

## RESEARCH ARTICLE

# Association between Ras association domain family 1A Promoter Methylation and Esophageal Squamous Cell Carcinoma: a Meta-analysis

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### Abstract

RASSF1A has been reported to be a candidate tumor suppressor in esophageal squamous cell carcinoma (ESCC). However, the association between RASSF1A promoter methylation and ESCC remains unclear. Eligible studies were identified through searching PubMed, Medline, Web of Science, and the China National Knowledge Infrastructure database. Studies were pooled and odds ratios (ORs) with corresponding confidence intervals (CIs) were calculated. Funnel plots were also performed to evaluate publication bias. Twelve studies involving 859 cases and 675 controls were included in this meta-analysis. A significant association was observed between RASSF1A methylation and ESCC overall (OR = 11.7, 95% CI: 6.59-20.9,  $z=8.36$ ,  $P<0.00001$ ). Subgroup analysis showed that the OR for heterogeneous tissues was 5.35 (95% CI = 2.95-9.71) while for autologous tissues it was 16.0 (8.31-30.96). For patient sample size, the OR for the <50 subgroup was 9.92 (95% CI = 2.88-34.2) and for the 50 case group was 13.1 (95% CI = 6.59-25.91). The OR for a relationship between RASSF1A methylation and TNM stages was 0.27 (95% CI=0.10-0.77), whereas there were no significant differences in RASSF1A methylation in relation to gender and differentiation among ESCC cases. This meta-analysis suggests a significant association between RASSF1A methylation and ESCC.

**Keywords:** RAS associations domain family 1A - methylation - esophageal squamous cell carcinoma - meta-analysis

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### Introduction

Esophageal carcinoma is the eighth most common cancer in the world and the seventh leading cause of cancer death worldwide (Jemal et al., 2011). Esophageal cancer may be divided into two major histological subtypes: esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma (EADC). ESCC is the predominant histological subtype, comprising 70% of esophageal cancer in the world and this tumor type is especially prevalent in East Asia, South Asia and South Africa (Parkin et al., 2000). Despite advances in multimodality therapy of ESCC, the overall 5-year survival rate was significant low (Lam, 2000). Thus, identification of biomarker for early detection of ESCC is great important. DNA methylation of tumor suppressor gene (TSG) leading to transcriptional inactivation has been identified as an important mechanism in many carcinogenesis including ESCC. The results of some studies also indicated that methylation of TSG was detected in tumor tissue and was associated with clinical features (Teodoridis et al., 2005; Bondurant et al.,

2011). Markers for methylation of TSG may represent a promising method for monitoring the occurrence and progression of cancer. Several potential tumors TSG, such as DUSP6 (Ma et al., 2013), CACNA2D3 (Li et al., 2013), hMLH1 (Chen et al., 2012), p16 (Taghavi et al., 2010), MGMT (Su et al., 2014), PTEN (Pan et al., 2013), RASSF1A (Wong et al., 2006), and so on, have been described as frequently silenced by hypermethylation in ESCC. In particular, RAS-association domain family 1 (RASSF1A) is widely investigated. RASSF1A is a putative tumor suppressor gene located at 3p21.3 and is implicated in the Ras signaling pathway, which plays a pivotal role in cell cycle control, microtubule stabilization, cellular adhesion, cell motility, and apoptosis (Agathangelou et al., 2005). Previous studies have reported the involvement of RASSF1A promoter methylation in several cancers, including prostate (Ge et al., 2013), Ovarian (Vo et al., 2013), endometrial (Fiolka et al., 2013), gastric (Zhou et al., 2013), lung (Liu et al., 2013), breast cancer (Jiang et al., 2012). Some studies have also reported differences in the methylation frequencies of RASSF1A between ESCC cancer tissues and non-

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cancerous tissues. Hypermethylation of the RASSF1A promoter in ESCC tissues was reported from 15% to 68%, indicating that RASSF1A is likely to be involved in the genesis of ESCC, and plays an important role in the progression of tumorigenesis (Kuroki et al., 2003; Wong et al., 2006; Cong et al., 2007; Ding et al., 2007; Zhang et al., 2007; Qin et al., 2009; Ren et al., 2009; He et al., 2010; Li et al., 2011; Mao et al., 2011; Wang et al., 2012; Zhou et al., 2013). However, they were mostly based on a small number of samples and showed inconsistent results. Therefore, we performed a meta-analysis to better identify the association between RASSF1A promoter methylation and ESCC.

