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Quantitative assessment of the relationship between *RASSF1A* gene promoter methylation and bladder cancer (PRISMA)

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Abstract

Background: Methylation of the Ras-association domain family 1 isoform A (*RASSF1A*) gene promoter region is thought to participate in the initiation and development of many different cancers. However, in bladder cancer the role of RASSF1A methylation was unclear. To evaluate the relationship between RASSF1A methylation and bladder cancer, a quantitative assessment of an independent meta-analysis was performed. In addition, a DNA methylation microarray database from the cancer genome atlas (TCGA) project was used to validate the results of the meta-analysis.

Methods: We searched published articles from computerized databases, and DNA methylation data were extracted from TCGA project. All data were analyzed by R software.

Results: The results of the meta-analysis indicated that the frequency of *RASSF1A* gene methylation in bladder cancer patients is significantly higher than in healthy controls. The hazard ratio (HR) was 2.24 (95% CI = [1.45; 3.48], P = 0.0003) for overall survival (OS), and the *RASSF1A* gene promoter methylation status was strongly associated with the TNM stage and differentiation grade of the tumor. The similar results were also found by the data from TCGA project.

Conclusion: There was a significant relationship between the methylation of the *RASSF1A* gene promoter and bladder cancer. Therefore, *RASSF1A* gene promoter methylation will be a potential biomarker for the clinical diagnosis of bladder cancer.

Abbreviations: HR = hazard ratios, OR = odds ratio, RASSF1A = Ras-association domain family 1A, TCGA = the cancer genome atlas.

Keywords: meta-analysis, promoter methylation, RASSF1A, TCGA

1. Introduction

Previous research has shown that allelic loss of chromosome 3p is frequent in malignant tumors.[1] Sekido et al[2] found that the deleted area was located at 3p21.3, which encompasses 120 kb DNA. Dammann et al[3] showed that its cDNA was highly homologous with the *NORE1/Maxp1* gene, and which was named Ras-association domain family 1 (RASSF1). RASSF1 has 8 exons, and one of the RASSF1 family members is Ras-association domain family 1A (RASSF1A). RASSF1A has been closely associated with several different cancers and has been identified as a candidate tumor suppressor. Current research showed that the role of the *RASSF1A* gene is to inhibit cell proliferation and also to promote cell apoptosis and aging. Functional analysis also showed that RASSF1A has a potential role in maintaining microtubule stability.[4] However, the RASSF1A protein is often absent in many tumor cells, as a consequence of the gene being inactivated/silenced. It is believed that the major mechanism of this silencing is RASSF1A promoter methylation.[5] Such methylation is a common means by which many normal genes are silenced, and indeed silencing of tumor suppressor genes is a part of normal homeostatic mechanisms. Recently, the *RASSF1A* gene has been highlighted as a gene most commonly methylated in tumors.

The use of biomarkers to detect cancer has attracted much attention in recent years, as it offers many advantages over routine techniques, which rely on biopsies to examine cell morphology and to look for signs of precancerous lesions. Methylation of *RASSF1A* gene promoter will be an ideal marker of tumor biology for several reasons. First, *RASSF1A* gene promoter methylation is rarely found in normal tissue, so it is a fairly unique marker. Second, unlike standard histological methods, the detection of methylation rely much less on the individual experience of a practitioner, as such a test can be automated. Third, the methylation of *RASSF1A* gene promoter occurs in many different types of cancer, so it can be developed as a broad-spectrum diagnostic test. Finally, the frequency of

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RASSF1A gene promoter methylation is linked to tumor grade, and thus can provide additional information regarding the tumor stage and prognosis.

Abnormal DNA methylation has been regarded participate in the early tumorigenesis and played an important role in the development of human tumors. Therefore, abnormal gene methylation can be detected early in patients. In addition, DNA methylation can be measured in serum and urine samples, as well as in tumor tissue, and thus that is very beneficial to clinical diagnosis of cancer. Although a previous study investigated the relationship between RASSF1A promoter methylation and bladder cancer risk,[6] the differences in gender proportion, age distribution, racial composition, test methods, and primers used in the studies would result in some differences in the results, and moreover, that study did not consider all of these fields. Meanwhile, the relationship between *RASSF1A* gene promoter and bladder cancer prognosis was not sure. Therefore, we carried out a meta-analysis based on lager data in order to further explore the relationship between *RASSF1A* gene promoter methylation and bladder cancer, which contained all of the above fields. As publication bias and heterogeneity can affect meta-analysis results, we downloaded DNA methylation data from the cancer genome atlas (TCGA) project to validate our meta-analysis results. The DNA methylation data from TCGA project contained genome-wide methylation status, and it would provide no publication bias and no heterogeneity in analyzing the relationship between *RASSF1A* gene promoter methylation and bladder cancer. Therefore, an integrated analysis with unbiased conclusions was conducted to come to regarding the relationship between *RASSF1A* gene promoter methylation and bladder cancer.

2. Materials and methods

2.1. Ethics Statement

This study was approved by the People's Hospital of Three Gorges University Ethics Committee. This study does not involve patients, so ethical approval was not required.

