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ORIGINAL ARTICLE

Association of *p16* gene methylation with prostate cancer risk: a meta-analysis

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Summary

Purpose: *The tumor suppressor gene p16 is frequently silenced and inactivated by hypermethylation in human cancers, including prostate cancer. However, the association between the methylation status of p16 and prostate cancer risk remains ambiguous. This study aimed to assess the association of p16 methylation with prostate cancer risk by a comprehensive metaanalysis.*

Methods: *Relevant studies were identified by searching PubMed, Embase and Web of Science databases before October 2014 with no restrictions. The strength of the association between p16 methylation and prostate cancer risk was assessed by combined odds ratio (OR) and 95 % confidence interval (CI). The between-study heterogeneity and the contributions of single studies to the final results were tested by chi-square-based Q test and sensitivity analyses, respectively. Publication bias was evaluated by funnel plots.*

Results: *A total of 1,296 samples from 12 independent studies were enrolled in the present metaanalysis. Overall, a significant association was observed between p16 methylation and prostate cancer risk (OR=3.06; 95% CI:1.34-6.98;p=0.008). Stratified analyses by ethnic groups further revealed that prostate cancer risk was increased for individuals carrying the methylated p16 compared with those with unmethylated p16 in Caucasian populations (OR=2.51;95% CI:1.01-6.26;p=0.047) and Asian populations (OR=9.50;95% CI:1.78-50.61;p=0.008).*

Conclusions: *This study identified a strong association of p16 methylation with prostate cancer risk and suggested that p16 methylation might be a potential biomarker for prostate cancer.*

Key words: *methylation, p16, prostate cancer, susceptibility*

Introduction

Prostate cancer is the second most commonly diagnosed cancer and the sixth leading cause of cancer-related deaths in males worldwide, accounting for 14% of the total new cancer cases and 6% of the total cancer deaths [1]. The highest incidence rate records are in the developed countries of Oceania, Europe, and North America. Results of ecological studies suggest that prostate cancer is associated with a western lifestyle, and in particular diet that includes a high intake of fat, meat, and dairy products [2]. In addition to environmental factors, genetic and epigenetic alterations play important roles in the tumorigenesis and progression of prostate cancer [3,4].

As an eminent tumor suppressor, *p16* (also known as MTS1, CDKN2A, CDK4I, p16INK4A) has gained widespread importance in cancer since its discovery. In humans, *p16* is encoded by cyclin-dependent kinase inhibitor 2A (CDKN2A) gene, located on 9p21, a region that undergoes frequent genomic deletion in many human cancer cell lines and primary tumors [5]. Aberrant *p16* expression is observed in many tumor tissues including head and neck cancer, lung cancer, breast cancer, colon cancer, and prostate cancer [6,7], and the silence of *p16* can be induced by a variety of genetic and

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epigenetic changes including homozygous deletion, mutation, and promoter hypermethylation [8]. The *de novo* methylation of *p16* gene is associated with inactivation of the gene indicating that *p16* methylation could be involved in the pathogenesis of cancer [9-11].

Since Jarrard et al. reported that the *p16* methylation was more frequently observed in prostate tumors than in the surrounding normal tissues [7], the diagnostic value of *p16* methylation in prostate cancer has been widely investigated. To date, multiple studies have evaluated the association of *p16* methylation with the risk of prostate cancer. However, the results from those studies remain ambiguous. Therein, we conducted a comprehensive metaanalysis to better assess the association between *p16* methylation and prostate cancer risk.

Methods

Search strategy

A comprehensive literature search was carried out before October 2014 using PubMed, Embase and Web of Science databases without language restrictions. The following keywords were used: [("cyclin-dependent kinase inhibitor 2A" OR "CDKN2A" OR "CDK4I" OR "p16" OR "P16INK4A" OR "MTS1") AND "methylation" AND "prostate" AND "cancer"]. Reference lists from relevant primary studies and review articles were also manually searched for tracing additional relevant publications.

Study selection and data extraction

Studies were selected in the present study if they met the following criteria: 1) the study had a case-control design; 2) the study must be focused on the relationship between *p16* methylation and prostate cancer susceptibility; 3) the frequency of the methylation status regarding *p16* gene had to be reported or could be calculated from the data presented; 4) if two or more studies had overlapping data, the studies with the largest number of subjects were selected. The following data from all eligible studies were extracted respectively by two researchers: the first author's last name, publication year, country where the study conducted, subject ethnicity, and numbers of cases and controls, the method for methylation detection, and testing materials in each study. The two investigators reached a consensus on all items.

