Original Article

Promoter methylation of *p16* and *DAPK* genes in brushing, blood, and tissue samples from patients with nasopharyngeal carcinoma: a systematic meta-analysis

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Background: Promoter methylation of tumor suppressor genes (TSGs) *p16* and death-associated protein kinase (*DAPK*) has been reported in various carcinomas. However, the correlation between *p16* or *DAPK* promoter methylation and the detection of nasopharyngeal carcinoma (NPC) in different sample types remains unclear.

Methods: A systematic literature research was conducted to identify available studies. The pooled odds ratios (ORs) and their 95% confidence intervals (CIs) were calculated and analyzed.

Results: Eleven eligible papers on *p16* promoter methylation and seven eligible papers on *DAPK* promoter methylation were included in this analysis. The pooled OR of *p16* and *DAPK* promoter methylation was significantly higher in samples of NPC tissues than in nontumorous (OR =5.49, P<0.001; OR =17.51, P<0.001; respectively). Moreover, the pooled OR of *p16* promoter methylation was significantly higher in NPC than in normal blood and noncancerous brushing samples (OR =19.37, P<0.001; OR =15.03, P<0.001; respectively). In NPC samples, the OR of *DAPK* promoter methylation were significantly higher than in normal blood and normal brushing samples (OR =7.23, P<0.001; OR =42.00, P=0.001; respectively). No significant correlation was found between *p16* or *DAPK* promoter methylation and clinical stage (P>0.1).

Conclusions: Our findings suggested that *p16* and *DAPK* promoter methylation may be associated with the carcinogenesis of NPC. Promoter methylation of *p16* or *DAPK* may become a noninvasive biomarker for NPC detection in blood and brushing samples. *DAPK* or *p16* promoter methylation was not correlated with tumor stage. Conducting additional studies is essential in the future to confirm our results.

Keywords: *p16*; death-associated protein kinase (*DAPK*); methylation; nasopharyngeal carcinoma (NPC); biomarker; blood; brushing

Submitted Aug 18, 2016. Accepted for publication Oct 31, 2016.
doi: 10.21037/tcr.2016.12.08
View this article at: http://dx.doi.org/10.21037/tcr.2016.12.08

Introduction

Nasopharyngeal carcinoma (NPC) is a unique epithelial cancer of the head and neck with an extremely geographical and ethnic distribution. NPC is a prevalent malignancy in Southeast Asia, where it occurs in 20–30 per 100,000 persons every year; it is particularly prevalent in Southern China and ranges from 15 to 50 cases per 100,000 individuals, whereas it is rare in the Western countries, with an annual incidence rate of less than 1 per 100,000 people (1,2). Radiotherapy alone or in combination with chemotherapy has been used as the primary treatment strategy, providing a generally satisfactory disease control in patients with early-stage NPC (3). However, approximately 60–70% of the patients are diagnosed at later stages (III–IV)

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with loco-regional lymph node metastases (4). Currently, distant metastasis is considered a typical failure pattern, particularly in advanced patients, with poor prognosis (5-7).

Previous studies have shown that certain etiological factors contribute to the initiation and development of NPC, including infection with Epstein-Barr virus, inflammation, dietary and lifestyle factors, and genetics and epigenetic alterations (8-11). Aberrant DNA methylation of tumor suppressor genes (TSGs), such as deleted in liver cancer 1 (*DLC1*), is a frequent event in NPC (12,13). The human *p16* gene, mapped to chromosome 9p21 and consisting of 3 exons and 2 introns, is a cyclin-dependent kinase inhibitor that plays a key role in cell cycle regulation (14,15). Localized on human chromosome 9p34, death-associated protein kinase (*DAPK*), a serine/threonine kinase, participates in the modulation of a variety of cellular processes, including apoptosis, autophagy, and inflammation (16,17). The loss of *p16* and *DAPK* expression as TSGs through promoter methylation has been shown to be associated with many carcinomas (18-20).

Nonetheless, there are conflicting findings concerning the relationship between p16 promoter methylation and NPC. For example, Challouf *et al.* reported the absence of significant association between *p16* promoter methylation and NPC (21), whereas the results of Nawaz *et al.* evidenced the presence of a significant association between *p16* promoter methylation and NPC (22). Similarly, there are controversial results on *DAPK* promoter methylation and its relation to NRC occurrence. For instance, Chang *et al.* established that the *DAPK* promoter methylation rate in NPC patients was similar to or even lower than that in healthy subjects (23). In contrast, the investigation of Tian *et al.* revealed that the level of *DAPK* promoter methylation was significantly higher in NPC cases than in healthy subjects (24). It is important to note that studies with a small sample size may lack strong statistical power (25,26). Therefore, to address these inconsistencies and evaluate the relationship between *p16* and *DAPK* promoter methylation and NPC, basing on several selection criteria, we integrated all eligible publications on this subject. In addition, we also determined whether *p16* and *DAPK* promoter methylation was linked with the clinical stage of NPC.