2.2. Published articles, search strategy, data extraction, and meta-analysis

We conducted a literature search (up to and including July 20th, 2015) of computerized databases, including PubMed, Cochrane Library, Web of Science, Google Scholar, and China National Knowledge Infrastructure, for articles published in both English and Chinese. The study used a subject and text word strategy with "bladder cancer or carcinoma of bladder or bladder carcinoma or bladder neoplasms or carcinoma of urinary bladder," "RASSF1A or Ras association domain family 1A or RASSF1," "methylation or hypermethylation or epigenetic." In addition, we searched the reference list of relevant original papers and review articles to identify additional eligible studies. We followed the standard guidelines for conducting and reporting meta-analyses of observational studies.[7] The included articles met the following criteria: Original study and the patients had to be diagnosis with bladder cancer; The subjects in every study comprised bladder cancer samples and healthy controls; and The studies had to be contained *RASSF1A* gene promoter methylation data. We excluded animal studies, clinical trials, reviews, commentaries, letters, and studies that examined other associations. The data were extracted from each study by 2 independent reviewers, using prespecified selection criteria. Decisions were made and disagreements about study selection were resolved by discussion with a 3rd reviewer. The following information was extracted from each study: the first author's last name, publication year, study location, mean age, tumor, node, metastasis (TNM) stage, differentiation grade, the methods and the primers used in the article, the number of *RASSF1A* gene promoter methylation in cancer samples, and normal controls. Ethnicity was categorized as "Caucasian," "Asian" or "mixed population" when a study did not state which ethnic groups were included.

All statistical tests were performed with R software (R version 3.1.2) including meta and metafor packages. The strength of the association between *RASSF1A* gene promoter methylation and bladder cancer was measured using a pooled odds ratios (ORs) and hazard ratios (HRs) with a 95% confidence interval (CI), and with $P<0.05$ considered statistically significant. Group analysis was performed and stratified by the study character of age, gender, smoking habit, TNM stage, and differentiation grade. The heterogeneity among studies was estimated by the Cochran Q test and I^2 statistic. Heterogeneity was considered statistically significant at $P\leq 0.05$. The I^2 statistic describes the percentage of total variation in point estimates that can be attributed to heterogeneity. For the I^2 metric, we considered low, moderate, and high I^2 values to be 25%, 50%, and 75%, respectively.[8] Tau-squared (τ^2) was used to determine how much any heterogeneity could be explained by subgroup differences. The data were pooled using the random-effects model ($I^2>50\%$, $P\leq 0.05$) or fixed-effects model ($I^2<50\%$, $P>0.05$) according to heterogeneity statistic I^2.[9] If there was no heterogeneity among included studies, the pooled OR estimates were calculated using the fixed-effects model.[10] Otherwise, the random-effects model was used.[9] The possibility of publication bias was assessed using the Begg and Egger regression asymmetry test.[11,12] For sensitivity analysis, we also used the random-effects model for all the above analyses. Additional sensitivity analyses were performed by omitting 1 study at a time, then calculating a pooled estimate for the remainder of the studies, to evaluate whether the results were markedly affected by a single study. Sensitivity (also called the true positive rate) measures the proportion of positives that are correctly identified as such, for example, the percentage of sick people who are correctly identified as having the condition; specificity (also called the true negative rate) measures the proportion of negatives that are correctly identified as such, for example, the percentage of healthy people who are correctly identified as not having the condition. Therefore, sensitivity and specificity were assessed in the meta-analysis of diagnostic tests.

2.3. TCGA data extraction and analysis

DNA methylation information for bladder cancer was downloaded from TCGA project (http://cancergenome.nih.gov/). The methylation signals of the 485,577 probes shared by 450K dataset was extracted and the methylation status of each probe was defined according to the beta-value (beta-value = [intensity value from the methylated bead type]/[the sum of intensity values from the methylated and unmethylated bead types + 100]). Any beta-value equal to, or greater than 0.6, was considered fully methylated, whereas a beta-value equal to, or less than 0.2, was considered unmethylated. Beta-values between 0.2 and 0.6 were considered partially methylated. To our knowledge, the CpG site will be considered methylated when the beta-value is greater than the empirical threshold of 0.3.[13]

3. Results

3.1. Study characteristics

For meta-analysis, 21 articles[14–34] were obtained according to the above standards, after we screened 104 potentially relevant articles for inclusion, on the basis of title, abstract, and full text (Fig. S1A, http://links.lww.com/MD/B554). The characteristics of the 21 articles (published between 2001 and 2014) are shown in Tables S1–8, http://links.lww.com/MD/B555 (Fig. S1B, http://links.lww.com/MD/B554). The 21 articles came from China, Korea, Pakistan, Brazil, USA, Germany, Denmark, Finland, and UK (Fig. S1C, http://links.lww.com/MD/B554). In total, 1588 bladder cancer samples and 720 normal controls were collected. According to the patient's information, we designated all patients from China, Korea, and Pakistan as Asian; patients who came from Brazil and the USA were termed Mixed-race; and patients from Germany, Denmark, Finland, and the UK were termed Caucasian. Among the 21 articles, 18 of 21 included articles used methylation-specific polymerase chain reaction (MSP), while others used quantitative methylation specific polymerase chain reaction (QMSP). The primers used in both methods are listed in Table S8 (http://links.lww.com/MD/B555). The promoter region and the CpG sites of RASSF1A as previously described.[3,35] Forty-nine percent of bladder cancer patients had the methylated RASSF1A allele, with a frequency ranging from 32.87% to 81.63%, in individual trials. However, only 4.44% of normal controls had the methylated RASSF1A allele, with a frequency ranging from 0% to 20.83%, in individual trials. All the 21 studies focused on the risk of bladder cancer; however, many had a different specific focus: 4 primarily focused on the prognosis in bladder cancer (Table S2, http://links.lww.com/MD/B555), 6 focused on the patients' age (Table S3, http://links.lww.com/MD/B555), 7 focused on gender differences (Table S4, http://links.lww.com/MD/B555), 2 examined smoking habits of patients (Table S5, http://links.lww.com/MD/B555), 13 (Table S6, http://links.lww.com/MD/B555), and 12 (Table S7, http://links.lww.com/MD/B555) examined the TMN stage and differentiation grade, respectively (Fig. S1D, http://links.lww.com/MD/B554). The different frequencies observed for *RASSF1A* gene promoter methylation between these different groups are listed in Table 1.