Statistics

The strength of the association between *p16* methylation and risk of prostate cancer was assessed by OR with the corresponding 95% Cl. The Z-test was used to estimate the statistical significance of pooled ORs. Subgroup analysis was also performed stratified by ethnicity, materials, and testing methods, respectively. Heterogeneity among studies was estimated by a chi-square-based *Q*-test, and the magnitude of the between-study heterogeneity was also quantified by the I^2 metric, which ranges from 0 to 100 % and is considered low for I^2 <25 %, modest for 25–50 %, and large for >50 % [12]. The summary of OR was calculated by the random-effects model (the DerSimonian and Laird method) when between-study heterogeneity was observed [13]. Otherwise, the fixed-effects model (the Mantel-Haenszel method) was selected [14]. Furthermore, sensitivity analysis, by which a single study in the metaanalysis was deleted each time to determine the influence of the individual data set to the overall pooled OR, was performed to assess the stability of the results. Begg's funnel plots and Egger's linear regression test were used to examine whether the results might have been affected by publication bias [15]. If publication bias appeared to exist, the nonparametric 'trim and fill' method was carried out for estimating the number of missing studies that might exist and the effect that these studies might have had on the outcome [16]. All statistical analyses were performed using the Stata software (Stata/SE, version 10.1 for Windows; Stata Corp, College Station, TX). All the p values were based on two-sided tests and $p<0.05$ was considered as statistically significant.

Results

Characteristics of included studies

According to our search strategy, 16 studies had investigated the relationship between *p16* gene methylation and prostate cancer risk. However, of these 16 studies, 4 articles didn't show the exact frequency of the gene's methylation [17-20] and were excluded. Eventually, 12 independent articles involving 845 cases and 451 controls were included in the pooled analysis [7,21-31]. The characteristics of these studies are summarized in Table 1.

The 12 studies enrolled in the present metaanalysis were published between 1997 and 2013. Among them, 11 studies were written in English and the other 2 were written in Chinese. Nine of the studies came from investigations involving Caucasian populations; 2 studies came from investigations involving Asian populations, and 1 involved more than two types of populations. DNA methylation status of *p16* was assessed in tumor tissues or urine samples. The methylated *p16* levels were detected using methylation sensitive restriction enzyme digestion (MSD), methylation specific PCR (MSP), or quantitative methylation specific PCR (QMSP).

Table 1. Characteristics of studies included in the present metaanalysis

First author	*Year*	*Country*	*Ethnicity*	*Cases (N)*	*Controls (N)*	*Methods*	*Materials*
Jarrard [7]	1997	US	Caucasian	36	36	MSD	Tissue
Maruyama [26]	2002	US	Mix	101	32	MSP	Tissue
Jeronimo	2004	Portugal	Caucasian	118	68	QMSP	Tissue
Yegnasubramanian [24]	2004	US	Caucasian	73	25	QMSP	Tissue
Hoque [23]	2005	US	Caucasian	52	91	QMSP	Urine
Yao [29]	2006	China	Asian	20	23	MSP	Tissue
Cho [22]	2007	Korea	Asian	179	30	MSP	Tissue
Kekeeva [25]	2007	Russia	Caucasian	73	40	MSD	Tissue
Roupret [28]	2007	France	Caucasian	95	38	QMSP	Urine
Ameri [21]	2011	Iran	Caucasian	42	21	MSP	Tissue
Murphy [27]	2011	Ireland	Caucasian	29	23	QMSP	Tissue
Yaqinuddin [30]	2013	Pakistan	Caucasian	27	24	MSP	Tissue

MSD: methylation sensitive restriction enzyme digestion, MSP: methylation-specific PCR, QMSP: quantitative methylation specific PCR

Table 2. Summary of *p16* methylation and prostate cancer risk

Variables	*Study number*	*Cases / Controls*	*OR (95% CI)*	P_H[a]	I^2 (%)
Total	12	845/451	3.06 (1.34-6.98)	0.007	57.5
Ethnicity					
Caucasian	9	545/366	2.51 (1.01-6.26)	0.009	61.0
Asian	2	199/53	9.50 (1.78-50.61)	0.652	0.0
Mixed	1	101/32	2.31 (0.12-45.91)		
Testing materials					
Tissue	10	698/282	2.18 (1.00-4.78)	0.058	45.2
Urine	2	147/129	13.08 (0.32-537.44)	0.021	81.3
Testing methods					
MSD	2	109/76	1.86 (0.17-19.98)	0.102	62.7
MSP	5	369/130	6.91 (2.01-23.79)	0.644	0
QMSP	5	367/245	2.93 (0.77-11.17)	0.010	69.9

