Could the methylation of *RASSF1A* be the potential epigenetic biomarker for nasopharyngeal carcinoma? – Systematic review and meta-analysis

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Research article

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Abstract

Background: *RASSF1A* is a tumor suppressor gene. The methylation of *RASSF1A* has been reported to be associated with the nasopharyngeal tumorigenesis. However, the heterogeneity was high among different studies. This meta-analysis aimed to evaluate the value of *RASSF1A* methylation for diagnosing and early screening NPC.

Methods: Relevant articles were identified by searching MEDLINE database. The frequency and Odds ratio (OR) were applied to estimate the effect of *CDH-1* methylation based on random-/fix-effect models. A meta-analysis was performed by using MedCalc® software. The subgroup analyses were performed by test-method, ethnicity, source of NPC samples to determine likely sources of heterogeneity.

Results: Total of 16 studies, included 1,548 samples: 1,095 samples from NPC samples, and 453 from non-cancerous samples, were enrolled in the meta-analysis. The overall frequency of *RASSF1A* methylation were 59.22% and 1.72% in case-group and control-group, respectively. By removing the poor relative studies, the heterogeneity was not observed among included studies. The association between the *RASSF1A* gene methylation and risk of NPC was also confirmed by calculating OR value of 37.74 (95%OR = 20.07-70.98) in fix-effect model (Q = 13.56, p = 0.48, I^2 = 0.00, 95% CI = 0.00-52.19). Additionally, the significant association was also found between the methylation of *RASSF1A* gene and subgroups.

Conclusion: This was the first meta-analysis provided scientific evidences to suggest the *RASSF1A* methylation was the potential diagnosis, prognosis and early screening biomarker for NPC.

Background

Nasopharyngeal carcinoma (NPC), a prevalent nasopharyngeal malignant tumor with the remarkable differences in distribution, gravitating toward Southern Asia [1-7]. According to the data from Global Cancer Observatory, updated to 2018, 129,079 new nasopharyngeal cases were recorded in the world, of which 72,987 nasopharyngeal death cases were occurred. Even though improvements in nasopharyngeal cancer treatment have been achieved, the diagnosis at an advanced stage led to reduce the success rate of treatment as well as the survival of patients. Thus, the early screening and diagnosis represents the beneficial opportunities to increase the survival of patients as well as the effects of nasopharyngeal cancer treatment. Because of the non-specific symptoms related to the early stage of NPC as well as the deeply seated location of nasopharynx, it leads to the major obstacle to early screening of NPC [8]. Therefore, effective biomarkers are truly needed [9]. Today, much efforts have been made for identification of early biomarkers based on focusing the etiological factors led to the nasopharyngeal tumorigenesis. The progression of NPC is a multiple steps associated with multiple factors, including the infection of Epstein-Barr Virus (EBV), environmental factors, as well as genetic and epigenetics alterations [10,11]. Among them, DNA hypermethylation has been postulated as the best-studied epigenetic alterations in tumorigenesis [12]. The phenomenon of tumor suppressor gene's (TSG) promoter cause the

transcriptional silencing, led to gene inactivation, which inhibit the functions of those genes, resulting in the cancer development [13,14].

Beside the detection of EBV DNA in plasma samples was considered as the useful method in screening for early asymptomatic nasopharyngeal carcinoma [15], up to date, many TSGs have been recorded to be involved in the critical cancer-related cellular pathways. Thus, the patterns of TSG promoters' methylation have been studied, and reported to be acted as the potential epigenetic biomarkers for NPC [9,12]. *RASSF1A* (RAS-association domain family 1 isoform A), located 3p21.31, belonged to the family of RAS effectors, is the well described tumor suppressor gene, encodes the scaffold protein member of the C-terminal RASSF family [16]. It functioned as the signaling protein that plays important roles in cell signal transduction through regulating the complex signal networks, includes ERK, Hippo, apoptotic and p53, death receptor signal pathways [17]. The RASSF1A network modulates different biological functions, includes apoptosis, cell cycle, DNA repair process, migration as well as autography [15-17]. The hypermethylation of two CpG islands located at *RASSF1A* gene' promoter mediated by DNA methyltransferase (DNMT), includes DNMT1, DNMT3A-3B, results in its loss of expression [17,18]. Increasing evidences demonstrated this aberrant hypermethylation is one of the most common events in human diseases, includes NPC, through the deregulation of its complex signal networks, and it is thought to be necessary for the development of disease. Increasing evidence indicated that the methylation of *RASSF1A* is strongly associated with NPC, but the value of *RASSF1A* methylation in NPC is uncertain. Challouf et al. reported that the methylation frequency of RASSF1A in NPC was 75.00%, but Tian et al. showed the methylation frequency was 17.5% [31,40]. The high heterogeneity was observed in different studies due to the different sensitivities and intra/inter-assay coefficients of variation of methods, the source of samples as well as the populations. Therefore, in current study, we performed the meta-analysis is to summarize the previously studies and identify the diagnosis, prognosis and early screening value of *RASSF1A* for NPC.