Methods

Search strategy and selection criteria

We conducted a comprehensive literature search of four online electronic databases, including PubMed, EBSCO, Cochrane Library, and EMBASE, to identify eligible studies published before August 8th, 2016. We used the following combination of keywords and free words: 'nasopharyngeal cancer OR nasopharyngeal neoplasm OR nasopharyngeal carcinoma OR nasopharyngeal tumor OR NPC', 'p16 OR INK4A OR CDKN2A OR cyclin-dependent kinase inhibitor 2A', 'DAPK OR death-associated protein kinase OR DAP-kinase', 'methylation OR hypermethylation OR promoter methylation OR epigenetic'.

To be included in our meta-analysis, the articles had to meet the following inclusion criteria: (I) the diagnosis of NPC was based on histopathological examination; (II) case-control or cohort design studies published in English; (III) studies provided sufficient information with regard to the methylation rate of *p16* or *DAPK* promoter methylation to calculate the pooled odds ratios (ORs) and corresponding 95% confidence intervals (CIs); (IV) when identical or overlapping data were used in multiple publications, only the most recent paper or the paper with the largest population was included; (V) the sample type was without restriction and included tissue, blood, and brushing samples from patients with NPC. A tumor stage of ≤1 was considered as an early stage, whereas a tumor stage of ≥2 was defined as a later stage.

Data extraction

Two independent reviewers extracted the following data from each available study using selection standards, including the last name of the first author, publication year, country, ethnicity, sample type, the participants of cases and controls, method for detection of methylation, the frequency of *p16* or *DAPK* promoter methylation, *p16* or *DAPK* expression status. Nontumorous specimens were used as control samples. Any disagreements concerning study selection and data extraction were resolved by consensus or by a third reviewer.

Statistical analysis

In the current meta-analysis, statistical analysis of the pooled data was performed using Stata software, version 12.0 (STATA Corp., College Station, TX, USA). The pooled OR and corresponding 95% CI were calculated to determine the strength of the association between *p16* or *DAPK* promoter methylation and NPC risk. In

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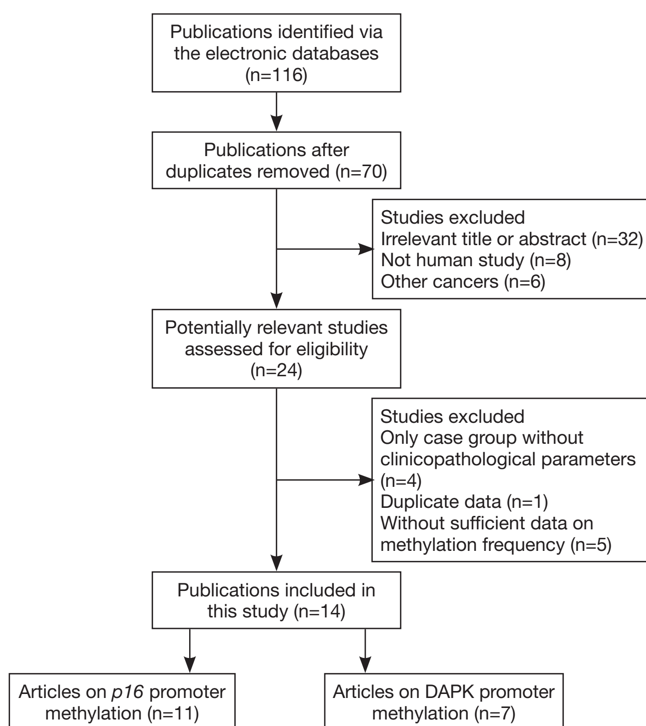

Figure 1 Flow diagram of the literature selection process.

addition, the correlation between *p16* or *DAPK* promoter methylation and the clinical stage of NPC was also established. The statistical heterogeneity among the studies was tested based on the Cochran's Q and I^2 tests (27). The random-effects model was employed when the *P value* was less than 0.1 in the Q-test, indicating the presence of substantial heterogeneity; otherwise, the fixed-effects model was applied when no evidence of the heterogeneity was observed (28,29). *P* value <0.05 was considered statistically significant.