According to previous studies[3] and the different primers used in the articles included in our meta-analysis, we analyzed 11 different probes located in or near the *RASSF1A* gene promoter region, and chose 4 of them (cg10580282, cg14943722, cg11607701, and cg06360465), which contained the transcription start site of *RASSF1A* gene. Ultimately, bladder cancer tissue samples (260 in total) and 21 adjacent cancer normal tissue samples were obtained from TCGA project database (Table S9, http://links.lww.com/MD/B556). Out of the 260 patients, 61.15% had *RASSF1A* gene promoter methylation, while there was no methylation of *RASSF1A* gene in normal tissue. Sensitivity and specificity analysis showed that the true positive rate was 0.61 and false positive rate was 0. The numbers of patients classified according to age, gender, smoker or nonsmoker, TNM stage, and differentiation grade are shown in Table 1.

3.2. The relationship between RASSF1A gene promoter methylation and bladder cancer risk

The results of this meta-analysis showed that the frequency of *RASSF1A* gene promoter methylation was significantly higher in bladder cancer patients than in normal controls, by fixed-effects model (OR=21.12; 95% CI=[14.51; 30.74]; z=15.93; $P<0.0001$) and by random-effects model (OR=18.46; 95% CI=[12.69; 26.85]; z=15.26; $P<0.0001$) with $\tau^2=0$ and $I^2=0.00\%$ (Fig. 1). This clearly indicated a statistically significant increase in the likelihood of methylation in bladder cancer compared to normal controls, with no heterogeneity in the 21 articles. Subgroup analysis by ethnicity demonstrated that methylation of the *RASSF1A* gene promoter was positively associated with an increased risk of bladder cancer, among Mixed-race (OR=23.36; 95% CI=[8.39; 65.05], z=15.93), Asians (OR=24.10; 95% CI=[15.01; 38.69], z=15.93), and Caucasians (OR=13.99; 95% CI=[6.47; 30.25], z=15.93) (all $P<0.0001$) (Fig. 2A) by the fixed-effects model. The similar meta-analysis results of the bladder cancer risk were found in tissue (OR=18.44; 95% CI=[11.66; 29.16]) and urine samples (OR=19.82; 95% CI=[9.25; 42.45]) (Fig. 2B). In addition, subgroup analysis by methods and by primer types showed that the OR was 22.68 (95% CI=[15.04; 34.21], $P<0.0001$) in MSP, 14.11 (95% CI=[5.55; 35.87], $P<0.0001$) in

Table 1

Characteristics of eligible studies and TCGA project considered in the report.

	Meta-analysis			TCGA project		
	N	M	OR; 95%CI; *P*	N	M	OR; 95%CI; *P*
Gender			1.43; [0.88; 2.32]; 0.15			0.48; [0.23; 1.03]; 0.062
Male	290	163		164	109	
Female	99	50		51	30	
Age			1.16; [0.72; 1.87]; 0.55			0.99; [0.52; 1.92]; 0.991
≥60	243	124		164	106	
<60	112	60		51	33	
TNM stage			2.51; [1.87; 3.37]; <0.0001			1.82; [1.01; 3.22]; 0.044
I	642	254		3	1	
II				69	33	
III	356	239		73	43	
IV				66	43	
Differentiation grade			2.68; [1.93; 3.74]; <0.0001			3.38; [1.23; 9.32]; 0.018
Low	299	96		19	7	
High	510	275		241	152	
Smoking status			0.86; [0.37; 2.00]; 0.73			1.40; [0.78; 2.51]; 0.254
Smoking	121	46		105	76	
Nonsmoking	32	16		101	71	

CI=confidence interval, OR=odds ratio, TCGA=the cancer genome atlas, TNM=tumor, node, metastasis.

