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ORIGINAL RESEARCH

RASSF1A promoter methylation correlates development, progression, and poor cancer-specific survival of renal cell carcinoma: trial sequential analysis

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Background: This meta-analysis evaluated the clinicopathologic and prognostic significance of *RASSF1A* promoter methylation in renal cell carcinoma (RCC).

Materials and methods: The ORs or HRs and their 95% CIs were calculated. Trial sequential analysis was conducted.

Results: Twenty-two articles that included 1,421 patients with RCC and 724 controls were identified. *RASSF1A* promoter methylation correlated with RCC in tissue, blood, and urine samples. On multivariate analysis, *RASSF1A* promoter methylation was associated with tumor grade (grade 3–4 vs 1–2: OR=3.59), clinical stage (stage 3–4 vs 1–2: OR=2.15), T classification (pT2–4 vs pT1: OR=2.66), histologic subtypes (papillary vs clear cell: OR=2.91), and cancer-specific survival (HR=1.78), but it was not linked to age, gender, lymph node status, distant metastasis, or overall survival. The Cancer Genome Atlas data also showed that *RASSF1A* methylation was significantly more likely to be seen in papillary vs clear-cell RCC (OR=23.19).

Conclusion: *RASSF1A* promoter methylation may be associated with the development and progression of RCC, as well as poor cancer-specific survival. Methylation was more frequent in papillary vs clear-cell RCC. More studies are needed to confirm these findings in blood or urine samples.

Keywords: RAS association domain family protein 1A, methylation, survival, clinical features

Introduction

Renal cell carcinoma (RCC) is the most common malignant tumor affecting the kidneys, accounting for about 90% of kidney carcinomas.[1] Approximately 63,990 new RCC cases were diagnosed in the USA in 2017, and these were associated with an estimated 14,400 deaths.[2] There are two common histological subtypes of RCC. Clear-cell RCC (ccRCC) is the most common, accounting for 70%–80% of all renal cancer cases. Papillary RCC (pRCC) represents another 10%–20% of cases.[3,4] Approximately 25%–30% of patients with RCC present with advanced or metastatic disease, and the 5-year survival rate is poor.[5]

DNA methylation within the promoter regions is an important mechanism underlying epigenetic modifications, which may cause inactivation of gene expression and play a crucial role in the carcinogenesis, progression, and prognosis of various human cancers.[6–8] Previous studies have suggested that promoter methylation of some cancer-related genes is found in RCC, such as *HOXB13*[9] and *CDKN2A/2B*.[10] *RASSF1A* is a key isoform of *RASSF1* located on the chromosomal region 3p21.3.[11] An important

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tumor suppressor gene, *RASSF1A* is involved in cell cycle regulation, microtubule stabilization, cellular adhesion and motility, and cell apoptosis.[12–14]

RASSF1A promoter methylation has been reported in tissue, blood, and urine samples from patients with RCC.[15–17] There are, however, inconsistent results regarding the level of *RASSF1A* promoter methylation in patients with RCC and controls. For example, Ellinger et al reported that the *RASSF1A* promoter had a similar methylation rate in RCC and adjacent normal tissue samples.[18] In contrast, *RASSF1A* promoter methylation was more frequent in RCC than in adjacent normal tissue samples in a study by Loginov et al.[19] With this background of conflicting results, we conducted a meta-analysis to assess differences in *RASSF1A* promoter methylation between RCC and control tissue, blood, and urine samples. Moreover, we evaluated the association of *RASSF1A* promoter methylation with clinicopathologic features and prognosis in patients with RCC.

Materials and methods

Search strategy

A systematic literature search of the PubMed, EMBASE, EBSCO, Wanfang, and CNKI databases was conducted to identify eligible studies published through December 1, 2017, without any language restrictions. The following keywords and scientific search terms were used: (kidney OR renal) AND (cancer OR tumor OR neoplasm OR carcinoma) AND (methylation OR methylated OR hypermethylation OR epigene*) AND (RAS association domain family protein 1A OR RASSF1A OR RASSF1 OR RAS association domain family protein 1). We manually searched the relevant references from all eligible articles to find other potential publications.

Selection criteria

Articles that met the following inclusion criteria were selected for the meta-analysis: 1) patients were confirmed with adult RCC by histopathologic examination; 2) studies reported sufficient data to evaluate differences in *RASSF1A* promoter methylation between the RCC and control groups; 3) studies had sufficient data to assess the correlation of *RASSF1A* promoter methylation with clinicopathologic features; and 4) studies provided enough survival data to evaluate the prognostic effect of *RASSF1A* promoter methylation in RCC. When multiple papers using the same patient population were published, the study with more information was included in the meta-analysis.

Data extraction

The following information was extracted from the included publications: surname of the first author, year of publication, country, ethnic population, cancer stage, mean or median age, sample type, detection method, histologic type, number of cases and controls, survival data with multivariate analysis, and clinicopathologic features such as age (≥50 vs <50 years), gender (male vs female), tumor grade (3–4 vs 1–2), clinical stage (3–4 vs 1–2), T classification (pT2–4 vs pT1), histologic subtypes (pRCC vs ccRCC), lymph node metastasis (yes vs no), and distant metastasis (yes vs no).

The Cancer Genome Atlas (TCGA) dataset

Clinical information for RCC, which included two sets of samples (methylation 450 K dataset: 275 pRCCs and 319 ccRCCs), was downloaded from the TCGA data portal (https://portal.gdc.cancer.gov/repository). The cutoff value of *RASSF1A* promoter methylation was set by its median value. The association between clinicopathologic characteristics and *RASSF1A* promoter methylation was analyzed using logistic regression (R; v.3.4.3). Multivariate Cox analysis was used to analyze the impact of *RASSF1A* promoter methylation on overall survival (R; v.3.4.3).