[a]p value of Q test for heterogeneity. For other abbreviations see footnote of Table 1

Quantitative data analysis

We firstly detected the heterogeneity of the 12 studies included in the present study, and we found a strong heterogeneity among them (p=0.007, I^2=57.5%, Figure 1, Table 2). Therefore, we determined the association of *p16* methylation with risk of prostate cancer by the random-effect model. Overall, the pooled OR of *p16* methylation in prostate cancer patients, compared to non-cancer controls, was 3.06 (95% CI: 1.34-6.98; p=0.008; Figure 1). Next, we performed sensitivity analysis to assess the influence of individual studies on the overall effect. As shown in Table 3, the results revealed that omission of a single study changed the overall OR from 2.11 (95% CI: 1.04-4.25) to 4.13 (95% Cl:1.59-10.73), indicating that there was no single sensitive study. These results suggested that *p16* methylation was significantly associated with increased risk of prostate cancer.

Then, we stratified the association by ethnicity, material and method. Ethnic-specific OR showed an increased risk for individuals carrying the methylated *p16* compared with those without methylated *p16* in Caucasian populations (OR=2.51; 95% CI:1.01-6.26; p=0.047) and Asian populations (OR=9.50; 95% CI:1.78-50.61; p=0.008, Table 2). In the stratified analysis by material, significantly increased risk was found in tissue samples in the detection *p16* methylation in prostate