Results

Study characteristics

Initially, a total of 116 potential articles were collected by an extensive search of the databases used (*Figure 1*). After selection based on the eligibility criteria, , eleven available articles on *p16* promoter methylation (21-26,30-34) and seven eligible articles on *DAPK* promoter methylation (21,23,24,34-37) were eventually identified in this analysis. Seven of these studies evaluated the association between *p16* promoter methylation and NPC in NPC *vs.* nontumorous tissues (21-23,25,26,31,34). Three studies evaluated the relationship between *p16* promoter methylation and NPC in NPC *vs.* normal blood samples (23,24,32). In addition, three studies estimated the connection between *p16* promoter methylation and NPC in NPC *vs.* nontumorous brushing samples (23,30,33). Six other studies assessed the correlation between *DAPK* promoter methylation and NPC in NPC *vs.* nontumorous tissues (21,23,34-37), and two studies evaluated the association between *DAPK* promoter methylation and NPC in NPC *vs.* normal blood samples (23,24). The relationship between *DAPK* promoter methylation and NPC in NPC *vs.* normal brushing samples was estimated in one study (23) and the correlation of *p16* promoter methylation with tumor stage in NPC in four (24,31-33). Two studies assessed the association of *DAPK* promoter methylation with tumor stage in NPC (24,37). *Table 1* presents the main characteristics of the included studies.

Correlation of p16 promoter methylation in cancer vs. controls

No obvious heterogeneity was observed for *p16* promoter methylation in cancer *vs.* controls (P value of the heterogeneity >0.1), and the fixed-effects model was applied. A higher frequency of *p16* promoter methylation in NPC than in nontumorous tissues (OR =5.49; 95% CI, 2.39–12.63; P<0.001) was established in seven studies with samples from 244 NPC and 61 nontumorous tissues (*Figure 2*).

We also evaluated the association between *p16* promoter methylation and the risk of NPC in fluid samples (blood and brushing samples). The results from three studies including 111 NPC and 127 normal blood samples demonstrated that *p16* promoter methylation in NPC samples was notably higher than in normal blood samples (OR =19.37; 95% CI, 4.45–84.26; P<0.001) (*Figure 2*). Three other studies with 111 NPC and 102 noncancerous brushing samples evidenced that *p16* promoter methylation was significantly higher in NPC samples than in noncancerous brushing samples (OR =15.03; 95% CI, 5.92–38.17; P<0.001) (*Figure 2*).

Therefore, our results revealed that *p16* promoter methylation was significantly correlated with the increased risk of NPC in tissue, blood, and brushing specimens.

Correlation of DAPK promoter methylation in cancer vs. controls

There was no substantial evidence of heterogeneity in

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Table 1 The general characteristics of eligible studies

Gene	First author	Country	Ethnicity	Method	Sample	Control Sample	Case, N (M, %)	Control, N (M, %)	Stage 3–4, N [M]	Stage 1–2, N [M]	Expression
p16	Lo 1996 (26)	China	Asians	MPCR	Tissue	Normal	27 (22.2)	4 (0)	–	–	Loss
	Tong 2002 (33)	China	Asians	MSP	Brushing	Non-tumor	28 (46.4)	12 (0)	17 [7]	11 [6]	ND
	Kwong 2002 (34)	China	Asians	MSP	Tissue	Normal	33 (51.5)	6 (0)	–	–	ND
	Chang 2003 (23)	China	Asians	MSP	Blood	Normal	30 (0)	43 (0)	–	–	ND
	Chang 2003 (23)	China	Asians	MSP	Brushing	Normal	30 (16.7)	43 (0)	–	–	ND
	Chang 2003 (23)	China	Asians	MSP	Tissue	Normal	30 (33.3)	6 (0)	–	–	ND
	Wong 2003 (25)	China	Asians	MSP	Tissue	Normal	30 (23.3)	5 (0)	–	–	ND
	Wong 2004 (32)	China	Asians	MethyLight	Blood	Normal	41 (41.5)	43 (2.3)	24 [12]	17 [5]	ND
	Ayadi 2008 (31)	Tunisia	Caucasians	MSP	Tissue	Normal	44 (61.4)	3 (0)	32 [20]	12 [7]	ND
	Hutajulu 2011 (30)	The Netherlands	Caucasians	MSP	Brushing	Non-tumor	53 (66)	47 (12.8)	–	–	ND
	Challouf 2012 (21)	Tunisia	Caucasians	MSP	Tissue	Non-tumor	36 (33.3)	19 (21.0)	–	–	ND
	Tian 2013 (24)	China	Caucasians	MSP	Blood	Normal	40 (22.5)	41 (2.4)	36 [7]	4 [2]	ND
	Nawaz 2015 (22)	Sweden	Caucasians	MSP	Tissue	Non-tumor	44 (45.5)	18 (0)	–	–	ND
DAPK	Kwong 2002 (34)	China	Asians	MSP	Tissue	Normal	33 (72.7)	6 (0)	–	–	ND
	Wong 2002 (37)	China	Asians	MSP	Tissue	Normal	32 (75.0)	3 (0)	25 [18]	7 [6]	ND
	Chang 2003 (23)	China	Asians	MSP	Blood	Normal	30 (3.3)	43 (2.3)	–	–	ND
	Chang 2003 (23)	China	Asians	MSP	Brushing	Normal	30 (50.0)	43 (2.3)	–	–	ND
	Chang 2003 (23)	China	Asians	MSP	Tissue	Normal	30 (76.7)	6 (0)	–	–	ND
	Kong 2006 (36)	China	Asians	MSP	Tissue	Non-tumor	46 (76.1)	6 (0)	–	–	Loss
	Fendri 2009 (35)	Tunisia	Caucasians	MSP	Tissue	Normal	68 (88.2)	9 (0)	–	–	Loss
	Challouf 2012 (21	Tunisia	Caucasians	MSP	Tissue	Non-tumor	36 (47.2)	19 (15.8)	–	–	ND
	Tian 2013 (24)	China	Caucasians	MSP	Blood	Normal	35 (51.4)	41 (9.8)	36 [16]	4 [2]	ND