## Materials and Methods

### Study Selection

A comprehensive literature search was performed using the PubMed, Medline, Web of Science, and China National Knowledge Infrastructure database for relevant articles published (last search updated in Dec. 2013) with the following key words: oesophageal cancer", "esophageal squamous cell carcinoma", "ESCC", "RAS association domain family protein 1A", "RASSF1A", "methylation" and "hypermethylation". Additional studies were found via the reference lists of the identified articles. Two independent reviewers screened the search results to reduce the possibility of missing relevant published papers. Where data were missing, we contacted the authors for the relevant information. The search was limited to human studies, without language and geographical location restrictions.

### Inclusion and Exclusion Criteria

Studies were selected for meta-analysis if they met the following criteria: 1) Studies which evaluated the association of RASSF1A methylation with Esophageal Squamous Cell Carcinoma; 2) the studies had to report the RASSF1A promoter methylation frequency from the ESCC tissue and normal tissue samples. Exclusion criteria were: review papers, animal experiments, case reports and studies with insufficient data.

### Data extraction

For each eligible study, two independent investigators extracted following information according to the inclusion criteria listed above: first author's name, year of publication, country of origin, ethnicity, source of controls, sample size, the measuring methods of methylation, and modulation frequencies RASSF1A in the case and the control groups.

### Statistical Analysis

Odds ratios (ORs) with the corresponding 95% confidence intervals (95% CIs) was used to assess the strength of association between RASSF1A methylation and ESCC risk. To assess heterogeneity across the studies, a statistical test for heterogeneity was performed. The chi-square-based Q-statistic test and I<sup>2</sup> statistics were used to test the heterogeneity among the included studies (Higgins et al., 2002). When a significant I<sup>2</sup> >50% or P < 0.05

indicated heterogeneity across studies, the random effects model with the DerSimonian and Laird (DL) method was used for meta-analysis, or else the fixed effects model with the Mantel-Haenszel method as used. Subgroup analyses were performed according to control type (autogenous or heterogeneous), patients sample size (<50 or 50) and publication language (English or Chinese) in consideration of the source of heterogeneity. The meta-regression was performed to explore the source of heterogeneity based on publication year, control type, patients sample size. Sensitivity analyses were also performed to assess the stability of the results. The influence of each study on the pooled estimate was assessed by omitting one study at a time. The potential publication bias was investigated with a funnel plot. In addition, Egger's linear regression was used to quantitatively analyze the potential publication bias (Egger et al., 1997). All statistical tests were two-sided and the significance level was set at P < 0.05. All P values were two-sided. Meta-analysis was performed using the Review Manager version 5.2 (provided by The Cochrane Collaboration) and STATA package version 12.0 (Stata Corporation, College Station, TX, USA).

## Results

### Study Characteristics

We identified 46 potentially relevant articles by our predefined search strategy in the database. Forty-two articles were obtained after duplicates removed. After reviewing these titles and abstracts, we obtained 22 potential eligible studies. By scanning the full texts, 10 articles were excluded according to the selection criteria. Finally, twelve studies from 2003 to 2013, with 859 tumor tissues and 675 controls, were involved in the meta-analysis (Figure 1). The frequencies of RASSF1A promoter methylation ranged from 14.89% to 67.50% (median, 44.70%) in ESCC tissues and 0.0% to 16.13% (median, 6.28%) in normal tissues, respectively. The pooled OR for RASSF1A methylation in cancer tissues compared with normal tissues was 11.73 (95%CI 6.59-20.89, z=8.36, P<0.00001) under the random-effects model, indicating an increased likelihood of methylation