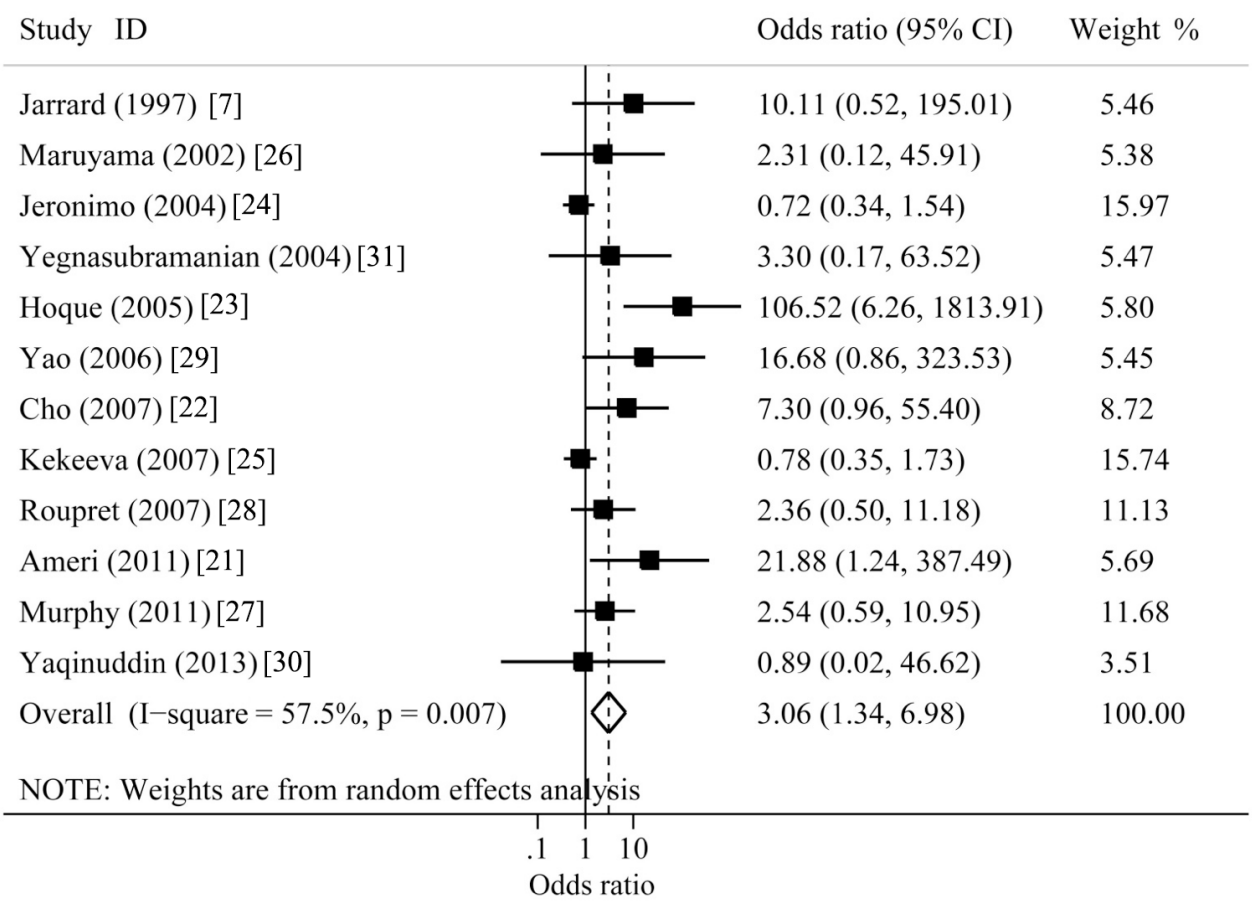

Figure 1. Metaanalysis for the association of *p16* methylation with prostate cancer risk. The squares and horizontal lines represent the study-specific OR and 95% CI, and the diamond represents the pooled OR and 95% CI.

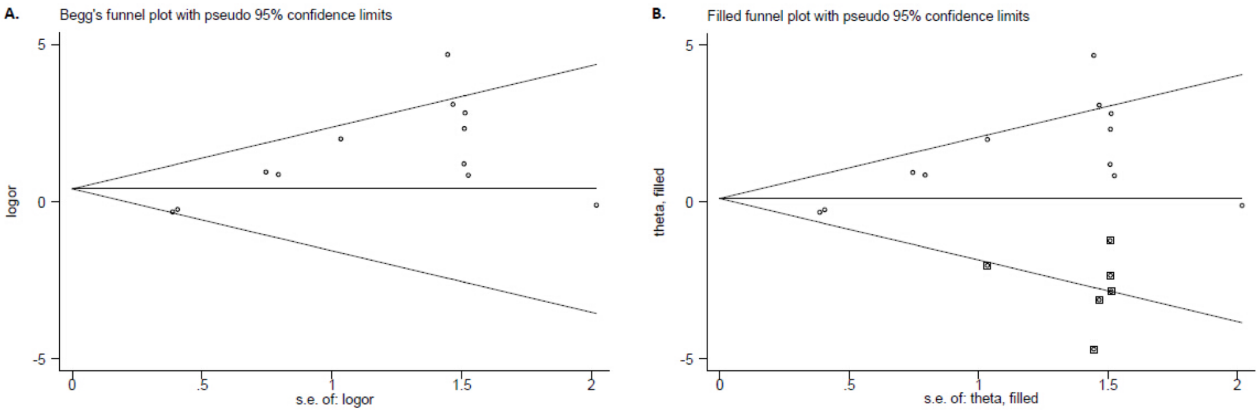

Figure 2. Begg's funnel plot of publication biases on the relationship between *p16* methylation and prostate cancer susceptibility. Each point represented a separate study for the indicated association. **A:** Begg's funnel plot of publication bias test. **B:** Begg's funnel plot of publication bias test after trim-and-fill method.

cancer (OR=2.18; 95% CI:1.00-4.78; p=0.050, Table 2). Stratified analysis by method showed significantly increased risk in methylation-specific PCR (MSP) (OR=6.91; 95% CI:2.01-23.79; p=0.002, Table 2).

Publication bias

The shape of the funnel plots seemed asymmetrical in the overall analysis (Figure 2A), suggesting the presence of publication bias. In fact, the result of Egger's test provided statistical evi-