Statistical analysis

All data analyses were performed using Stata software 12.0 (StataCorp LP, College Station, TX, USA). Differences in *RASSF1A* promoter methylation between RCC and control samples and the correlation of *RASSF1A* promoter methylation with the clinicopathologic characteristics of patients with RCC were calculated using pooled ORs and the corresponding 95% CIs. Overall HRs with their 95% CIs were also calculated to determine the prognostic role of *RASSF1A* promoter methylation, using multivariate analysis if possible. The Cochran's Q statistic was used to estimate possible heterogeneity among studies.[20,21] A random-effects model was applied in the meta-analysis. When substantial heterogeneity was measured ($P<0.1$), a sensitivity analysis was carried out to determine the influence of an individual study on the pooled OR and heterogeneity by deleting one study at a time.[22,23] For results covered by more than nine studies, possible publication bias was detected with Egger's test.[24] We performed trial sequential meta-analyses (TSA) to reduce type I error and to calculate the estimated required sample size information.[25,26] For significant results with more than one study, the type I error rate was set at

5% and the type II error rate was considered to be 20% (a statistical test power of 80%). The relative risk reduction was set at 20% in the meta-analysis. If the cumulative Z-curve crossed the trial sequential monitoring boundary or the required information size, the statistical evidence was deemed conclusive. Otherwise, additional studies would be needed for a definitive result.[27,28]

Results

Study characteristics

Figure 1 summarizes the details of the study selection procedure; 22 publications with a total of 1,421 patients with RCC and 724 controls fulfilled the inclusion criteria and were selected for the meta-analysis.[15–19,29–45] Of the included publications, 15 assessed differences in *RASSF1A* promoter methylation between RCC and control samples using tissue samples and 6 used blood or urine samples. Sixteen studies evaluated the relationships between *RASSF1A* promoter methylation and the clinicopathologic characteristics of patients with RCC. Two studies reported information on survival in patients with RCC using multivariate analysis. The baseline characteristics of the included publications are presented in Tables 1 and S1.

Correlation between *RASSF1A* promoter methylation and RCC in cancer vs control tissue samples

In 15 studies that included the comparison of 829 patients with RCC and 467 adjacent/normal tissue samples (Figure 2), *RASSF1A* promoter methylation was notably higher in RCC than in adjacent/normal tissue samples (OR=5.64, 95% CI=1.82–17.51, P=0.003).

Subgroup and sensitivity analyses in cancer vs control tissue samples

We conducted subgroup analyses by ethnicity (Asians and Caucasians) and testing method (methylation-specific polymerase chain reaction [MSP] and non-MSP) (Table 2). In the ethnicity analysis, *RASSF1A* promoter methylation was associated with RCC in Caucasians (OR=4.90, 95% CI=1.45–16.64, P=0.011), but not in Asians (OR=9.27, 95% CI=0.35–243.63, P=0.182). In the testing method analysis, *RASSF1A* promoter methylation was associated with RCC in the MSP subgroup (OR=16.32, 95% CI=5.25–50.69, $P<0.001$), but not in the non-MSP subgroup (OR=1.85, 95% CI=0.27–12.48, P=0.527).

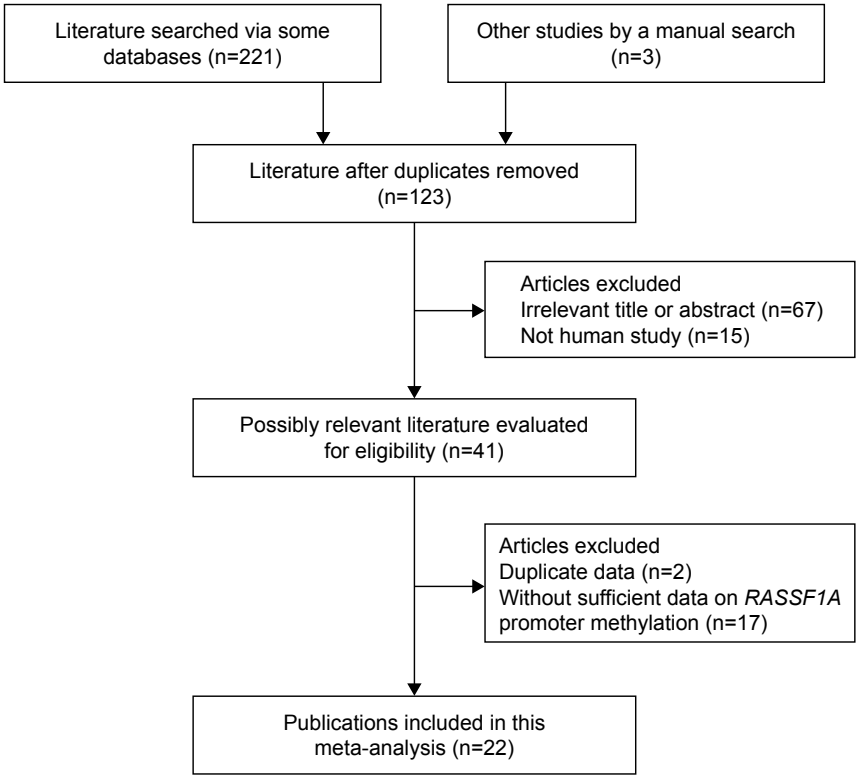

Figure 1 Flow diagram of the study selection procedure.