Methods

Search strategy and inclusion/exclusion criteria

We conducted a comprehensive search strategy towards the guidelines of Preferred Reporting Items for Systematics Reviews and Meta-Analyses [21]. By using separation or combination of following keywords: "Nasopharyngeal carcinoma", "methylation", "RASSF1A", "diagnosis", "prognosis", "epigenetic", "hypermethylation", were applied to search reveal published articles in MEDLINE database (Updated on December, 2019). Additional studies were also identified via the references listed in the articles.

Studies were deeply considered eligible only when they met all of the following inclusion criteria: i) The articles were limited to studies written in English; ii) case-control study designed; iii) provided that data about the frequency of *RASSF1A* methylation as well as the sample size in both case and control group. Exclusion criteria were as follows: i) The articles were written in other languages; ii) abstracts, case

reports, letter to editor or unpublished articles were eliminated; iii) studies were related to other tumors and not specific for NPC; iv) studies lacked vital information for analysis.

Data extraction

The eligibility of each study, the relevant data from the eligible studies were independently retrieved by two authors. Disagreements were resolved through discussion within the third author or our research team. The relevant data were extracted from each study according to the data form, including first Author's last name, year of publication, country where the study was performed, sample type, experimental methods to assess the methylation of *RASSF1A*, and number of cases and controls subjects.

Statistical analysis, publication bias and sensitivity analysis

MedCalc® software, by MedCalc Software Ltd, was applied to statistically analyze extracted data (https://www.medcalc.org/). The frequency of *RASSF1A* methylation was calculated in both case and control group. The strength of association between *RASSF1A* methylation and NPC was evaluated by Odds ratio (OR) with 95% confidence intervals (95%CI). In current study, the heterogeneity among the included studies was estimated by the Cochran Q test and I^2 statistics [22]. The cut-off point: p = 0.05 for the Q test and I^2 were used to test the heterogeneity between studies. The scale of I^2 value is classified as following: $I^2 < 25\%$: no heterogeneity, $25\% \leq I^2 \leq 50\%$: moderate heterogeneity, and $I^2 > 50\%$: strong heterogeneity [22-24]. The random-effect model was applied if the heterogeneity among studies existed ($p < 0.05$ for Q test, $I^2 > 50\%$). In the case of no between-study heterogeneity, a fixed-effects model was applied to compute the pooled ORs. In order to determine the presence of publication bias, the symmetry of the funnel plots in which ORs were plotted against their corresponding standard errors were assessed by the Begg's funnel plot and Egger's test ($p < 0.05$) indicates statistically significant [25,26]. Additionally, the sensitivity analysis was also performed by sequential omission of individual studies to evaluate stability of the results.

Results

Study characteristics

After exclusion of studies that not met the inclusion criteria, finally, 16 studies, published from 2001 to 2015, included 1,548 samples: 1,095 samples from NPC samples, and 453 samples from non-cancerous samples, were enrolled in the meta-analysis. The characteristics of included studies of *RASSF1A* methylation and risk of NPC were summarized in Table 1.

The number of NPC samples included studies ranged from 21 to 368 (mean: 68.44 ± 86.50), and number of control samples included studies ranged from 3 to 172 (mean: 30.20 ± 42.35). The patients' ethnicity in 16 studies, comprised of 14 studies from Asian countries: China, Hong Kong, Singapore, Taiwan (counting for 87.50%), and 2 studies from African country: Tunisia (counting for 12.5%). Regarding to the

test-method of *RASSF1A* methylation, thirteen studies used MSP (counting for 81.25%), one study used COBRA (counting for 6.25%), one study used RT-qPCR (counting for 6.25%), and one study used MS-HRM counting for 6.25%).

Meta-analysis: The frequency of *RASSF1A* promoter methylation, and the association between *RASSF1A* gene methylation and NPC

The differences of *RASSF1A* gene methylation between the case-group and control-group in 16 studies, including 1,095 samples from NPC samples, and 453 samples from non-cancerous samples, were accessed. Concerning to the heterogeneity between studies, as there was heterogeneity across studies in the case-group (Q = 259.78, $p < 0.0001$, I^2 = 94.32%, 95%CI for I^2 = 92.04-95.81), and no heterogeneity across studies in the control-group (Q = 6.01, $p = 0.97$, I^2 = 0.00%, 95%CI for I^2 = 0.00-0.00), thus, the random-effect model and fix-effect model were applied to calculate the frequency of *RASSF1A* gene in case-group and control-group, respectively (Fig. 1, Fig. 2). As the results, the frequency of *RASSF1A* gene methylation in case-group and control-group were 59.22% (ranged from 4.88% (95%CI = 0.60-16.53) to 82.76% (95% CI = 64.23-94.15)) and 1.72% (ranged from 0.00 (95%CI = 0.00-7.11) to 4.88% (95% CI = 0.60-16.53)), respectively. The meta-analysis result also indicated that the frequency of *RASSF1A* gene methylation in case-group was significant higher than control group.