DAPK, death-associated protein kinase; “-” stands for no data; MSP: methylation-specific polymerase chain reaction; MPCR, multiplex PCR; M, methylation positive status; N, the number of methylation and unmethylation; ND, not done.

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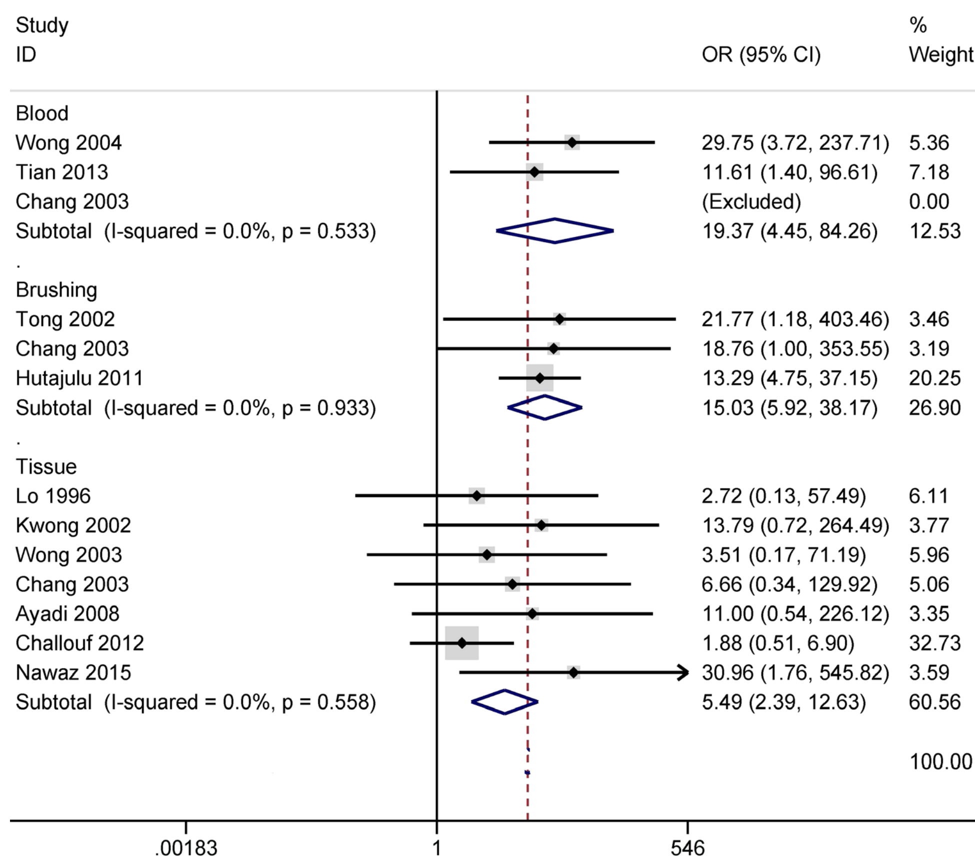

Figure 2 Forest plot for the correlation of *p16* promoter methylation the risk of NPC showing the pooled OR obtained by the fixed-effects model in cancer vs. controls. Tissue: OR =5.49, P<0.001, including seven studies with 244 NPC and 61 nontumorous tissues; blood: OR =19.37, P<0.001, including three studies with 111 NPC and 127 normal blood samples; brushing: OR =15.03, P<0.001, including three studies with 111 NPC and 102 noncancerous brushing samples.

cancer *vs.* controls; thus, the fixed-effects model was used for *DAPK* promoter methylation (P value of the heterogeneity >0.1). The results concerning six studies with 245 NPC and 49 nontumorous tissue samples indicated that a higher *DAPK* promoter methylation rate was observed in NPC tissues in comparison to that in nontumorous tissues (OR =17.51; 95% CI, 7.08–43.32; P<0.001) (*Figure 3*).