Table 3. Sensitivity analysis

First author (year) [Ref. No.]	*OR*	*95% CI*
Jarrard (1997) [7]	2.85	1.22-6.64
Maruyama (2002) [26]	3.19	1.33-7.62
Jeronimo (2004) [24]	4.11	1.63-10.35
Yegnasubramanian (2004) [31]	3.11	1.31-7.42
Hoque (2005) [23]	2.11	1.04-4.25
Yao (2006) [29]	2.73	1.19-6.24
Cho (2007) [22]	2.80	1.19-6.58
Kekeeva (2007) [25]	4.13	1.59-10.73
Roupret (2007) [28]	3.31	1.32-8.28
Ameri (2011) [21]	2.63	1.16-5.95
Murphy (2011) [27]	3.29	1.31-8.25
Yaqinuddin (2013) [20]	3.27	1.39-7.72
Combined	3.06	1.34-6.98

dence of funnel plot asymmetry (t=4.34, p=0.001). To adjust this bias, a nonparametric trim-and-fill method developed by Duval and Tweedie was implemented [16]. As shown in Figure 2B, 6 missing studies were added to the dataset, and the filled dataset was much more symmetric than the original data and the plot showed no evidence of publication bias. This filled dataset moved the random-effects summary and the estimated OR changed from 3.06 (95% CI:1.34-6.98) to 3.04 (95% CI:1.60-13.86). The correction for publication bias didn't change the overall interpretation of the dataset, indicating that our results were statistically robust.

Discussion

To the best of our knowledge, this is the first metaanalysis of published studies to focus on the association of *p16* methylation and prostate cancer risk. Our analysis, combining 12 studies with 1,296 samples totally enrolled, revealed that the methylation of *p16* increased the risk of prostate cancer. In particular, the overall OR for *p16* methylation status in prostate cancer vs normal samples was 3.06 (95% CI:1.34-6.98; Figure 1), indicating a strong positive association of the methylation of *p16* gene with prostate cancer. Notably, although only 2 studies regarding Asian populations were enrolled, subgroup analysis by ethnicity showed a strong association between *p16* methylation and prostate cancer risk in both Asian and Caucasian populations, suggesting a parallel effect of *p16* methylation on prostate cancer among different ethnicities.

Assessment of the heterogeneity within studies is regarded as an essential component of metaanalyses [32]. To better achieve this, we used two widely used methods, chi-square-based *Q* test and I^2 test to determine the between-study heterogeneity in the present metaanalysis. We also preformed sensitivity analyses to determine the effects of individual studies on the overall effect and found that there was no single sensitive study in our metaanalysis. In addition to between-study heterogeneity, a key concern in robust metaanalyses is publication bias. We therefore used funnel plot and Egger's test to assess whether the studies could be affected by publication bias, and found presence of publication bias. To ameliorate the effect of publication bias on the results of the current metaanalysis, we next performed the nonparametric 'trim and fill' method to adjust the bias. Finally, 6 missing studies were added to the dataset and the filled dataset shows no evidence of publication bias. Importantly, the correction for publication bias didn't change the overall result effect of *p16* methylation on the risk of prostate cancer. Taken together, these results suggested a reliable association of *p16* methylation with prostate cancer risk.

It is well documented that *p16* gene is an important tumor suppressor in various human malignancies including prostate cancer, and the major biochemical effect of *p16* is induction of cell growth arrest by halting cell-cycle progression at the G1/S boundary [33,34]. Promoter methylation is a frequent epigenetic event and a vital mechanism leading to silencing and dysfunction of *p16* gene, which could further result in unregulated cell proliferation and tumor initiation and development [8,35]. These findings support our findings in the current study that *p16* gene methylation increased the risk of prostate cancer.

It is well known that prostate cancer is a common malignancy and a leading cause of cancer-related deaths in men. Early detection of human prostate cancer include digital rectal examination and serum prostate-specific antigen (PSA) determination [36]. However, due to the limited sensitivity and specificity of these examinations, these methods cannot identify early-stage prostate cancer. In recent years, molecularly-based diagnostic approaches for prostate cancer have been extensively investigated and increasing epigenetic markers have been identified [4,37]. Based on the investigations on *p16* methylation and prostate cancer, the data of the present metaanal-

ysis showed a strong positive association of *p16* methylation with risk of prostate cancer, which is consistent with previous findings that *p16* methylation could be used as an independent prognostic factor for several types of cancer, including breast, lung, gastric, and colorectal cancer [38-41]. Moreover, in combination with the notion that *p16* methylation has been reported in various cancer types, *p16* methylation has the potential to be a molecular marker for early detection and monitoring in certain types of cancer, for example, prostate cancer.

In conclusion, the present metaanalysis provides evidence to support a strong association of *p16* methylation with increased risk of prostate cancer. Although further investigations with larger sample size are still needed to provide more convincing statistical analyses and to confirm the correlation between *p16* methylation and prostate cancer susceptibility, our findings suggest that *p16* methylation is a potentially useful biomarker for predicting prostate cancer diagnosis.

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