In the meta-analysis, no heterogeneity between studies based on the Q-test (Q = 13.56, p = 0.48) and I^2 (I^2 = 0.00%) was observed in the calculation of OR. Therefore, the methylation of *RASSF1A* gene was significantly associated with an increased NPC risk with a pooled of Odds ratio (OR) of 37.74 (95%CI = 20.07-70.98) based on the fix-effect model (Q = 13.56, $p = 0.48$, I^2 = 0.00%, 95%CI = 0.00-52.19) (Fig. 3).

The subgroup analysis, which based on the fix-effect model, was applied to evaluate the *RASSF1A* promoter methylation including ethnicity, test-method, as well as source of sample (p > 0.05 for Q test, I^2 < 50%) (Table 2). With respect to the subgroups categorized by ethnicity, the significantly association between *RASSF1A* methylation and risk of NPC was observed among the Asian country and non-Asian countries in the fix-effect model (Asian countries: OR = 25.71, 95%CI = 14.54-45.46; non-Asian countries: OR = 135.96, 95%CI = 16.03-1,153.41). The subgroup analysis by source of NPC samples, there was a strong association between *RASSF1A* methylation and NPC among the NPC biopsy tissue group, swab sample and other samples, including nasopharyngeal swab, mouth and rinsing fluid, plasma, blood in the fix-effect model (Biopsy tissue: OR = 66.29, 95%CI = 29.20-150.53; Swab sample: OR = 35.21, 95%CI = 6.72-184.36; Others: OR = 5.56, 95%CI = 2.20-14.15). Additionally, significant association between *RASSF1A* methylation and NPC risk among test-method subgroup was found in fix-effects model (MSP: OR = 22.35, 95%CI = 12.23-40.86; Other methods: OR = 73.79, 95%CI = 17.93-303.73).

Sensitivity analysis and publication bias

The sensitivity analysis was done to evaluate the stability and reliability of the conclusions according to the leave-one-out method by excluding one study. As the results, the pooled OR was ranged from 32.80

(95%CI = 17.08-62.99) to 48.83 (95%CI = 24.03-99.22) under the fix-effects model within the I^2 = 0.00-4.67 (*p > 0.05*) (Table 3), which confirmed the stability and reliability of the results. Regarding to the publication bias, the publication bias of the included studies was assessed through the Begg's funnel plot and Egger's test. The shape of funnel plot did not reveal potential asymmetry (Fig. 4, p for Egger's test > 0.05). Therefore, the results, and conclusion of present meta-analysis, which was to evaluate the association between methylation of *RASSF1A* and NPC risk, were stable and reliable.

Discussion

It is important to find out the effective biomarker for early screening of NPC. Based on the etiological factors of NPC, the methylation of TSGs promoter has been recognized as the common mechanism of the inactivation of TSG, leading to the tumorigenesis of NPC [13,43]. According to Xiong et al. (2004), they identified the susceptibility locus at the chromosome 3p21 linked to the NPC tumorigenesis based on the genome-wide linkage analyses of high-risk Chinese NPC patients [44]. *RASSF1A*, belonged this region, is reported as the tumor suppressor gene. The inactivation of *RASSF1A* via the methylation has been recognized as highly associated with the progression of NPC [45,46]. Notably that the methylation of TSGs promoter is reported as one of the earlier molecular modification during the human epithelial cells transformed to malignancy, and often occurred earlier than the changes of morphology of cancer [47]. Hence, the analysis of TSG hypermethylation, including *RASSF1A*, may be served as the potential epigenetic biomarker for early screening of NPC. As previous reports, the frequency of *RASSF1A* methylation in NPC was is highly variable, to determine whether *RASSF1A* methylation could be served as potential biomarkers for NPC, the meta-analysis and the subgroup analysis by ethnicity, source of samples, and test-methods were performed.

To our knowledge, this was the first meta-analysis of previous published studies, revealed that methylation of *RASSF1A* does the increasing of NPC, to evaluate the relationship between *RASSF1A* promoter and NPC tumorigenesis. In this meta-analysis, 1,095 samples from NPC samples, and 453 from non-cancerous samples, were enrolled. As the results, the overall frequency of *RASSF1A* methylation in the NPC and control-group were 59.22% (ranged from 4.88% to 82.76%) and 1.72% (ranged from 0.00 to 4.88%), respectively. It could be observed that the individuals with *RASSF1A* gene methylation was significantly associated with NPC based on the calculation of pooled OR (OR = 37.74; 95%CI = 20.07-70.98) based on the fix-effect model (Fig. 3). The resulting OR indicated that a 37.74-fold increased odds of a positive outcome in the positive of *RASSF1A* methylation, and this increase was statistically significant at the 5% level. Therefore, the methylation of *RASSF1A* was significantly associated with the NPC tumorigenesis. It suggested the methylation of *RASSF1A* might play a crucial role in the pathogenesis of NPC.