Additionally, the pooled OR of two studies with 65 NPC and 84 normal blood samples showed that *DAPK* promoter methylation was significantly greater in NPC than in normal blood samples (OR =7.23; 95% CI, 2.44–21.45; P<0.001) (*Figure 3*). The combined OR in one study exhibited a significant association between *DAPK* promoter methylation and NPC based on the results from the analysis of 30 NPC and 43 normal brushing samples (OR =42.00; 95% CI, 5.10–345.85; P=0.001) (*Figure 3*).

Our findings revealed that the methylation status of *DAPK* promoter was significantly associated with the increased risk of NPC in tissue, blood, and brushing specimens. However, the analyses of brushing and blood samples should be cautiously interpreted as only one or two studies with small sample sizes were involved in this meta-analysis.

Subgroup analysis by ethnic population in cancer vs. nontumorous tissues

Further, we conducted subgroup analysis by ethnicity to evaluate the different degree of association of the stratified population in tissue samples. Subgroup analysis based on ethnicity showed that *p16* promoter methylation was significantly correlated with an increased risk of NPC in both among both populations investigated, Asians (OR =5.90; 95% CI, 1.35–25.82; P=0.018) and Caucasians

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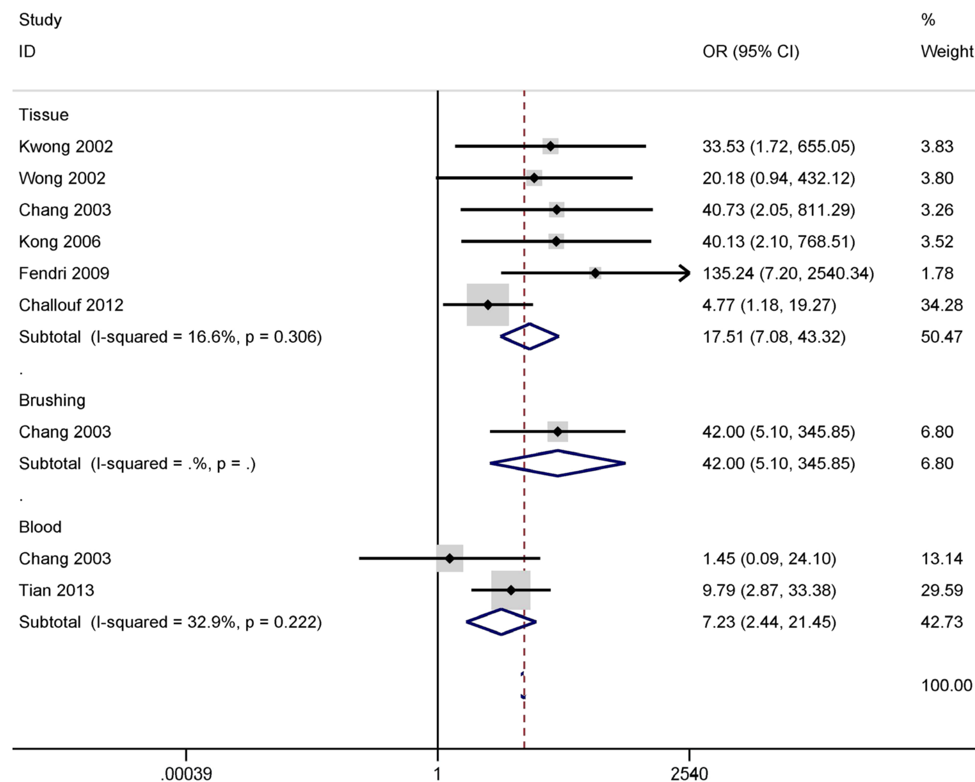

Figure 3 Forest plot for the association of *DAPK* promoter methylation with the risk of NPC displaying the pooled OR under the fixed-effects model in cancer *vs.* controls. Tissue: OR =17.51, P<0.001, including 6 studies with 245 NPC and 49 nontumorous tissues; blood: OR =7.23, P<0.001, including 2 studies with 65 NPC and 84 normal blood samples; brushing: OR =42.00, P=0.001, including 1 study with 30 NPC and 43 normal brushing samples.

(OR =5.28; 95% CI, 1.93–14.43; P=0.001) (*Figure 4*).

DAPK promoter methylation also was significantly correlated with the increased risk of NPC in the Asian (OR =33.25; 95% CI, 7.48–147.89; P<0.001) and the Caucasian population (OR =11.21; 95% CI, 3.58–35.09; P<0.001) (*Figure 5*). However, the results of subgroup analysis should be carefully considered as only small sample sizes were included in this study, especially in the Caucasian population subgroup.

Correlation of p16 or DAPK promoter methylation with tumor stage in NPC

We also determined whether *p16* or *DAPK* promoter methylation was correlated with tumor stage in NPC using the fixed-effects model (*Figure 6*). The overall OR from four studies involving 109 advanced NPC patients and 44 early NPC patients demonstrated that *p16* promoter methylation was not correlated with tumor stage (OR =1.06; 95% CI, 0.51–2.20; P=0.876). The overall OR from two other studies including 61 advanced NPC patients and 11 early NPC patients indicated that *DAPK* promoter methylation was not associated with tumor stage (OR =0.59; 95% CI, 0.13–2.65; P=0.491). Nonetheless, the results regarding the relationship between *p16* or *DAPK* promoter methylation and tumor stage should be cautiously interpreted as only a small sample size of NPC patients were analyzed in the current research.