Table 1 Baseline characteristics of the included publications

First author	Country	Ethnicity	Age	Stage	Method	Histology	Sample	Control type	Cancer	Control	Clinical features	MA (survival)
									n (M %)	n (M %)		
Morrissey et al, 2001[43]	UK	Caucasians	NA	NA	#	RCC	Tissue	Adjacent normal	211 (28)	80 (2.5)	Yes	NA
Yoon et al, 2001[42]	USA	Caucasians	NA	NA	BSQ	RCC	Tissue	Normal	32 (56.3)	10 (0)	Yes	NA
Battagli et al, 2003[41]	USA	Caucasians	NA	1–4	MSP	RCC	Tissue	Normal	50 (52)	15 (0)	Yes	NA
Battagli et al, 2003[41]	USA	Caucasians	NA	1–4	MSP	RCC	Urine	Nonmalignant	50 (50)	24 (0)	Yes	NA
Yano et al, 2004[40]	Japan	Asians	NA	NA	MSP	RCC	Tissue	Adjacent normal	29 (65.5)	29 (6.9)	NA	NA
Dulaimi et al, 2004[39]	USA	Caucasians	NA	NA	MSP	RCC	Tissue	Normal	99 (45.5)	15 (0)	Yes	NA
Hoque et al, 2004[17]	USA	Caucasians	NA	NA	QMSP	RCC	Urine	Nonmalignant	26 (65.4)	91 (11)	NA	NA
Hoque et al, 2004[17]	USA	Caucasians	NA	NA	QMSP	RCC	Blood	Nonmalignant	18 (11.1)	30 (3.3)	NA	NA
Tokinaga et al, 2004[38]	Japan	Asians	NA	1–4	COBRA	ccRCC	Tissue	Adjacent normal	50 (78)	39 (97.4)	Yes	NA
Loginov et al, 2004[37]	Russia	Caucasians	NA	1–4	MSRA	ccRCC	Tissue	Adjacent normal	53 (94.3)	30 (33.3)	Yes	NA
Gonzalgo et al, 2004[36]	USA	Caucasians	61.1	>1	QMSP	RCC	Tissue	Adjacent normal	38 (78.9)	22 (90.9)	Yes	NA
Peters et al, 2007[34]	Germany	Caucasians	NA	NA	COBRA	RCC	Tissue	Adjacent normal	45 (97.8)	45 (97.8)	NA	NA
Costa et al, 2007[33]	Portugal	Caucasians	61	1–4	QMSP	RCC	Tissue	Adjacent normal	85 (80)	62 (100)	Yes	NA
Hori et al, 2007[35]	Japan	Asians	NA	NA	MSP	RCC	Tissue	NA	42 (97.6)	NA	Yes	NA
Duan et al, 2007[44]	China	Asians	55	1–4	MSP	RCC	Tissue	Adjacent normal	26 (65.4)	26 (0)	NA	NA
Yuan et al, 2008[45]	China	Asians	NA	NA	MSP	RCC	Tissue	Adjacent normal	19 (52.6)	19 (0)	NA	NA
Loginov et al, 2009[19]	Russia	Caucasians	NA	1–4	MSP	RCC	Tissue	Adjacent normal	39 (74.4)	39 (15.4)	Yes	NA
Onay et al, 2009[32]	Turkey	Caucasians	59.2	1–3	MSP	RCC	Tissue	Adjacent normal	21 (52.4)	21 (38.1)	Yes	NA
Kawai et al, 2010[31]	Japan	Asians	65	1–4	COBRA	ccRCC	Tissue	NA	179 (49.7)	NA	Yes	Yes
Ellinger et al, 2011[18]	Germany	Caucasians	60.5	1–3	QMSP	pRCC	Tissue	Adjacent normal	32 (100)	15 (93.3)	Yes	NA
de Martino et al, 2012[30]	Austria	Caucasians	64.7	1–3	QMSP	RCC	Blood	Nonmalignant	157 (45.9)	43 (7)	NA	NA
Hauser et al, 2013[29]	Germany	Caucasians	66	NA	MSRA	RCC	Blood	Healthy	35 (22.9)	54 (1.9)	NA	NA
Klacz et al, 2016[16]	Poland	Caucasians	62.16	NA	MSHRM	ccRCC	Tissue	NA	58 (39.7)	NA	Yes	Yes
Skrypkina et al, 2016[15]	Ukraine	Caucasians	NA	2–3	QMSP	RCC	Blood	Healthy	27 (63)	15 (6.7)	Yes	NA

Note: "#" stands for bisulfite modification, direct sequencing, and restriction enzyme digestion.

Abbreviations: BSQ, bisulfite sequencing; ccRCC, clear cell RCC; COBRA, combined bisulfite restriction analysis; M, methylation-positive status; MA, multivariate analysis; MSHRM, methylation-sensitive high-resolution melting analysis; MSP, methylation-specific polymerase chain reaction; MSRA, methylation-sensitive restriction enzyme analysis; NA, not applicable; pRCC, papillary RCC; QMSP, quantitative methylation-specific polymerase chain reaction; RCC, renal cell carcinoma.