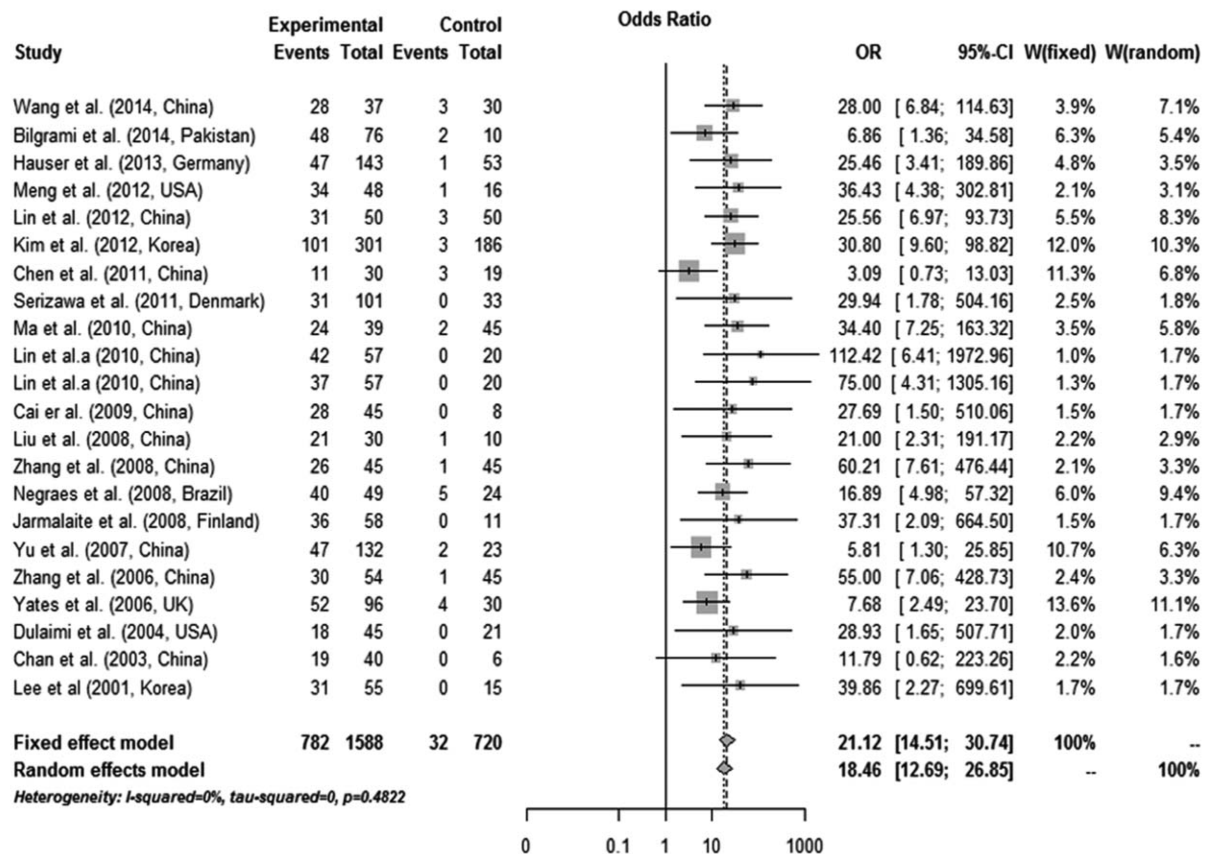

Figure 1. Combined estimates for the association between *RASSF1A* gene promoter methylation and bladder cancer risk, with forest plots. Author, year, country of the studies, and methylated (M) and total (T) number of samples in case and control, combined OR with 95% confidence region are indicated in the right column of the figure. The DerSimonian–Laird estimator and Mantel–Haenszel method were selected to conduct a combined estimation for the random-effects model and fixed-effects model, respectively. OR = odds ratio, RASSF1A = Ras-association domain family 1A.

QMSP, 21.15 (95% CI = [13.23; 33.80], $P<0.0001$) in primer located I, and 21.06 (95% CI = [11.27; 39.33], $P<0.0001$) in primer located II, with no heterogeneity (Fig. 2C, D).

Using data obtained from TCGA project, we were able to compare the frequency of *RASSF1A* gene promoter methylation in bladder cancer samples and normal samples, and found a significant difference (Fig. 3A). This significant difference was true for patients who were Asian, Black, African American, and White (Fig. 3B). This result therefore gave a similar result to that of the meta-analysis.

Next we performed bias analysis and sensitivity analysis of the 21 articles, which were focused on the relationship between *RASSF1A* gene promoter methylation and bladder cancer risk. The visual assessment of the Begg test ($Z=0.70$, $df=20$, $P=0.48$) and Egger test ($t=2.12$, $df=20$, $P=0.046$) did not reveal any evidence of obvious asymmetry in the 21 articles. Therefore, there did not appear to be any publication bias in the 21 studies (Fig. S2A, http://links.lww.com/MD/B554). Sensitivity analyses were conducted to determine whether modification of the inclusive criteria of the meta-analysis affected the final results, but no single study was found to affect the pooled OR (Fig. S2B, http://links.lww.com/MD/B554). The pooled sensitivity of the 21 articles was 0.96 (95% CI = [0.94–0.98]) and the specificity was 0.47 (95% CI = [0.39–0.55]) (Fig. S2C, http://links.lww.com/MD/B554), while the area under the curve of the receiver-operating characteristic curve (ROC) was 0.93 (95% CI = [0.90–0.95]) (Fig. S2C, http://links.lww.com/MD/B554). Hence, the diagnostic accuracy of the included studies was high, and the meta-analysis results were overall very reliable.