Discussion

The hypermethylation of TSGs and hypomethylation of oncogenes are two important molecular mechanisms in epigenomic regulation that play key roles in the initiation and progression of cancer (38-40). The promoter methylation of TSGs may affect cell proliferation, cell death, cell migration, and cell invasion (41). Aberrant promoter methylation of *p16* and *DAPK* genes has been

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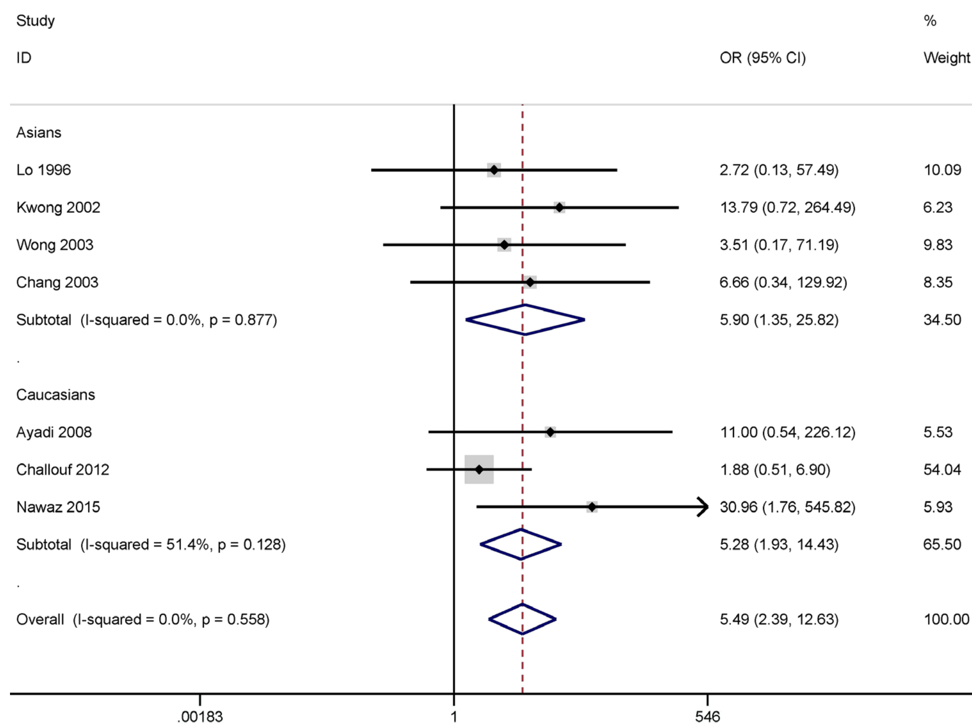

Figure 4 Forest plot for the correlation of *p16* promoter methylation with the risk of NPC by ethnic subgroup illustrating the pooled OR under the fixed-effects model in cancer *vs*. controls. Asians: OR =5.90, *P*=0.018; Caucasians: OR =5.28, *P*=0.001.