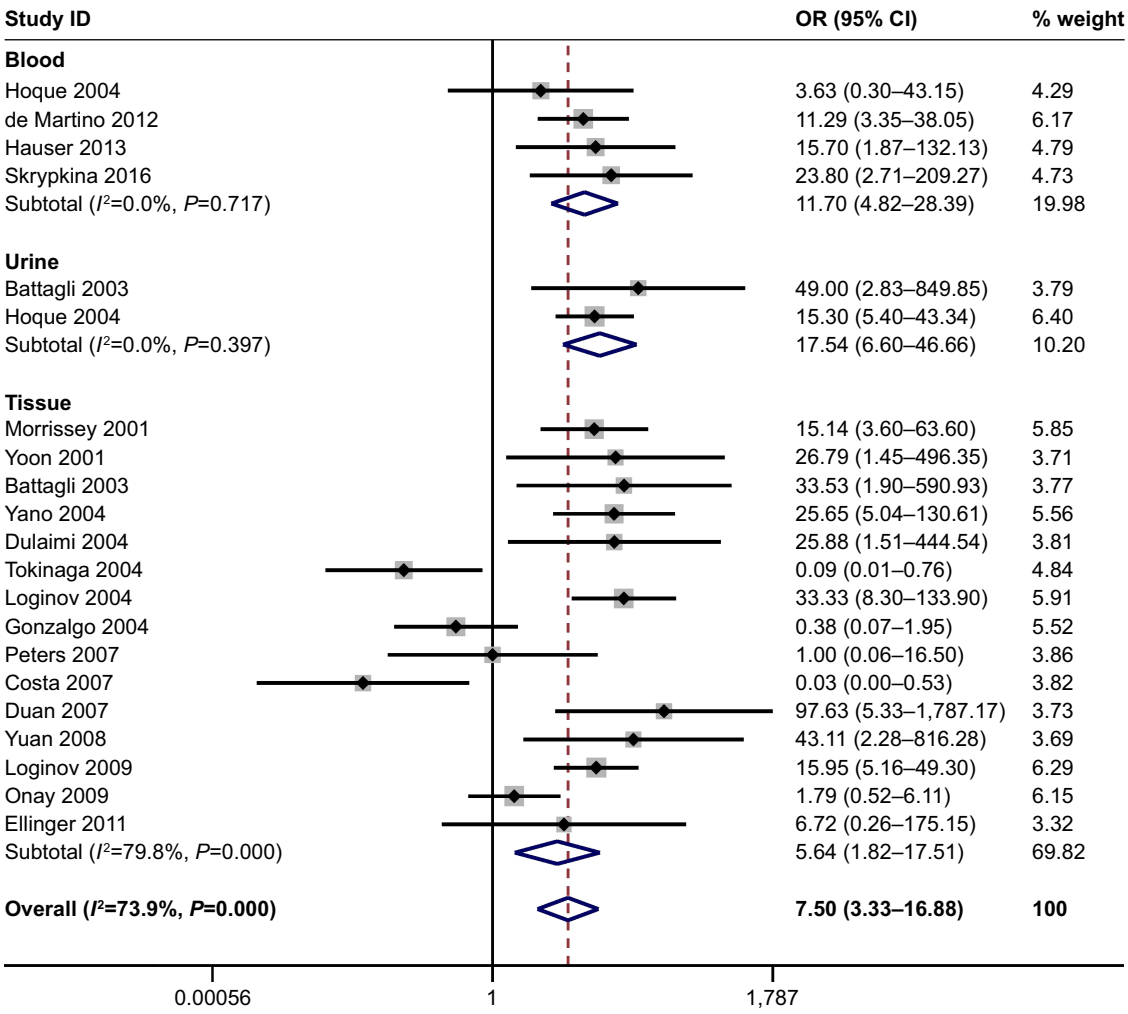

Figure 2 Forest plot of the association of *RASSF1A* promoter methylation in the RCC vs control group using tissue: OR=5.64, 95% CI=1.82–17.51, *P*=0.003; blood: OR=11.70, 95% CI=4.82–28.39, *P*<0.001; and urine: OR=17.54, 95% CI=6.60–46.66, *P*<0.001.
Note: Weights are from random-effects analysis.
Abbreviation: RCC, renal cell carcinoma.

There was evidence of significant heterogeneity in cancer vs control tissue samples, so we performed a sensitivity analysis. We successively removed four studies – Tokinaga et al[38] in Japan, Costa et al[33] in Portugal, Gonzalgo et al[36] in the USA, and Onay et al[32] in Turkey – and recalculated the overall OR (OR=19.78, 95% CI=11.09–35.29, *P*<0.001), resulting in no heterogeneity (*P*=0.693).

Correlation between *RASSF1A* promoter methylation and RCC in cancer vs control blood or urine samples

In four studies that included the comparison of 237 patients with RCC with 142 nonmalignant blood samples, *RASSF1A* promoter methylation was significantly more likely in RCC than in nonmalignant blood samples (OR=11.70,

Table 2 Subgroup analyses of *RASSF1A* promoter methylation in cancer vs control tissue samples

Subgroup analyses	Pooled OR (95% CI)	Heterogeneity (*P*)	*P*-value	Cases	Controls
Ethnicity					
Caucasians	4.90 (1.45–16.64)	<0.001	0.011	705	354
Asians	9.27 (0.35–243.63)	<0.001	0.182	124	113
Testing method					
Non-MSP	1.85 (0.27–12.48)	<0.001	0.527	546	303
MSP	16.32 (5.25–50.69)	0.028	<0.001	283	164

Abbreviation: MSP, methylation-specific polymerase chain reaction.

95% CI=4.82–28.39, $P<0.001$; Figure 2). In addition, in a comparison of 76 RCCs and 115 nonmalignant urine samples, *RASSF1A* promoter methylation was significantly higher in RCC than in nonmalignant urine samples (OR=17.54, 95% CI=6.60–46.66, $P<0.001$; Figure 2).

Correlation of *RASSF1A* promoter methylation with age and gender in RCC

Seven studies that included 321 patients with RCC demonstrated that *RASSF1A* promoter methylation was not correlated with age (OR=1.00, 95% CI=0.50–2.04, $P=0.99$; Figure 3). Eight studies that included 537 patients with RCC showed that *RASSF1A* promoter methylation was not correlated with gender (OR=0.95, 95% CI=0.51–1.76, $P=0.86$; Figure 3).

Correlation of *RASSF1A* promoter methylation with lymph node status and distant metastasis in RCC

In seven studies that included 438 patients with RCC, *RASSF1A* promoter methylation was not associated with lymph node metastasis (OR=1.72, 95% CI=0.76–3.87, $P=0.192$; Figure 4). Four studies that included 257 patients with RCC showed that there was no correlation between *RASSF1A* promoter methylation and distant metastasis (OR=1.66, 95% CI=0.75–3.69, $P=0.21$; Figure 4).

Correlation of *RASSF1A* promoter methylation with tumor grade and clinical stage in RCC

In 13 studies that included 686 patients with RCC, a significant relationship was observed between *RASSF1A* promoter methylation and tumor grade (OR=3.59, 95% CI=1.85–6.95, $P<0.001$; Figure 5). *RASSF1A* promoter methylation was also linked to clinical stage in eight studies that included 463 patients with RCC (OR=2.15, 95% CI=1.34–3.45, $P=0.001$; Figure 5).