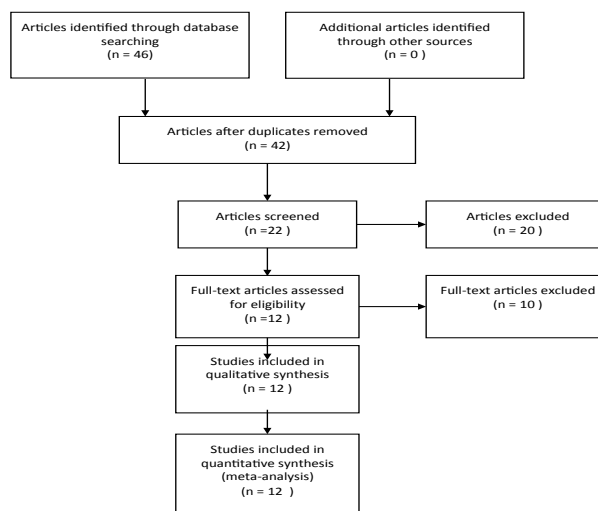


Figure 1. Flow Chart of Study Selection

**Table 1. Characteristics of the Included Studies**

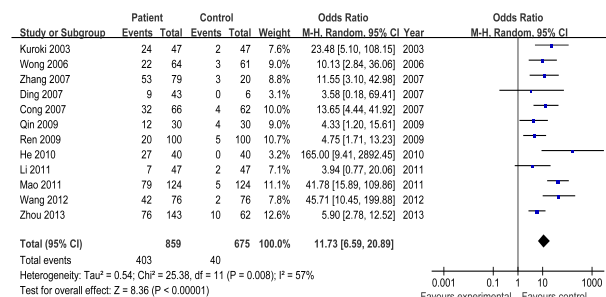
author	Year	Country	Patients		Control		Control Type	Method	Materials
			M+	M-	M+	M-			
Zhou et al	2013	China	76	67	10	52	Heterogeneous	MSP	Tissue
Wang et al	2012	China	42	34	2	74	Autologous	MSP	Tissue
Li et al	2011	China	7	40	2	45	Autologous	MSP	Tissue
Mao et al	2011	China	79	45	5	119	Autologous	RT-MSP	Tissue
He et al	2010	China	27	13	0	40	Autologous	MSP	Tissue
Ren et al	2009	China	20	80	5	95	Heterogeneous	MALDI-TOF MS	Tissue
Qin et al	2009	China	12	18	4	26	Autologous	MSP	Tissue
Ding et al	2007	China	9	34	0	6	Heterogeneous	MSP	Tissue
Cong et al	2007	China	32	34	4	62	Autologous	MSP	Tissue
Zhang et al	2007	China	53	26	3	17	Autologous	MSP	Tissue
Wong et al	2006	China	22	42	3	61	Autologous	MSP	Tissue
Kuroki et al	2003	Japan	24	23	2	45	Autologous	MSP	Tissue

M+, The number of tissues with methylation; M-, The number of tissues with no methylation

**Table 2. Subgroup Analysis**

Group	Patients		Controls		M-H pooled OR			D+L pooled OR			Heterogeneity		
	M+	M-	M+	M-	OR (95%CI)	Z	P	OR (95%CI)	Z	P	I <sup>2</sup> (%)	P	τ <sup>2</sup>
Total	403	456	40	635	12.64 (8.99-17.77)	14.61	<0.0001	11.87 (6.66-21.15)	8.39	<0.0001	56.8	0.008	0.54
Control type													
Heterogeneous	105	181	15	153	5.35 (2.95-9.71)	5.51	<0.0001	5.38 (2.97-9.74)	5.55	<0.0001	0	0.911	0.00
Autologous	298	275	25	482	18.67 (12.19-28.61)	13.44	<0.0001	16.04 (8.31-30.96)	8.27	<0.0001	51.1	0.038	0.50
Patients sample size													
<50	79	128	8	162	11.76 (5.74-24.09)	6.74	<0.0001	9.92 (2.88-34.17)	3.64	<0.0001	54.2	0.068	1.02
50	324	328	32	473	12.92 (8.78-19.01)	12.98	<0.0001	13.06 (6.59-25.91)	7.35	<0.0001	63.6	0.011	0.53
Publication language													
English	208	217	22	319	12.69 (7.96-20.24)	10.67	<0.0001	12.23 (4.88-30.65)	5.34	<0.0001	68.0	0.014	0.71
Chinese	195	239	18	316	12.58 (7.65-20.70)	9.98	<0.0001	11.62 (5.09-26.50)	5.83	<0.0001	53.7	0.044	0.61