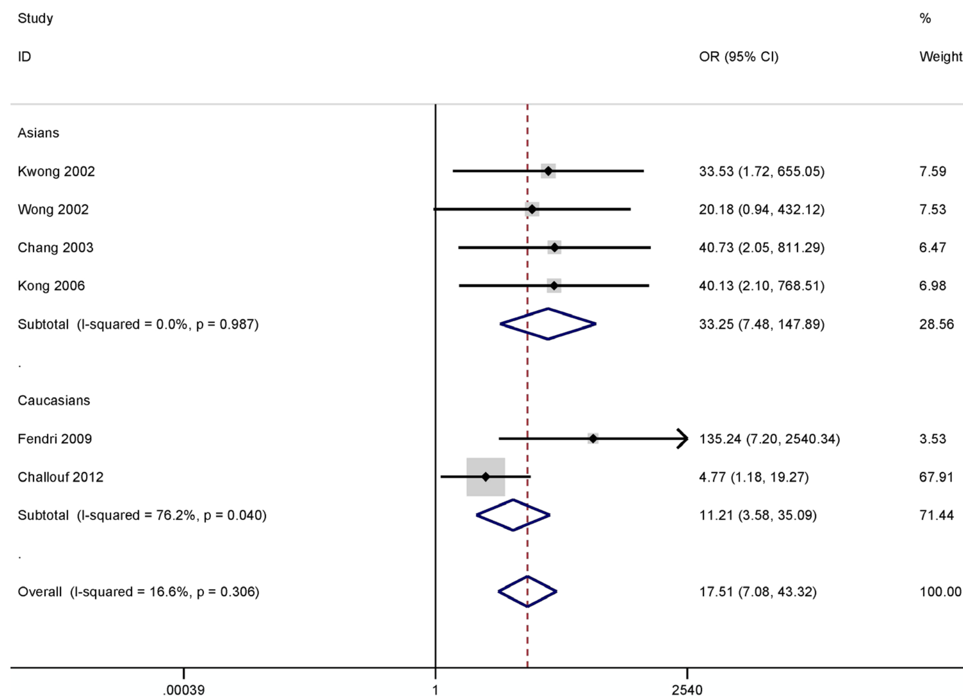

Figure 5 Forest plot for the correlation of *DAPK* promoter methylation with the risk of NPC by ethnic subgroup showing the pooled OR under the fixed-effects model in cancer *vs*. controls. Asians: OR =33.25, P<0.001; Caucasians: OR =11.21, P<0.001.

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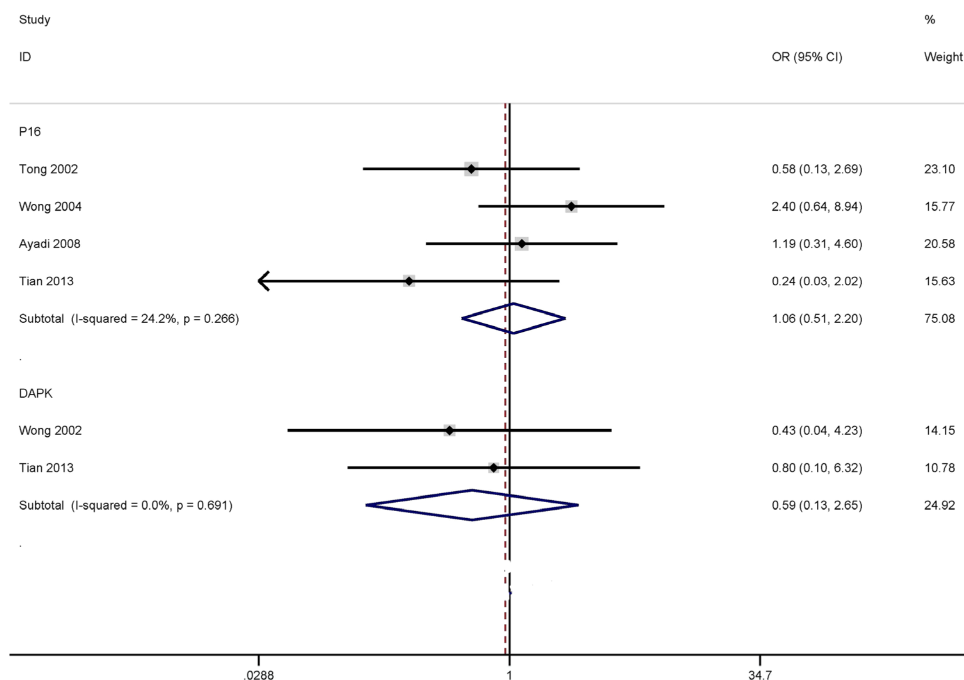

Figure 6 Forest plot for the association of *p16* and *DAPK* promoter methylation with tumor stage showing the pooled OR under the fixed-effects model in cancer. *p16*: OR =1.06, P=0.876, including four studies with 109 advanced NPC patients and 44 early NPC patients; *DAPK*: OR =0.59, P=0.491, including two studies with 61 advanced NPC patients and 11 early NPC patients.

reported in NPC (21,23,34). As a key cell cycle regulator, the *p16* gene is involved in the inhibition of cell cycle progression, and the restoration of its expression may induce G0/G1 arrest and suppress the tumorigenic growth of NPC cells (42). A correlation was found between *p16* promoter methylation and its expression in NPC, with the absence of *p16* expression (26). Earlier studies showed that the loss of *DAPK* expression was associated with its promoter methylation in NPC (35,36). The inactivation of *p16* and *DAPK* through promoter methylation may play a key in NPC tumorigenesis (43).

However, there are still inconsistent and controversial results regarding the methylation rate of *p16* and *DAPK* promoter in NPC specimens. For example, different promoter methylation rates of the *p16* gene, ranging from 22.2% (26) to 61.4% (31), were established in NPC tissues. Inconsistent findings on the frequency of *DAPK* promoter methylation in NPC tissues, which ranged from 47.2% (21) to 88.2% (35) were also reported previously. Therefore, we systematically investigated studies of *p16* and *DAPK* promoter methylation in NPC samples to estimate the association between *p16* and *DAPK* promoter methylation and NPC.

p16 and *DAPK* promoter methylation rates were shown to be significantly higher in NPC than in nontumorous tissue samples from the nasopharynx, suggesting that *p16* or *DAPK* promoter methylation may play a pivotal role in the tumorigenesis of NPC. No significant heterogeneity was found in our study, indicating the stability of our results.