Correlation of *RASSF1A* promoter methylation with T classification and histologic subtypes in RCC

In seven studies that included 306 patients with RCC, a significant correlation was found between *RASSF1A*

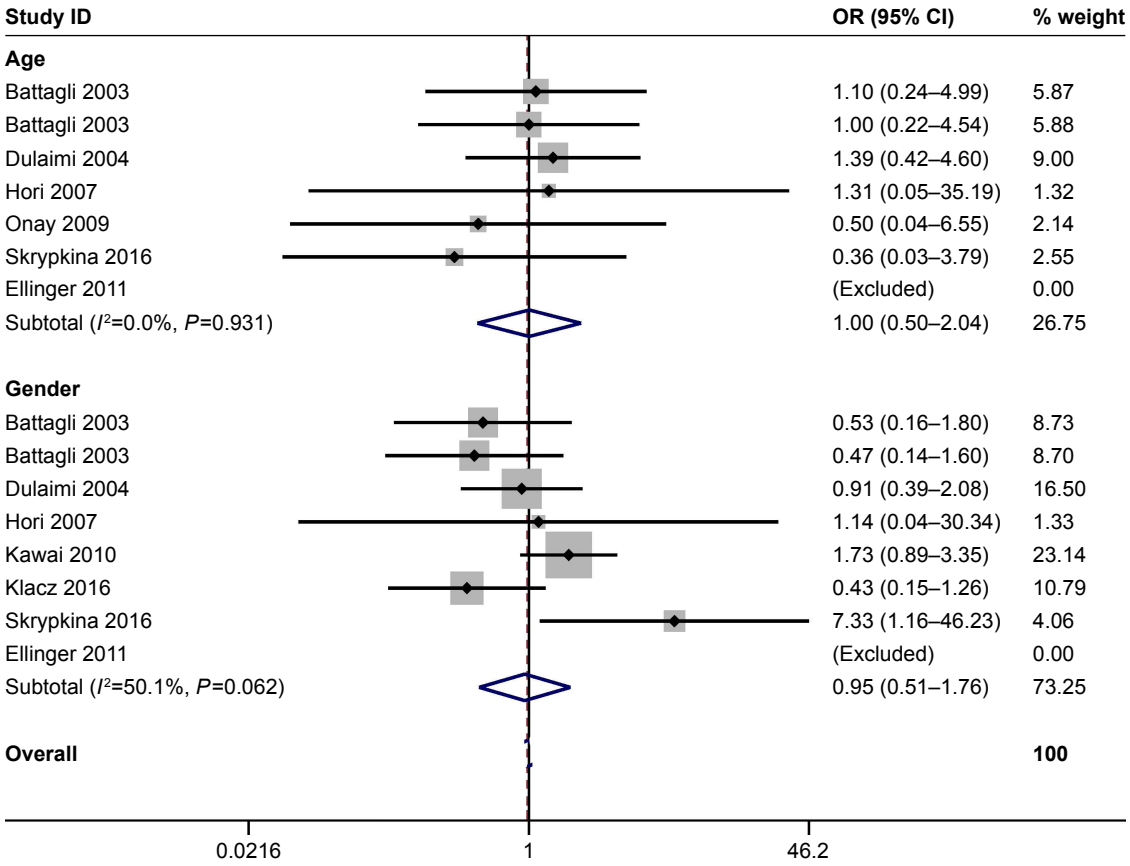

Figure 3 Forest plot of the association of *RASSF1A* promoter methylation with age (OR=1.00, 95% CI=0.50–2.04, $P=0.99$) and gender (OR=0.95, 95% CI=0.51–1.76, $P=0.86$) in RCC.
Note: Weights are from random-effects analysis.
Abbreviation: RCC, renal cell carcinoma.

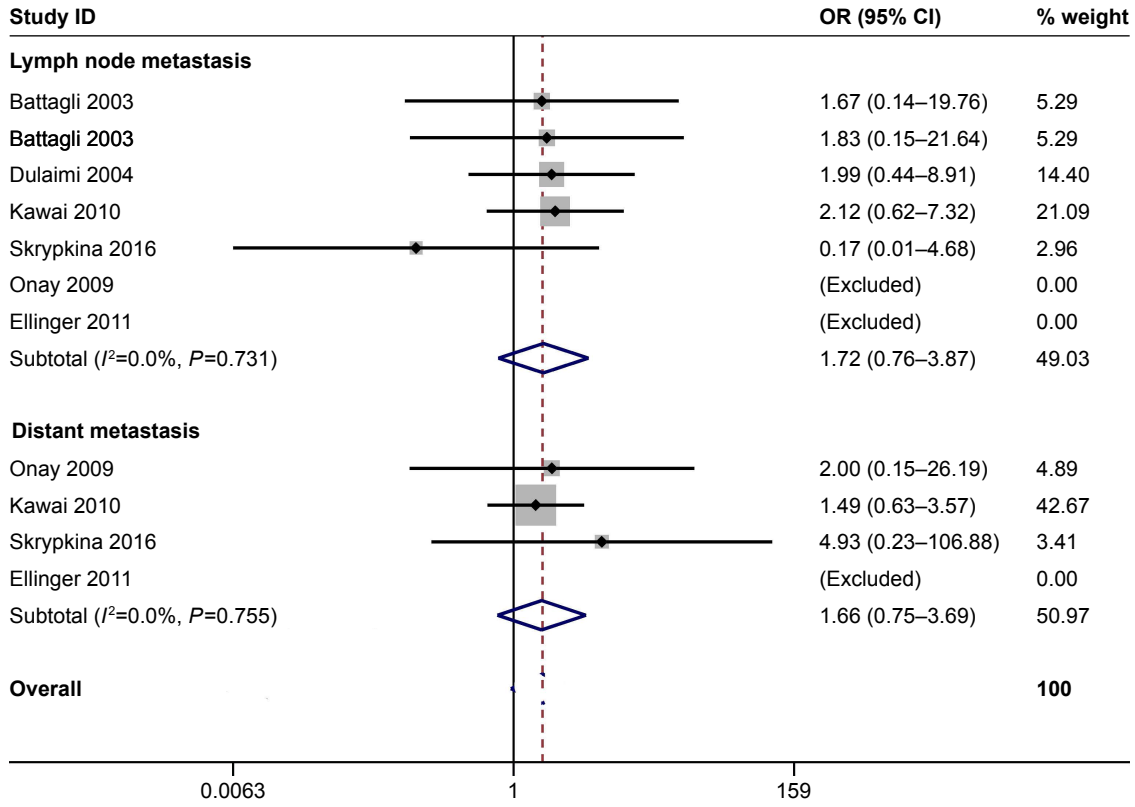

Figure 4 Forest plot of the association of *RASSF1A* promoter methylation with lymph node metastasis (OR=1.72, 95% CI=0.76–3.87, *P*=0.192) and distant metastasis (OR=1.66, 95% CI=0.75–3.69, *P*=0.21) in RCC.
Note: Weights are from random-effects analysis.
Abbreviation: RCC, renal cell carcinoma.

promoter methylation and T classification (OR=2.66, 95% CI=1.11–6.39, *P*=0.029; Figure 6). *RASSF1A* promoter methylation was also significantly associated with histologic subtypes in eight studies that included 472 patients with RCC (OR=2.91, 95% CI=1.61–5.23, $P<0.001$; Figure 6).