By subgroup analysis, the significant association between *RASSF1A* gene methylation and NPC risk were found among in all subgroups, including ethnicity, source of samples and test-method. Regarding to the ethnicity, a significant association between methylation of *RASSF1A* and NPC was found among the Asian region and the Non-Asian region. Additionally, most studies was performed in Asian region (14 of

16 studies), only two studies was done in Africa, thus, one again confirmed the nasopharyngeal cancer is native and posed to Asian region. The subgroup analysis by source of cancer samples revealed a significant correlation in subgroups: biopsy samples, swab samples and other samples. It indicated that the type of biopsy was more suitable to apply to evaluate the methylation of *RASSF1A* gene. However, the source of other samples, including nasopharyngeal swab, mouth and rinse, plasma – type of non/less-invasive source of sample, could be reflect alterations in the NPC and facility of collecting NPC samples led it a potential biomarker for diagnosis and early screening of NPC. Therefore, it should be focused on in the future to find out the non/less-invasive biomarker for NPC. The MSP method was used in thirteen studies (counting for 81.25%). It could be explained MSP is the “gold standard method” of evaluation of methylation. The MSP shows the useful tool for the qualitative DNA methylation analysis within the ease of design and execution, sensitivity in the ability to detect small quantities of methylated DNA [48]. Moreover, in which MSP products are run on a gel, and the results are reported as methylated or unmethylated at the target DNA sequence [49]. In recent year, the method of HS-HRM has been developed to detect the methylation status of *RASSF1A* in clinical samples, due to the cost-effective method for the reliable quantification of DNA methylation by unique primer, which are enable to detect 0.1–1% methylated alleles in an unmethylated background [50]. Overall, these results are in accordance with that documented *RASSF1A* gene methylation to be the common and early epigenetic event in the progression of NPC tumorigenesis. So its epigenetic event might could be used as the potential epigenetic biomarker for diagnosis and early screening of NPC. However, the current meta-analysis exhibited some limitations due to the number of current enrolled studies of 16, the data of non-English language studies may contribute to some bias, as well as the evaluation of the correlation between methylation of *RASSF1A* gene and clinicopathological features, age and the TNM stage which are differences in *RASSF1A* methylation between cases and controls.

Conclusion

This meta-analysis highlighted the significant association between the methylation of *RASSF1A* gene and risk of NPC based on the evaluation of frequency of both case-group, counting for 59.22% and control-group, counting for 1.72%, as well as OR value of 37.74. Additionally, our findings underscore the correlation among *RASSF1A* gene methylation and all subgroups, including region, source of samples, and test-method. The finding in present meta-analysis emphasized that the methylation of *RASSF1A* gene was recorded as the early epigenetic event in the progression of NPC tumorigenesis based on the literature-based meta-analysis. Here, we reported that the *RASSF1A* methylation was the potential diagnosis, prognosis and screening biomarker for NPC.

Declarations

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City, Vietnam.

Author contribution

TDL conceived, designed, performed, analyzed data, wrote the draft manuscript and acted as primary author for the project, HHT, DHN contributed to perform and analyze data. TAHL designed, performed, analyzed data, oversaw the research and edited the article. All authors have read and approved the manuscript.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Ethics approval and consent to participate

This is an observation and review study, no ethics and consent applied

Consent for publication

Not applicable

Competing interests

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article

Abbreviations

COBRA: Combined bisulfite restriction analysis

MMSP: Multiplex methylation-specific PCR

MS-HRM: Methylation-Sensitive High Resolution Melting

MSP: Methylation-specific PCR

NPC: Nasopharyngeal carcinoma

OR: Odds ratio

RASSF1A: RAS-association domain family 1 isoform A

RT-qPCR: Real-time quantitative PCR

TSG: tumor suppressor gene

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Tables

Table 1. The characteristics of studies included in the meta-analysis of *RASSF1A* methylation and risk of NPC

Author, Reference	Year	Region	Case		Control		Method	Source of	
			N	P	N	P		Case	Control
Chow et al. [27]	2004	Asia	26	20	-	-	COBRA	B	-
Chang et al. [28]	2003	Asia	30	20	6	0	MSP	B	B
			30	10	37	0	MSP	S	S
			30	11	43	0	MSP	MT	MT
			30	1	43	1	MSP	Pl	Pl
			30	0	43	1	MSP	Bf	Bf
Kwong et al. [29]	2002	Asia	29	24	6	0	MSP	B	B
Wong et al. [30]	2004	Asia	41	2	43	0	RT-qPCR	Pl	Bl
Challouf et al. [31]	2012	Africa	36	27	19	0	MSP	B	B
Zhou et al. [32]	2005	Asia	28	23	8	0	MSP	B	B
Wang et al. [33]	2009	Asia	38	27	14	0	MSP	B	B
Hutajulu et al. [34]	2011	Asia	53	40	25	1	MSP	B	S
Fendri et ai. [35]	2009	Africa	68	62	9	0	MSP	B	B
Tong et al. [36]	2002	Asia	28	11	12	0	MSP	S	S
	2002	Asia	16	11	-	-	MSP	B	-
Zhang et al. [37]	2012	Asia	49	39	20	0	MSP	B	B
	2012	Asia	49	29	20	0	MSP	S	S
Wong et al. [38]	2003	Asia	28	13	5	0	MSP	B	B
Qiu et al. [39]	2004	Asia	27	20	3	0	MSP	B	B
Tian et al. [40]	2013	Asia	40	7	41	2	MSP	Bl	Bl
Lo et al. [41]	2001	Asia	21	14	6	0	MSP	B	B