The subgroup analysis by ethnic population comparing p16 and DAPK promoter methylation in NPC and nontumorous tissues from the nasopharynx revealed that the promoter methylation of *p16* or *DAPK* was significantly correlated with the risk of NPC in both the Asian and Caucasian populations, indicating that *p16* and *DAPK* promoter methylation may be susceptible genes for Asian and Caucasian populations. In addition, the Asian population had a higher OR of *DAPK* promoter methylation than the Caucasian population subgroup (33.25 *vs*. 11.21), which suggested that the Asian population may be more susceptible to *DAPK* promoter methylation. However, due to the limitation of the small sample size, the analysis of the Caucasian population subgroup in the current study should be cautiously interpreted.

Chang *et al.* reported that the promoter methylation rate of *p16* was 0% in NPC blood samples and 16.7% in NPC

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brushing samples, respectively (23). On the other hand, Wong *et al.* reported that a *p16* promoter methylation rate of 41.5% detected in NPC blood samples (32), and Hutajulu *et al.* discovered that *p16* promoter had a methylation frequency of 66% in NPC brushing samples (30). Detection of DNA methylation in brushing or blood samples, shows a great potential to be applied as an invasive biomarker for early detection of NPC (44). Previous studies suggest that *p16* and *DAPK* promoter methylation identified in brushing or blood samples may become a useful biomarker in NPC (24,33). Our findings demonstrate that *p16* promoter methylation established in blood and brushing samples is significantly associated with the risk of NPC and its rate is significantly higher in blood or brushing samples of NPC patients than in those of healthy subjects, suggesting that *p16* promoter methylation may be used as a noninvasive biomarker for NPC detection in blood and brushing samples.

Chang *et al.* discovered that *DAPK* promoter methylation frequency was 3.3% in NPC blood samples (23), but a much higher value (51.4%) was reported by Tian *et al.* (24). On the other hand, a frequency of *DAPK* promoter methylation in NPC brushing samples that reaches 50% has been reported only in the study of Chang *et al.* (23). Our results indicated that *DAPK* promoter methylation in blood and brushing samples was significantly correlated with the risk of NPC and its level was significantly higher in the blood or brushing samples of NPC patients than in those of healthy subjects, indicating that *DAPK* promoter methylation is a potential noninvasive biomarker for the detection of NPC in blood or brushing samples. Additionally, Tian *et al.* evidenced that the combined *P16* and *DAPK* promoter methylation did not significantly increase the potential capacity for NPC detection, but the combination of four-gene marker can be applied as a promising tool for the diagnosis of NPC (24). However, more clinical research studies are required to further validate these findings in the future. Only the OR of one study with brushing samples was included in this study. Thus, we should carefully consider the results from the analysis of blood and brushing samples conducted for the detection of *p16* and *DAPK* promoter methylation. Additional studies with larger sample sizes are needed to confirm our results.

Finally, we also investigated whether *p16* or *DAPK* promoter methylation was correlated with the clinical stage of NCR and found that *p16* and *DAPK* promoter methylation was not associated with tumor stage. More studies are exceedingly essential to validate that finding in the future.

Some limitations of this meta-analysis should be acknowledged. First, only articles published in English were included in this research. Articles in other languages and publications of other types, such as conferences abstracts, were excluded due to their unreadable contents or insufficient information, which might have led to a selection bias. Second, due to the limitation of insufficient data, we did not assess the relationship between *p16* and *DAPK* promoter methylation and other clinicopathological features, such as tumor grade, sex status, and lymph node status. Third, further studies with larger sample sizes should be done to validate our results, especially those concerning *DAPK* promoter methylation in blood and brushing specimens.

In conclusion, our findings suggest that *p16* and *DAPK* promoter methylation may play an important role in NPC development. The promoter methylation levels of *p16* or *DAPK* are potential useful biomarkers for NPC detection in blood and brushing samples in clinical settings. No significant association was found between *p16* or *DAPK* promoter methylation and tumor stage. Due to the limitations of the sample size in the present analysis, further large-scale studies with larger sample sizes of subjects are necessary to investigate more comprehensively the clinical effects of *p16* and *DAPK* promoter methylation in NPC patients.

Acknowledgments

Funding: None.

Footnote

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at http://dx.doi.org/10.21037/tcr.2016.12.08). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Cite this article as: Xiao L, Jiang L, Hu Q, Li Y. Promoter methylation of p16 and DAPK genes in brushing, blood, and tissue samples from patients with nasopharyngeal carcinoma: a systematic meta-analysis. Transl Cancer Res 2016;5(6):827-837. doi: 10.21037/tcr.2016.12.08

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