3.3. The relationship between RASSF1A gene promoter methylation and the clinical features of bladder cancer

DNA methylation is thought to be linked to certain clinical characteristics, such as whether the patient was a smoker or not, and the tumor differentiation grade. Therefore, meta-analyses were conducted based on age, gender, smoking status, TNM stages, and differentiation grade, and revealed that methylation of the *RASSF1A* gene promoter was not implicated in the incidence of bladder cancer based on age (OR = 1.16, 95% CI = [0.72; 1.87], $P=0.55$) (Fig. 4A), gender (OR = 1.43, 95% CI = [0.88; 2.32], $P=0.15$) (Fig. 4C), and smoking status (OR = 0.86, 95% CI = [0.37; 2.00], $P=0.73$) (Fig. 4E). This result was similar to the 1 data from TCGA project (Fig. 4B/D/F). However, when we compared the TNM stage I–II (low grade) and TNM stage III–IV (high grade), by meta-analysis and by using TCGA project data, a significant difference was found (Fig. 4G, H). The same was true for the differentiation grade (Fig. 4I, J), suggesting that advanced bladder cancer has a high frequency of *RASSF1A* gene promoter methylation.

Although there was no heterogeneity between age, gender, smoking habits, TNM stage, and differentiation grade (Table 2),

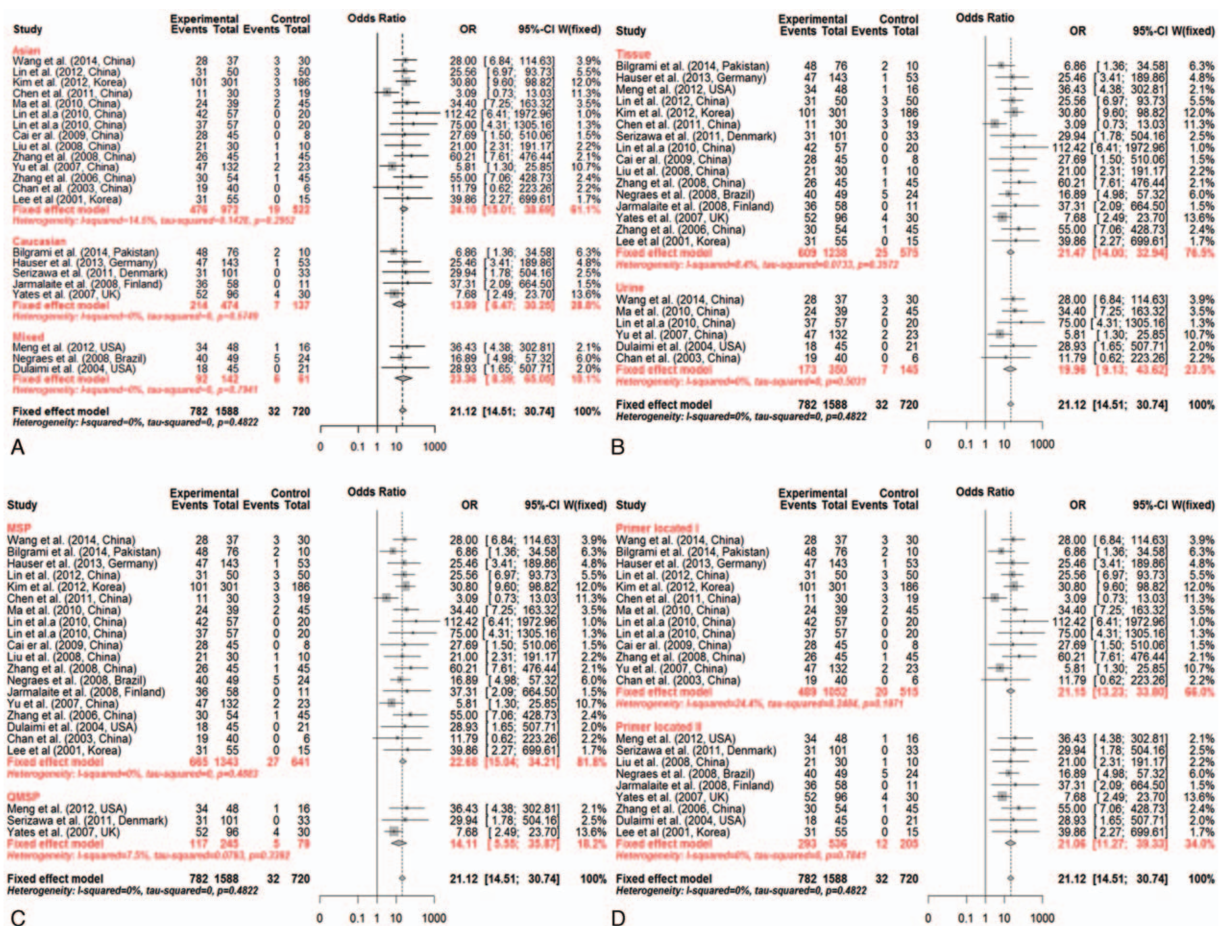

Figure 2. Subgroup meta-analysis of the relationship between Ras-association domain family 1A (*RASSF1A*) gene promoter methylation and risk of bladder cancer. (A–D) Subgroup meta-analysis based on race, sample, different test methods, and different primers, by fixed-effects model.

a bias and sensitivity analysis for these 5 parameters was implemented. As a result of the small amount of data for patients grouped according to age, gender, and smoking habits, the assessment was only carried out between patients grouped according to the TNM stage and differentiation grade. Therefore, a larger and higher quality study should be undertaken in the future. As a result, we only found publication bias in the studies of TNM stages and differentiation grade; the sensitivity analysis found that no single study could affect the pooled OR of TNM stage and differentiation grade (Table 2).