Prognostic role of *RASSF1A* promoter methylation using multivariate analysis

Kawai et al reported that *RASSF1A* promoter methylation was a poor prognostic factor in terms of cancer-specific survival among 179 patients with ccRCC (HR=1.78, 95% CI=1.18–2.78).[31] Klacz et al reported that *RASSF1A* promoter methylation was not associated with overall survival using multivariate analysis among 58 patients with ccRCC.[16] More studies with large patient population are needed to further investigate the prognostic role of *RASSF1A* promoter methylation in RCC.

Publication bias

There was no evidence of publication bias using Egger's test for the comparison of RCC vs control tissue samples (*P*=0.782) or in relation to tumor grade (*P*=0.547; Figure 7).

Trial sequential meta-analysis

As shown in Figures 8 and 9, based on the a priori anticipated information size method for significant results, when cancer was compared with control tissue samples, the cumulative Z-curve crossed the trial sequential monitoring boundary and the required information size (Figure 8), suggesting conclusive results. When cancer was compared with control blood or urine samples, the cumulative Z-curve was more than the conventional boundary, but did not cross the trial sequential monitoring boundary (Figure 8), which suggests that more studies are needed to inform these two results. In relation to tumor grade, clinical stage, and histologic subtypes, the cumulative Z-curve was more than the trial sequential monitoring boundary (Figures 8 and 9), which suggests that additional studies are not necessary. In relation to T classification, the cumulative Z-curve crossed the conventional boundary, but did not cross the trial sequential monitoring boundary (Figure 8), suggesting that further studies are essential.

TCGA dataset

After adjusting for tumor stage (stage 3–4 vs stage 1–2) and tumor histology (pRCC vs ccRCC), *RASSF1A* promoter

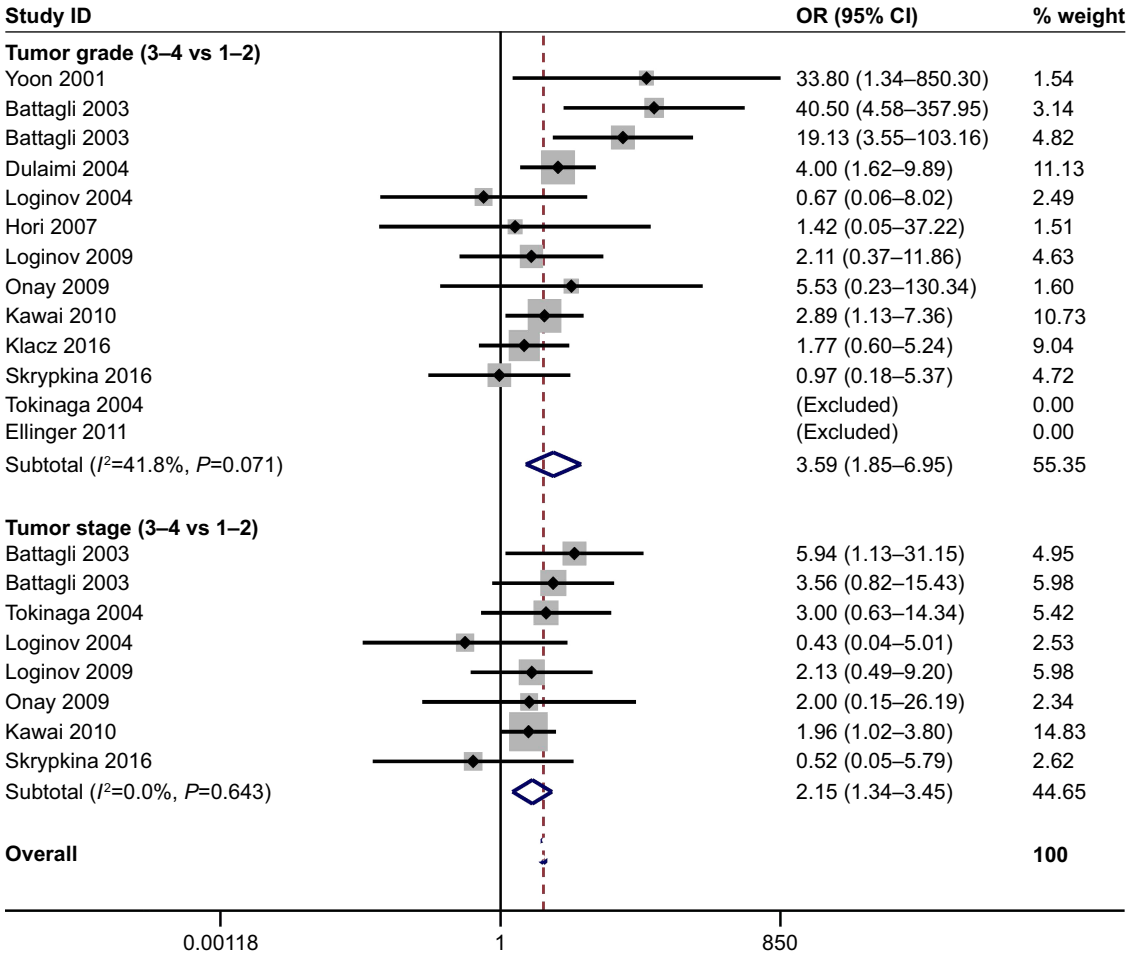

Figure 5 Forest plot of the correlation of *RASSF1A* promoter methylation with tumor grade (OR=3.59, 95% CI=1.85–6.95, $P<0.001$) and clinical stage (OR=2.15, 95% CI=1.34–3.45, $P=0.001$) in RCC.
Note: Weights are from random-effects analysis.
Abbreviation: RCC, renal cell carcinoma.

methylation was not associated with overall survival using multivariate analysis (HR=0.921, $P=0.687$) in 567 RCCs.