Yang et al. [42]	2015	Asia	52	36	50	0	HS-HRM	B	Pl
			96	66	-	-	HS-HRM	S	
			220	53	-	-	HS-HRM	Pl	

Note: MSP: Methylation-specific PCR; COBRA: combined bisulfite restriction analysis; HS-HRM: Methylation-sensitive high resolution melting; RT-qPCR: Real-Time Quantitative PCR;B: tumor biopsy; Pl: plasma, Bf: buffy coat; MT: Mouth-throat rinsing fluid; S: nasopharyngeal swab; Bl: blood; -: not recorded; P: positive; N: total samples

Table 2. Summary of subgroup analysis in meta-analysis of *RASSF1A* methylation and NPC risk

Group	Case		Control		Model, OR, 95% CI (Fix-effects model)	Heterogeneity	
	N	P	N	P		I^2 (%)	p
Ethinicity							
Asia	633	327	425	5	25.71, 14.54 - 45.46	25.13	*0.16*
Non-Asia	104	89	28	0	135.96, 16.03 - 1153.41	0.00	*0.82*
Sources of sample							
Biopsy sample	459	345	171	1	66.29, 29.20 - 150.53	0.00	*0.95*
Swab sample	107	50	69	0	35.21, 6.72 - 184.36	0.00	*0.83*
Others	171	21	213	4	5.56, 2.20 - 14.15	28.03	*0.25*
Test-methods							
MSP	546	310	320	5	22.35, 12.23 - 40.86	24.98	*0.18*
Other test-method	191	106	133	0	73.79, 17.93 - 303.73	16.85	*0.31*

Table 3. Sensitivity analysis of methylation of *RASSF1A* and NPC risk by the fix-effects model

	OR, 95% CI	Heterogeneity	
		I^2, 95% CI	*p*
Omitting Chang et al. 2003	38.94, 15.15 - 65.49	4.67, 0.00 - 57.21	*0.40*
Omitting Kwong et al. 2002	37.27, 19.59 - 70.91	3.26, 0.00 - 56.58	*0.41*
Omitting Wong et al. 2004	40.16, 20.98 - 76.88	0.00, 0.00 - 52.23	*0.51*
Omitting Challouf et al. 2012	35.78, 18.73 - 68.34	0.00, 0.00 - 54.39	*0.46*
Omitting Zhou et al. 2005	36.96, 19.41 - 70.34	2.22, 0.00 - 56.11	*0.43*
Omitting Wang et al. 2009	36.69, 19.22 - 70.02	2.13, 0.00 - 56.07	*0.43*
Omitting Hutajulu et al. 2011	35.81, 18.52 - 69.22	0.00, 0.00 - 54.91	*0.45*
Omitting Fendri et al. 2009	35.91, 18.86 - 68.34	0.00, 0.00 - 52.34	*0.51*
Omitting Tong et al. 2002	38.54, 20.17 - 73.65	4.11, 0.00 - 56.96	*0.41*
Omitting Zhang et al. 2012	32.80, 17.08 - 62.99	0.00, 0.00 - 50.64	*0.54*
Omitting Wong et al. 2003	39.78, 20.80 - 76.07	0.00, 0.00 - 54.79	*0.46*
Omitting Qiu et al. 2004	38.43, 20.16 - 73.25	3.24, 0.00 - 56.57	*0.42*
Omitting Tian et al. 2013	48.83, 24.03 - 99.22	0.00, 0.00 - 8.95	*0.93*
Omitting Lo et al. 2001	38.26, 20.05 - 72.98	3.98, 0.00 - 56.90	*0.41*
Omitting Yang et al. 2015	34.74, 18.42 - 65.50	0.38, 0.00 - 55.29	*0.44*