3.4. The relationship between RASSF1A gene promoter methylation and prognosis of bladder cancer patients

The role of *RASSF1A* gene promoter methylation has been examined for several different tumor types, such as lung

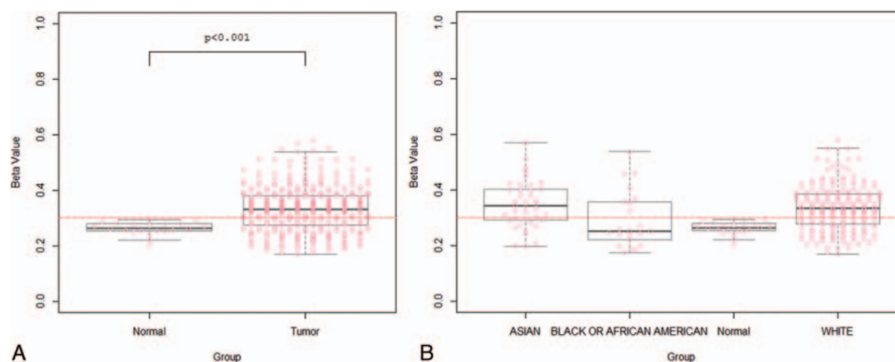

Figure 3. Statistical analysis of the relationship between *RASSF1A* gene promoter methylation and risk of bladder cancer in TCGA project. (A, B) Evaluation of the methylation of *RASSF1A* gene promoter in bladder cancer, including different race, in TCGA project. The $\beta = 0.3$ indicates by red dotted line. RASSF1A = Ras-association domain family 1A, TCGA = the cancer genome atlas.

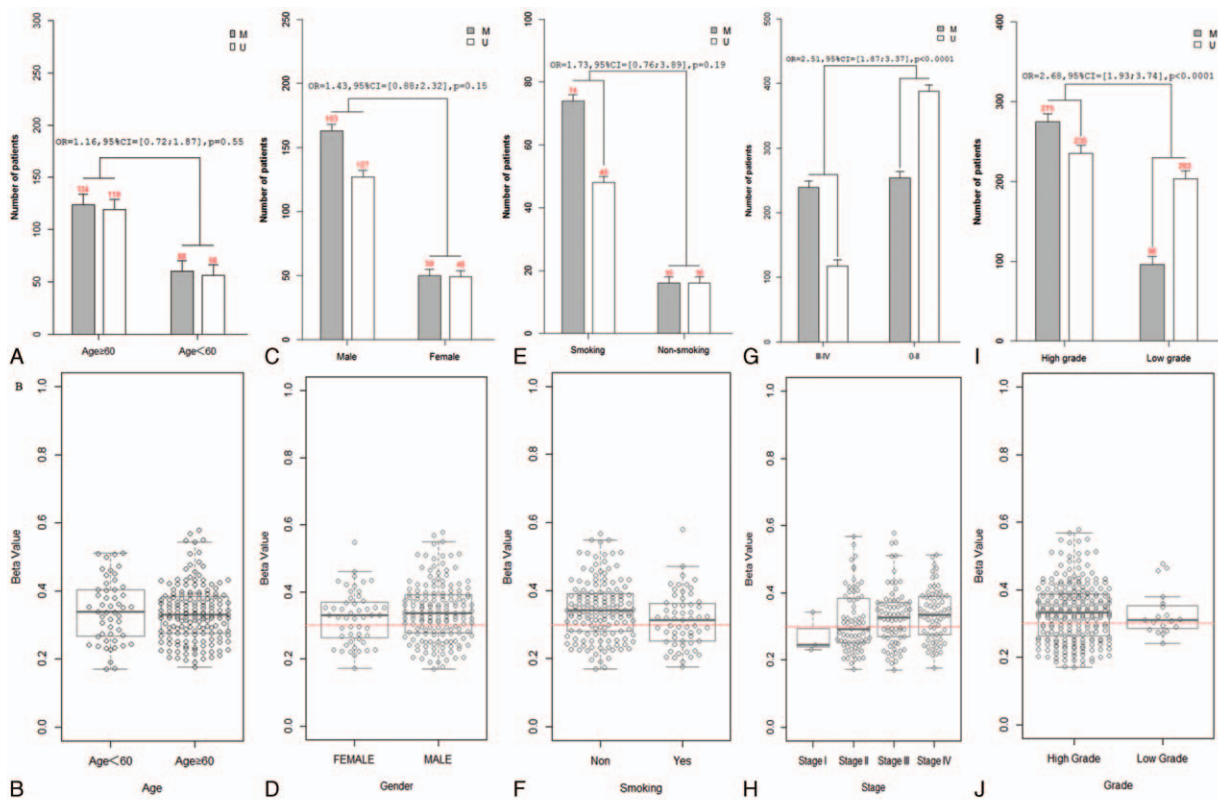

Figure 4. Quantitative assessment of the relationship between *RASSF1A* gene promoter methylation and patient age, gender and smoking habit, TNM stage and differentiation grade, in bladder cancer. (A, C, E, G, I) Meta-analysis for the relationship between *RASSF1A* gene promoter methylation and patient age, gender and smoking habit, TNM stages and differentiation grade, in bladder cancer. (B, D, F, H, J) Assessment of the relationship between *RASSF1A* gene promoter methylation and patient age, gender and smoking habit, TNM stages and differentiation grade, in bladder cancer by TCGA project. The $\beta=0.3$ indicates by red dotted line. RASSF1A = Ras-association domain family 1A, TCGA = the cancer genome atlas.

