 95 % CI, 16.71~56.23; z=11.0591; $p<0.0001$) in fixed effects model and (OR, 32.06; 95% CI, 14.27~72.01; z=8.3984; $p<0.0001$) in random effects model pooled (Figure 2), demonstrating a statistically significant increasing in likelihood of methylation in NPC comparing to controls. When stratifying for sample of nasopharyngeal cancer, the increased risk of *RASSF1A* methylation in case and controls was found tissue group (OR, 26.29, 95% CI 13.62~50.76) and in the NP brushings group (OR, 75.94, 95% CI 15.48~372.54) by fixed effects model with no between-study heterogeneity (Figure 3).

Bias Diagnosis

Begg's funnel plot and Egger's test were carried out to assess the publication bias of the studies. Visual assessment of the Begg's test did not reveal any evidence of obvious asymmetry in the overall analysis (t=0.8615, $p=0.4058$) and Egger's test to provide a result did not show evidence of publication bias (t=1.796, $p=0.09769$), too. Therefore, there are any publication bias of the studies.

Sensitive analysis

Sensitive analyses were conducted to determine whether modification of the inclusive criteria of the meta-analysis affected the final results. According to sensitivity analysis the odds ratio ranged from 26.7264 (95% CI, 14.0673~50.7775) to 45.0297 (95% CI, 21.2412~95.4596) by omitting a single study under the random-effect model (Table 2). No single study was found to affect the pooled OR as indicated by sensitivity analysis.

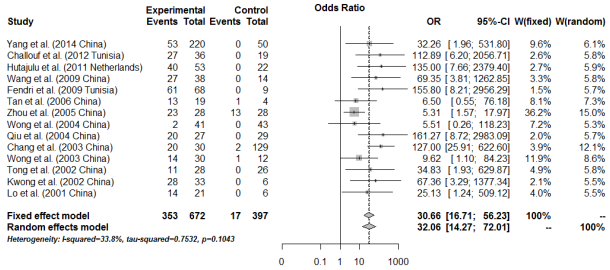

Figure 2. Combined Estimates for the Association between *RASSF1A* Promoter Methylation and NPC with Forest Plot. Author, year, country of the studies and methylated (M) and total number of the sample (T) in case and control, combined odds ratio (OR) with 95% confidence region were labeled in the right column of the figure. The DerSimonian-Laird estimator and Mantel-Haenszel method were selected to conduct combination estimation for the random effects model and fixed effects model, respectively

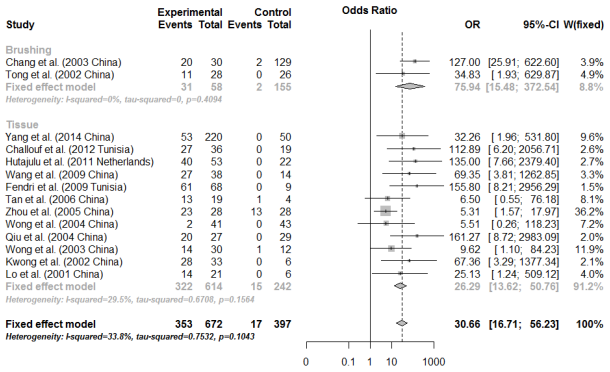

Figure 3. Subgroup Meta-analysis Estimation for the Relationship between *RASSF1A* Promoter Methylation and NPC. Subgroup meta-analysis based on material source are show. Author, year, country of the studies and methylated (M) and total number of the sample (T) in case and control, combined odds ratio (OR) with 95% confidence region were labeled in the right column of the figure. The DerSimonian-Laird estimator and Mantel-Haenszel method were selected to conduct combination estimation for the random effects model and fixed effects model, respectively

Discussion

NPC is generally only diagnosed at advanced stages of cancer progression because of its location deep inside the head and its vague symptoms which leading to poor prognosis and low survival rate of patients (Xie et al., 2014). Hence, it is very importance to find biomarker for early diagnosis of NPC. As an early epigenetic change in carcinogenesis, DNA methylation has been considered as a possible biomarker for detection of cancer. To determine whether *RASSF1A* methylation can serve as a biomarker for risk of NPC, we undertook a systematic review and meta-analysis of the literature.

To our knowledge, this is the first meta-analysis of published studies to evaluate the relationship between *RASSF1A* promoter methylation and NPC risk. Our analyses, combining 14 independent studies, revealed that the methylation of *RASSF1A* promoter does increase the risk of NPC. In particular, the overall OR for methylation status in nasopharyngeal cancer versus normal nasopharyngeal sample was 30.66 and 32.06 (95

% CI, 16.71~56.23; p<0.0001. 95% CI, 14.27~72.01; p<0.0001; Figure 2) by fixed and random effect model, suggesting a strong association of the methylation of *RASSF1A* promoter with nasopharyngeal cancer. Next, we stratified the association between *RASSF1A* promoter methylation and nasopharyngeal cancer risk by tissue and NP brushings, found subgroup analysis show similar results by tissue group (OR, 26.29, 95% CI 13.62~50.76) and NP brushings group (OR, 75.94, 95% CI 15.48~372.54), indicating a parallel effect of *RASSF1A* promoter methylation on NPC risk among different sample.

Assessment of the between-study heterogeneity is an essential requirement in meta-analyses (Joseph Lau, 1998). In our study, heterogeneity within the subjects was demonstrated by chi-square-based Q test. After systematically examined, we found that no significant heterogeneity between the enrolled 14 studies. In sensitivity analyses we found that there was no single sensitive study in our meta-analysis. In addition to between-study heterogeneity, publication bias has also been recognized as a major concern in robust meta-analyses. Thereby, we used Begg's and Egger's test to assess whether the studies included could be affected by publication bias. As a result, no evidence of publication bias was found. Taken together, these results indicated a credibly related of *RASSF1A* methylation with nasopharyngeal carcinoma.

Nasopharyngeal carcinoma is a distinct type of head and neck cancer with a remarkable geographic and racial distribution worldwide (Lo et al., 2004). However, NPC is prevalent among ethnic southern Chinese and the native Eskimos living in Greenland and Alaska. High incidence of NPC is also seen in some areas in North Africa (Adham et al., 2012). Our result showed a strong positive association of *RASSF1A* methylation with risk of nasopharyngeal cancer of Chinese, which is consistent with previous findings that *RASSF1A* methylation could be used as an independent clinical diagnosis factor for nasopharyngeal cancer (Wang et al., 2009; Challouf et al., 2012; Yang et al., 2014). Although our data have two studies of Tunisia and one about Netherlands, took account of the small size of the three data, we didn't have further elaborated. Therefore, *RASSF1A* methylation is a potential to be used as a molecular marker for early detection and monitoring in NPC of Chinese.

The present study has several limitations, such as ethnic, gender, histologic type, age and the TNM stage which are differences in *RASSF1A* methylation between cases and controls. Therefore, a meta-analysis including more high-quality designed epidemiology studies and a stratified analysis targeting different status are necessary in the future in this field.

In conclusion, there exists a strong association between methylation of the *RASSF1A* promoter and risk of nasopharyngeal cancer. Although further investigations with large number of samples are required to confirm the associations between *RASSF1A* promoter methylation and nasopharyngeal cancer, the findings in the present study highlight a promising potential for *RASSF1A* promoter methylation in nasopharyngeal cancer risk prediction.

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