Figures

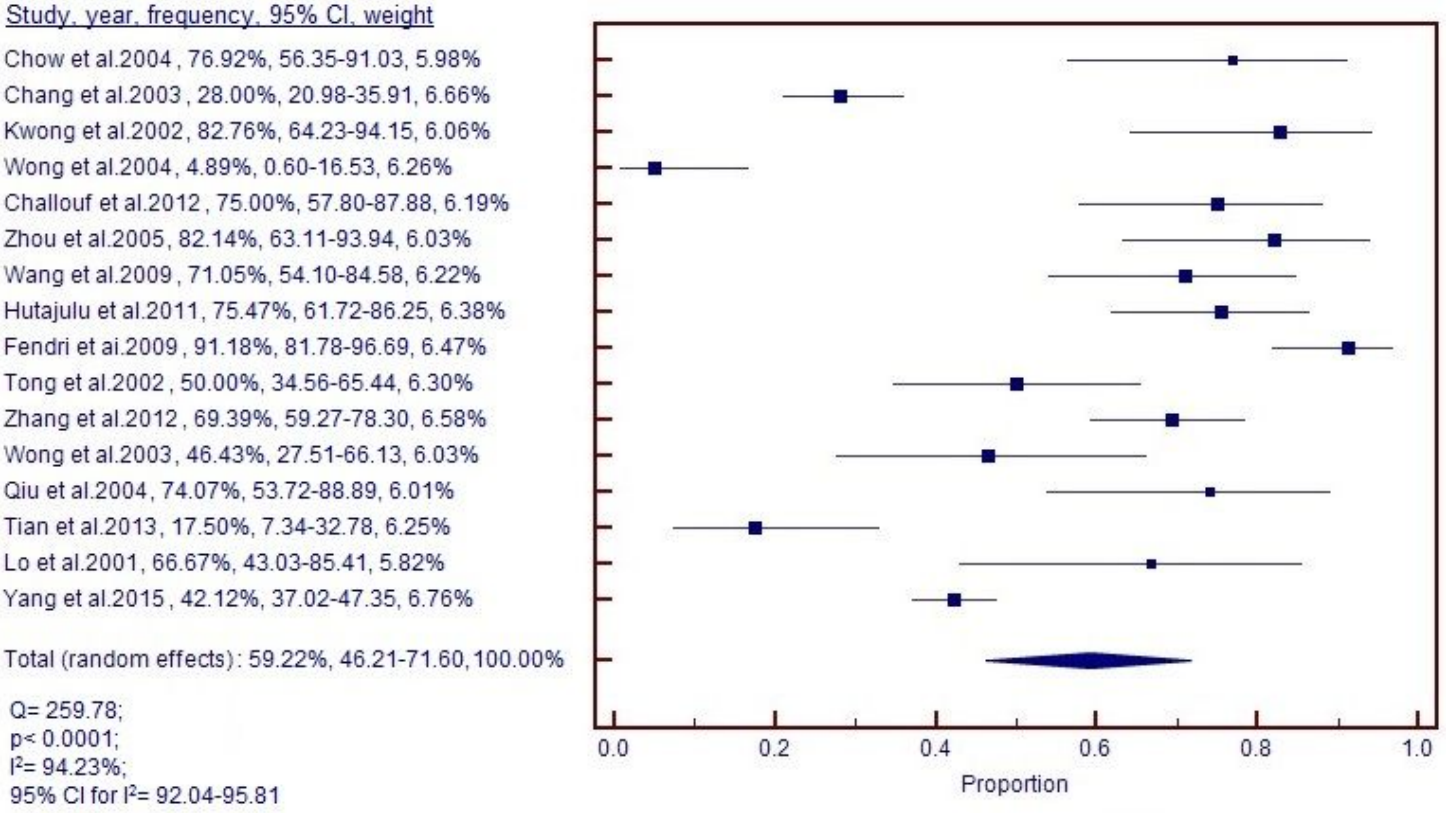

Figure 1

Forest plot of frequency of RASSF1A gene methylation detected in NPC samples

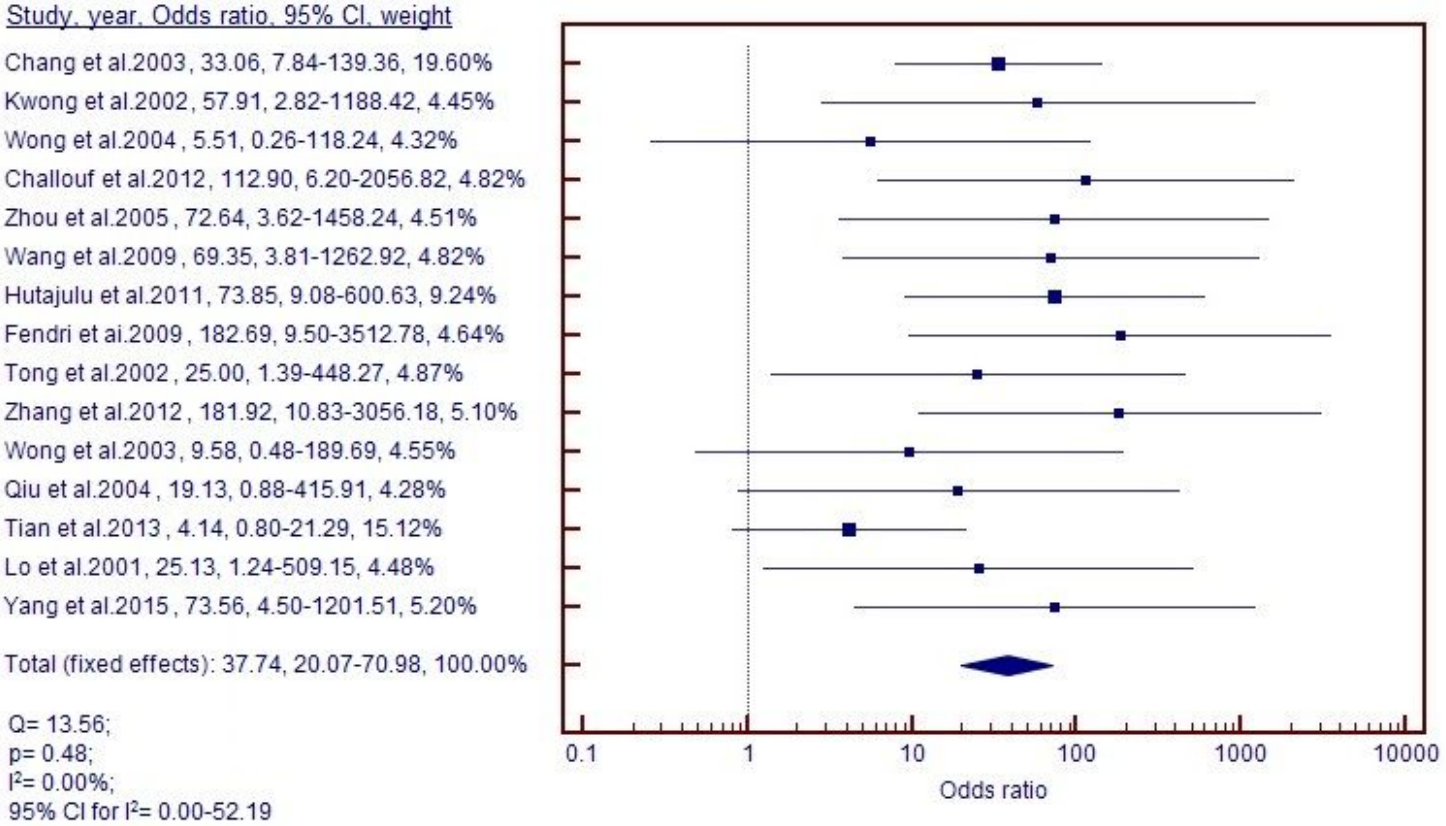