cancer,[36] breast cancer,[37] and liver cancer.[38] However, the role of *RASSF1A* gene promoter methylation in the prognosis of bladder cancer was not known. Here, an analysis, basing on 4 articles including 503 bladder cancer patients in total (Table S2, http://links.lww.com/MD/B555) and data extracting from TCGA project, was carried out. The HR was found to be 2.24 (95% CI = [1.45; 3.48], $P=0.0003$) for the 4 articles and 1.83 for TCGA project data (95% CI: 1.03–3.25, $P=0.040$) (Fig. 5A) for overall survival when we used 199 bladder cancer patients analyzed by Kaplan–Meier method, suggesting that bladder cancer patients with *RASSF1A* gene promoter methylation have a poor prognosis. The HR of 172 bladder cancer patients analyzed for disease-free survival was 1.97 (95% CI = [1.04; 3.72], $P=0.037$), which demonstrated that bladder cancer patients with *RASSF1A* gene promoter methylation may have a 97% chance of recurrence after surgery or other treatment (such as chemotherapy and combined treatment) (Fig. 5B).

4. Discussion

Modern tumor molecular biology studies have shown that tumors can be caused by genetic and epigenetic mechanisms. The instability of the genome has long been considered an important mechanism driving bladder cancer.[39] Multiple molecular genetics studies have found that many gene loci experience loss of heterozygosity and lack of homozygosity, and a deficiency in tumor suppressor genes is thought to play an important role in the development of bladder cancer. In addition, epigenetic

Table 2

Heterogeneity, bias analysis, and sensitivity analysis.

	Test of heterogeneity			Quantifying heterogeneity		Begg test		Egger test		Sensitivity analyses	
Group	Q	df	P	τ^2	I^2	t	P	t	P	Low (OR; 95%CI)	High (OR; 95%CI)
Risk	20.62	21	0.48	0.00	0%	0.97	0.34	2.12	0.05	19.80 [13.34; 29.37]	23.42 [15.80; 34.72]
Gender	5.79	7	0.56	0.00	0%	NA	NA	NA	NA	1.28 [0.76; 2.15]	1.61 [0.94; 2.76]
Age	2.66	6	0.85	0.00	0%	NA	NA	NA	NA	1.07 [0.64; 1.78]	1.11 [0.67; 1.85]
TNM stage	21.8	13	0.06	0.24	40.40%	−0.12	0.90	0.71	0.38	2.36 [1.74; 3.18]	2.79 [2.05; 3.79]
Grade	25.31	12	0.01	0.49	52.60%	−1.79	0.101	−1.00	0.34	2.24 [1.57; 3.20]	3.10 [2.18; 4.40]
Smoking	0.00	1	0.99	0.00	0%	NA	NA	NA	NA	NA	NA

CI = confidence interval, NA = not available due to the small data, OR = odds ratio, TNM = tumor, node, metastasis.

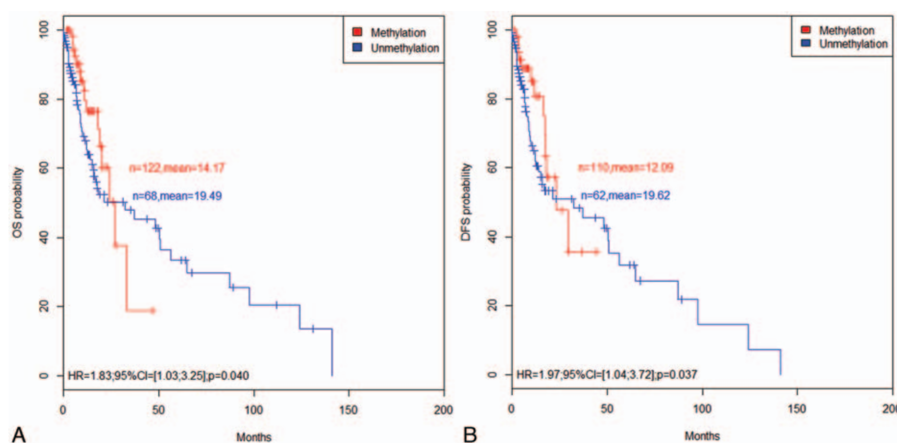

Figure 5. Association of patient survival and Ras-association domain family 1A (*RASSF1A*) gene promoter methylation status by Kaplan–Meier method. (A) Survival curves by methylation status of *RASSF1A* gene promoter. The number of censored cases with and without methylation was 122 and 68, respectively, and the mean survival time was 14.17 and 19.49, respectively. (B) Kaplan–Meier survival analysis of recurrent bladder cancer showing the association between tumor progression and *RASFF1A* gene methylation status. The number of censored cases with and without methylation was 110 and 62, respectively, and the mean survival time was 12.09 and 19.62, respectively.