M+, The number of tissues with methylation; M-, The number of tissues with no methylation



**Figure 2. Forest Plot of RASSF1A Promoter Methylation in ESCC Tissues and Normal Tissues**

in ESCC tissue, compared with normal tissue (Figure 2). There were two control styles, nine studies were autogenous control (the tissues from the patients themselves) and three studies were heterogeneous control (the tissues from other non-cancerous individuals). In these studies, only one study was conducted in Japan, others were all conducted in China. Among the 12 included studies, 5 studies were published in English and 7 in Chinese. For the methylation method, 10 studies used methylation-specific polymerase chain reaction (MSP), 1 study used matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS), 1 study used real-time methylation-specific polymerase chain reaction (RT-MSP). The main characteristics of these studies were presented in Table 1.

**Subgroup Analysis and meta-regression**

In the subgroup analysis, the OR in the heterogeneous tissue subgroup was 5.35 (95% CI = 2.95–9.71) under

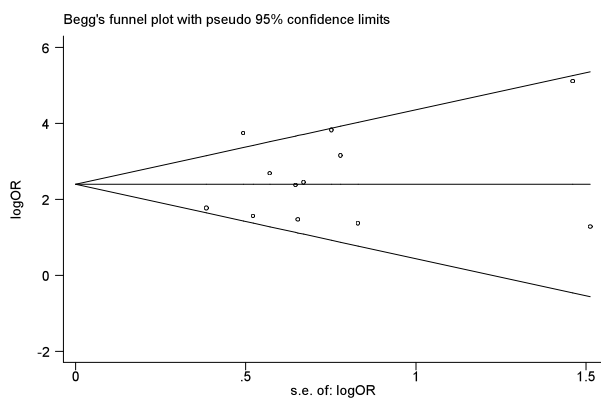
**Table 3. RASSF1A Promoter Methylation in Relation to Gender, TNM Stages, Differentiation Among ESCC Patients**

Patients characteristics	N <sup>a</sup>	Methylation (%)	OR (95%)	Heterogeneity test (I <sup>2</sup> , P value)	Publication bias test (p value)
Gender	6				
Male		50.00	1.17(0.79,1.75)	0.0%, 0.433	0.707
Female		47.85			
TNM stages	7				
I+II		32.13	0.27 (0.10,0.77)	83%, 0.013	1.000
III+IV		59.78			
Differentiation	7				
High+Middle		47.25	0.63(0.27,1.47)	66.7%, 0.287	1.000
Low		56.84			

<sup>a</sup>Number of comparisons

the fixed-effects model and that in the autologous tissue subgroup was 16.04 (8.31-30.96) under the random-effects model. In the subgroup analysis of the patients sample size, the OR for the <50 subgroup was 9.92 (95% CI = 2.88-34.17) and for the 50 case group was 13.06 (95% CI = 6.59-25.91) under the random-effects model. Similarly, the OR for the Publication language subgroup was 12.23 (95% CI =4.88-30.65) in the English subgroup and 11.62 (95% CI =5.09-26.50) in the Chinese subgroup under the random-effects model (Table 2).

Heterogeneity existed across all the included studies (I<sup>2</sup>=56.8%, P=0.008), we therefore conducted meta-regression to estimate potential sources of heterogeneity. The multiple regression model with four variables (such as publication year, control type, patients sample size and publication language) was conducted. As the result, no significant heterogeneity was found.