RASSF1A promoter methylation was not significantly linked to gender (594 patients: OR=1.35, 95% CI=0.95–1.91, $P=0.094$), but it did correlate with clinical stage (568 patients: $P=0.023$) and tumor histology (594 patients: pRCC vs ccRCC: OR=23.19, 95% CI=15.07–35.7, $P<0.001$; Table 3).

Discussion

Tumor suppressor genes are commonly inactivated via promoter methylation within the CpG islands, which may affect several biological processes, including cell proliferation, cell death, cell migration, and cell invasion, and contribute to the initiation and progression of human cancers.[46,47] Studies have indicated that methylation of the promoter of the tumor suppressor gene *RASSF1A* reduces its expression, which may play an important role in RCC carcinogenesis.[40,43] However, potential differences in methylation between RCC and control tissue samples have remained unclear owing to conflicting evidence from previous studies. Two studies showed that *RASSF1A* promoter methylation correlated negatively with RCC.[33,38] Four studies reported no association between *RASSF1A* promoter methylation and RCC.[18,32,34,36] Also, nine other studies showed that *RASSF1A* promoter methylation correlated positively with RCC.[19,37,39–45] The present meta-analysis including all eligible publications with large patient populations demonstrated that *RASSF1A* promoter methylation was notably higher in RCC than in adjacent or normal tissue samples; TSA revealed that the result was conclusive. This suggests that *RASSF1A* promoter methylation is significantly associated with RCC carcinogenesis.

When RCC was compared with control tissue samples, a subgroup analysis of ethnicity showed that *RASSF1A* promoter

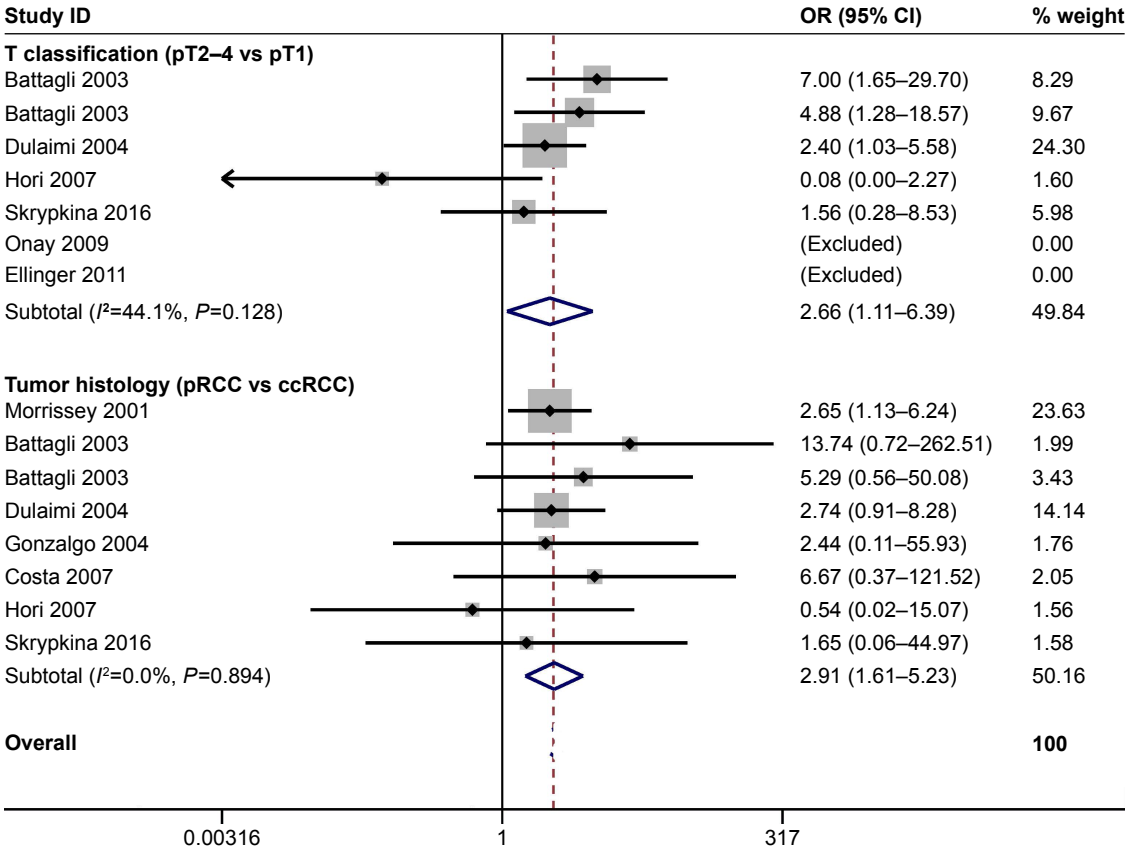

Figure 6 Forest plot of the correlation of *RASSF1A* promoter methylation with T classification (OR=2.66, 95% CI=1.11–6.39, *P*=0.029) and histologic subtypes (OR=2.91, 95% CI=1.61–5.23, *P*<0.001) in RCC.
Note: Weights are from random-effects analysis.
Abbreviations: ccRCC, clear cell RCC; pRCC, papillary RCC; RCC, renal cell carcinoma.

methylation was associated with RCC in Caucasians, but not in Asians, which suggests that only the Caucasian population is susceptible to *RASSF1A* promoter methylation. A subgroup analysis of detection method demonstrated that *RASSF1A* promoter methylation correlated with RCC in the MSP subgroup, but not in the non-MSP subgroup, which indicates that the MSP method may be sensitive to the detection of *RASSF1A* promoter methylation. We performed a sensitivity analysis because substantial heterogeneity was measured in the comparison of cancer and control tissue samples. When four studies[32,33,36,38] were successively removed and the pooled OR was recalculated, remaining significant, there was no evidence of heterogeneity (*P*=0.693). It is possible that the main cause of heterogeneity in this meta-analysis was contamination of adjacent normal tissue samples by cancer cells in these four studies. In addition, Egger's test showed no publication bias. The relevant analyses supported the stability and credibility of our results.