Figure 2

Forest plot of frequency of RASSF1A gene methylation detected in control samples

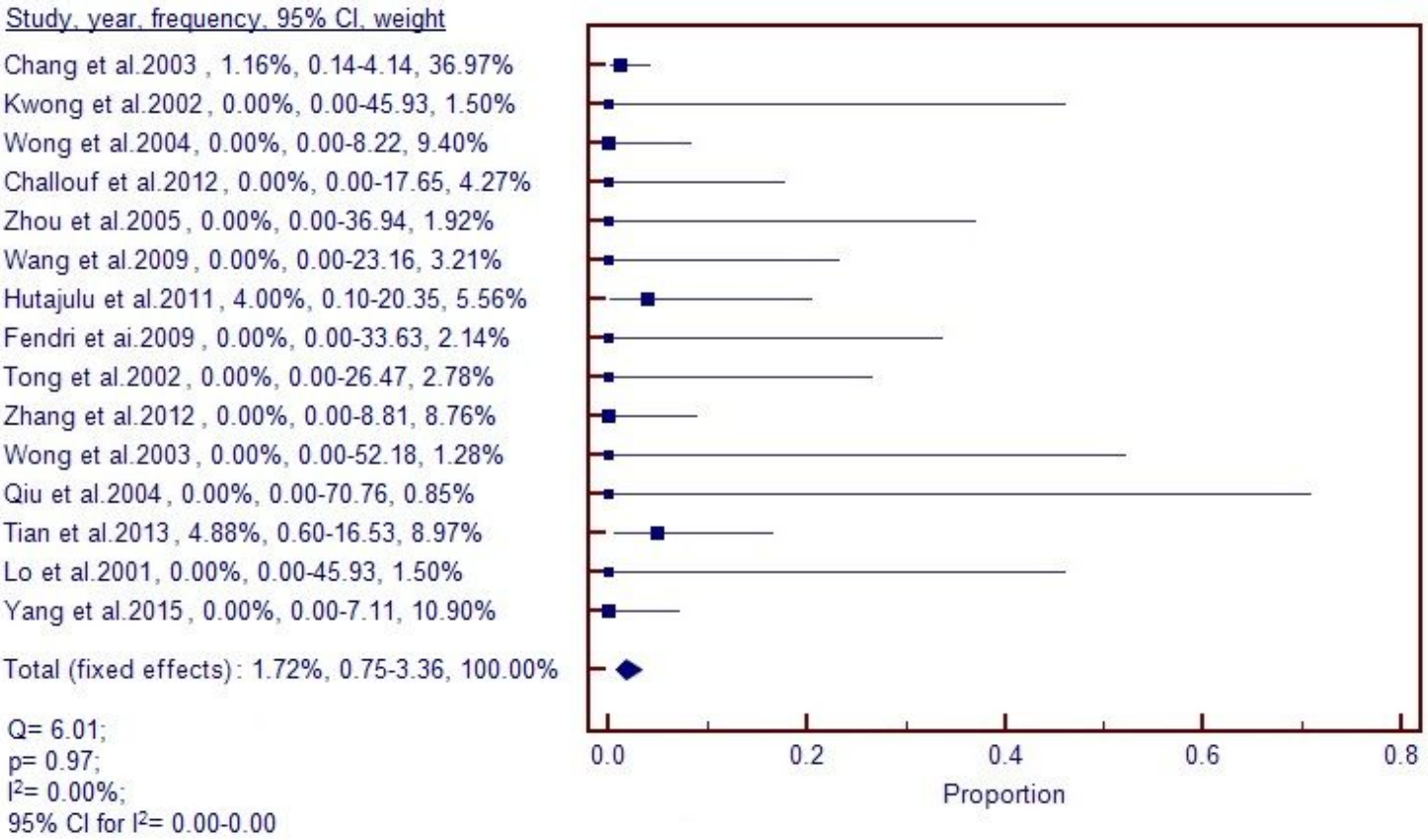

Figure 3

Forest plot of RASSF1A