modifications, such as DNA methylation and histone acetylation, are also responsible for the development of tumors.[40] Abnormal DNA methylation patterns were identified 10 years ago as one of the molecular characteristics all tumors have in common,[41] and are now known to be the most important form of genetic modification in mammals.[42] Many researchers[43] believed that DNA methyltransferase mediated methylation in overall genomic DNA, and high levels of methylation were a sign of tumorigenesis. Indeed, DNA methylation is implicated in the silencing of tumor suppressor genes and is suggested to lead to the development of tumors. Some studies[5] found that gene promoter region methylation patterns were not random: some genes in certain tumor types were commonly methylated, but unmethylated in other tumor types. Tumor suppressor gene promoter regions often show abnormal methylation, resulting in gene inactivation and thus tumorigenesis. The CpG islands within the gene promoter region are the targets for methylation, as this prevents gene transcription. Methylation of tumor suppressor genes can lead to permanent gene silencing, such that the proteins are never expressed and cannot inhibit growth and differentiation.

Methylation leads to epigenetic silencing of tumor suppressor genes is now known to be common in many human tumors, including bladder cancer.[39] RASSF1A is a tumor suppressor gene, and its inactivation can occur due to methylation of the promoter region, gene mutation or loss of heterozygosity, and lack of homozygosity, although studies have shown that abnormal methylation of the promoter region is the major mechanism. In most human epithelial tumors, the RASSF1A promotor is highly methylated. For example, 94% of small cell lung cancer,[44] 87% of breast cancer,[45] 74% of prostate cancer,[46] 76% of renal cell carcinoma,[47] and 91% of nasopharyngeal carcinoma[48] had abnormal methylation of the *RASSF1A* gene promoter. Although a previous study investigated the relationship between RASSF1A promoter methylation and bladder cancer risk,[6] the differences in gender proportion, age distribution, racial composition, test methods, and primers used in the studies would result in some differences in the results, and moreover, that study did not consider all of these fields. Therefore, an integrated analysis to quantify the ability to test for such methylation in bladder cancer was performed. A significant association was identified between methylation of the *RASSF1A* gene promoter and the risk of bladder cancer ($P<0.0001$) as well as prognosis ($P<0.05$), using a meta-analysis. Subgroup analysis by race, sample type, methods, and primers used also showed that *RASSF1A* gene promoter methylation was associated with bladder cancer risk.

Meta-analysis involves a merger effect on the results between multiple studies, whereas it should be stressed that only homogeneous studies would merge. Therefore, if the difference between the studies is too big, they cannot merge together. In other words, meta-analysis results may be affected by heterogeneity. The heterogeneity in meta-analysis is mainly caused by methodological differences and biological effects resulting from different subject group characteristics in each study, such as age, gender, and race composition. In our meta-analysis, no heterogeneity in the analysis of bladder cancer risk (including subgroup analysis by race, sample source, method, and primers), also in the group analysis of age, gender, and smoking status, whereas there was heterogeneity in the analysis of TNM stage and differentiation grade. However, the number of articles matched by age, gender, and smoking status was very small, which could affect the veracity of the meta-analysis results. Therefore, we decided to use the data from TCGA project to further support our meta-analysis results. The data from TCGA project can avoid the heterogeneity produced by methodological differences and biological effects. Moreover, we did not have to consider the human factor and the bias between different researchers. We therefore analyzed the data from TCGA project and found a significant association between *RASSF1A* gene promoter methylation and the risk, the prognosis, the TNM stage, and the differentiation grade of bladder cancer. Hence, these results confirm the results of the meta-analysis.

Analysis was performed to assess the influence of publication bias on the random-effects model in the meta-analysis of bladder cancer risk. We found no obvious asymmetry in the 21 articles and no single study was found to affect the pooled OR. The pooled sensitivity of the 21 articles was 0.96 and the specificity was 0.47 with the area under the curve of 0.93. Hence, the diagnostic accuracy of the included studies was high and the

meta-analysis results were overall very reliable. This therefore indicates a strong association between RASSF1A promoter methylation and bladder cancer risk. It should be noted that although no association could be made between *RASSF1A* gene promoter methylation and age, gender, or smoking status of the bladder cancer patients, publication bias could not be fully eliminated. This is therefore a major limitation of the study, although it is reassuring that TCGA project data analysis reached the same conclusion.

RASSF1A can inhibit cell proliferation, control cell cycle, promote cell apoptosis, and aging. However, whether methylation of the *RASSF1A* gene promoter contributes to TNM stage in tumors and their differentiation grade remains unclear. In all of the data selected for this analysis, the TNM stage III–IV groups indicated a higher significance for *RASSF1A* gene promoter methylation in bladder cancer ($P < 0.0001$) than TNM stage 0–II. When high and low grade bladder cancers were compared for *RASSF1A* gene promoter methylation, a significant difference was identified. These results were similar to the results of several studies previously published,[15,18,19] indicating that advanced cancer has a higher frequency of *RASSF1A* gene promoter methylation.

5. Conclusion

In conclusion, this integrated analysis of pooled data provides strong evidence that the methylation status of the *RASSF1A* gene promoter is strongly associated with both the risk of developing bladder cancer and patient prognosis. In addition, RASSF1A promoter methylation is strongly associated with an advanced TNM stage and differentiation grade of bladder cancer. Therefore, methylation of the *RASSF1A* gene promoter will be a promising diagnostic assay for the clinical diagnosis of bladder cancer.

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