**Figure 3. The Begg's Funnel Plot for Assessment of Publication Bias in the Meta-analysis**

#### *Associations between RASSF1A promoter methylation and pathologic features in ESCC patients*

We also conducted an analysis of the relationship between pathologic features and RASSF1A promoter methylation among ESCC patients (Table 3). Six studies have sufficient information to perform analysis for gender, and seven studies for differentiation and TNM stage. The gender and differentiation were not found significant associations with RASSF1A methylation. However, there was a relationship between RASSF1A and TNM stages (OR=0.27, 95% CI, 0.10-0.77).

#### *Sensitivity analysis*

To assess the effect of individual study on the pooled estimate, we performed a sensitivity analysis by omitting each study in turn. There was almost no change of the ORs and 95% CIs after each deletion.

#### *Publication bias*

The potential publication bias of literatures was evaluated by Begg's funnel plot and Egger's test. The obvious asymmetry was not found in the Shape of funnel plot among studies investigating RASSF1A promoter methylation and risk of ESCC (Figure 3). And the results of Egger's test didn't not suggest any evidence of publication bias ( $P=0.46$ )

## **Discussion**

Methylation of the RASSF1A promoter is one of the most common methylation events detected in human cancer and leads to silencing of RASSF1A expression. Hypermethylation of RASSF1A was frequently found in most major types of human tumors including lung, breast, prostate, pancreas, kidney, liver, cervical, thyroid and many other cancers (Pfeifer et al., 2005). We therefore performed a meta-analysis to estimate the association between RASSF1A promoter methylation and ESCC.

Our meta-analysis included 12 studies with 859 tumor tissues and 675 controls. The frequencies of RASSF1A methylation ranged from 0.0% to 67.09% (median, 46.92%) in ESCC tissues and 0.0% to 16.13% (median, 5.93%) in the normal tissues, respectively. RASSF1A methylation level of the ESCC group was significantly higher than the control group. The results of our meta-

analysis showed that RASSF1A methylation had an increased risk in tumor tissues (OR = 11.73; 95% CI: 6.59, 20.89) in comparison with non-cancerous tissue. This finding was consistent with other studies (Mao et al., 2013; Zhou et al., 2013).

The subgroup analysis showed that the OR in the heterogeneous tissues was 5.35 (95% CI = 2.95–9.71) and that in the autologous tissues was 16.04 (8.31–30.96). This indicated an increased likelihood of RASSF1A methylation in ESCC cases compared with heterogeneous controls than autologous controls. For patients sample size, the OR for the <50 subgroup was 9.92 (95% CI = 2.88–34.17) and for the 50 case group was 13.06 (95% CI = 6.59–25.91). This result showed that the difference in frequency of RASSF1A promoter methylation between the ESCC tissues and the normal tissues in studies of large sample size was greater than that in studies of small sample size. However, there was no significant difference of the OR in studies published in English and in Chinese. In meta-regression analysis, the factors we conducted, including publication year, control type, patients sample size and publication language, were not identified as sources of heterogeneity.

There were no significant differences between RASSF1A methylation in ESCC tissues and the following pathologic features: gender and differentiation status. However, association was found with TNM stage. Although some previous studies have showed significant difference in methylation status in ESCC and differentiation, the results of our meta-analysis failed to support the existence of such a relationship (Yamaguchi et al., 2005).

To our knowledge, this meta-analysis is firstly available for comprehensively evaluating the associations between RASSF1A promoter methylation and ESCC risk. However, there are also some limitations should demonstrate. First, due to the limited availability of published results, the number of studies included in our meta-analysis was relatively small, and majority of studies that estimated the relationship of RASSF1A promoter methylation and ESCC were conducted in Chinese, while studies in other ethnicity were scarce. Second, although we performed the analysis with strict criteria for study inclusion and precise data extract, significance study heterogeneity existed in all comparisons. Third, although no significant publication bias was found according to Egger's test, negative and unpublished studies may lead to some bias. Otherwise, we only included the studies published in English and Chinese because it was difficult to get the all articles published in various language.

In conclusion, despite the above limitations, RASSF1A promoter hypermethylation was found to be associated with ESCC according to our meta-analysis. Large-scale and well-designed case-control studies are needed to validate the associations identified in the present meta-analysis.

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