methylation and NPC risk using fix-effects model.

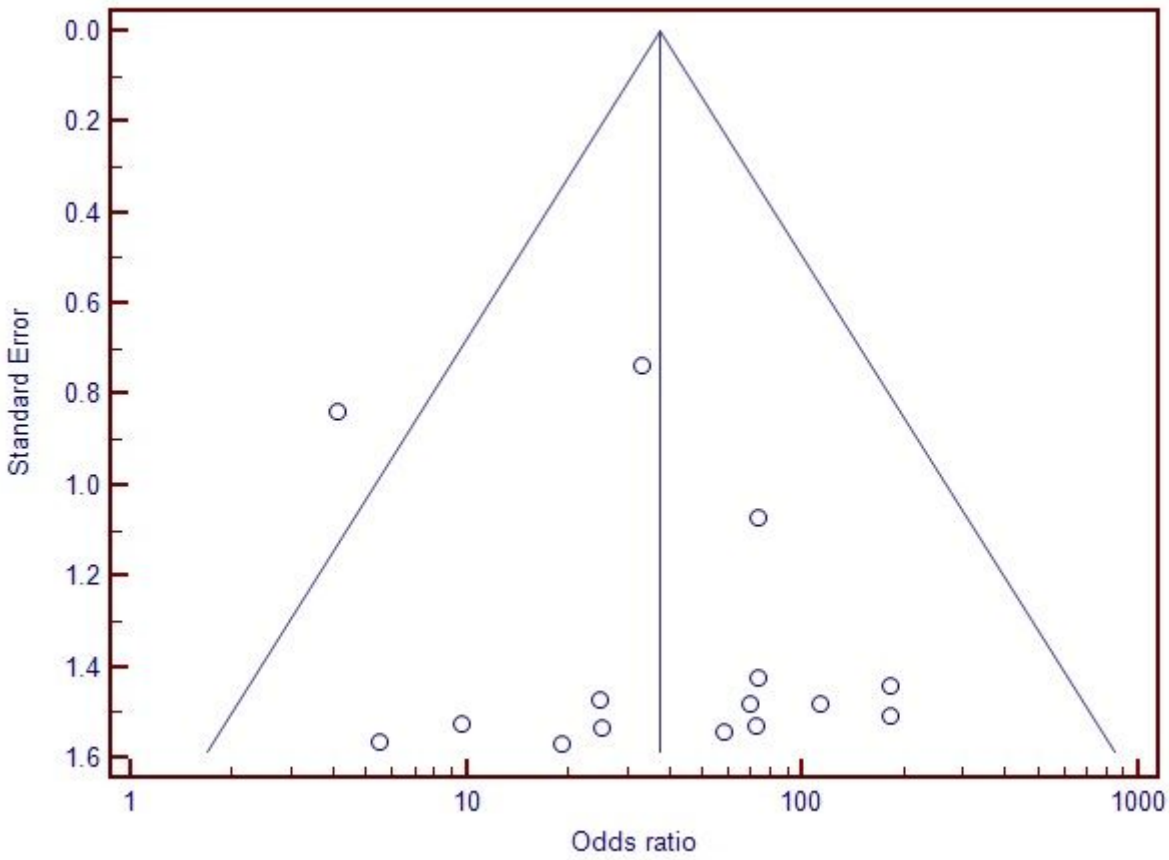

Figure 4

Funnel plot of RASSF1A methylation and NPC risk based on the fix-effects model