RASSF1A promoter methylation was associated with RCC in blood and urine samples (cancer vs nonmalignant controls), which suggested that *RASSF1A* promoter methylation may become a promising noninvasive biomarker for the detection of RCC in the future. According to the results of TSA, additional prospective clinical studies with large sample sizes are required to further investigate whether *RASSF1A* promoter methylation could be used as a biomarker for the diagnosis of RCC based on blood or urine samples.

Finally, we evaluated whether *RASSF1A* promoter methylation was linked to clinicopathologic characteristics and prognosis in patients with RCC. *RASSF1A* promoter methylation did not correlate with age, gender, lymph node status, or distant metastasis. Significant relationships were observed between *RASSF1A* promoter methylation and tumor grade, clinical stage, and T classification, with methylation notably higher in high-grade vs low-grade tumors, advanced vs early-stage patients, and high (pT2–4) vs low (pT1) T classification. These analyses suggest that *RASSF1A* promoter methylation may be closely associated with RCC progression. TSA showed that additional studies are essential to

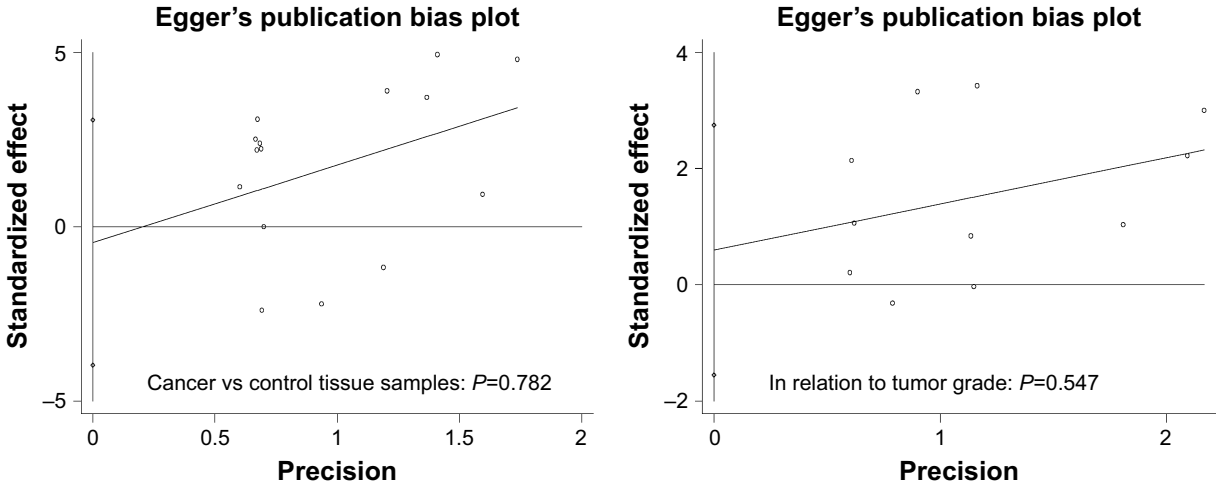

Figure 7 Forest plot of potential publication bias using Egger's test in RCC vs control tissue samples (*P*=0.782) and in relation to tumor grade (*P*=0.547).
Abbreviation: RCC, renal cell carcinoma.

inform the analyses of T classification, but that the analyses of tumor grade, clinical stage, and tumor histology were robust. Additionally, TCGA data showed that *RASSF1A* promoter methylation remained significantly associated with pRCC vs ccRCC (OR=23.19, $P<0.001$), suggesting that it may play a more important role in the pathogenesis of pRCC. On multivariate analysis, *RASSF1A* promoter methylation was associated with poorer cancer-specific survival among 179 patients with ccRCC patients,[31] but did not correlate with overall survival among 58 patients with ccRCC patients.[16] Further analysis using TCGA data showed that no correlation was found between *RASSF1A* promoter methylation and overall survival on multivariate analysis (HR=0.921, *P*=0.687) in 567 RCCs. More studies using multivariate analysis will be crucial to confirm the prognostic impact of *RASSF1A* promoter methylation on cancer-specific survival.

The current results compare favorably with the previous meta-analyses by Yu et al[48] and Huang et al.[49] Yu et al only analyzed whether *RASSF1A* promoter methylation was correlated with RCC in cancer vs nontumor controls,[48] and *RASSF1A* promoter methylation did not correlate with RCC in tissue samples.[48] Our result involving a greater number of eligible studies with a larger population (15 studies with 1,296 tissue samples) showed that *RASSF1A* promoter methylation was significantly associated with RCC in tissue samples. In addition, Yu et al[48] did not report whether *RASSF1A* promoter methylation was linked to clinical features (eg, gender, tumor grade, clinical stage, T classification, histologic subtypes, lymph node metastasis, and distant metastasis) and did not include an analysis of overall survival. Huang et al only analyzed whether *RASSF1A* promoter methylation was linked to tumor stage (five studies with 252 cases) and grade (four studies with 190 cases), showing that *RASSF1A* promoter methylation had a borderline significant correlation with tumor stage (*P*=0.051) and a significant association with tumor grade (*P*=0.001).[49] Our meta-analysis involving more patients suggested that *RASSF1A* promoter methylation was significantly linked to tumor grade (13 studies with 686 patients with RCC, $P<0.001$) and clinical stage (8 studies with 463 patients with RCC, *P*=0.001). Additionally, Huang et al[49] did not analyze whether *RASSF1A* promoter methylation was associated with prognosis (cancer-specific survival or overall survival) or other clinical features such as gender, T stage, and lymph node status.

The current meta-analysis had several limitations. First, the size of the population with blood or urine samples was small. Second, the populations of the included studies mainly consisted of Asians and Caucasians, with limited numbers of other ethnic subgroups, such as Africans. Third, only two studies reported the prognostic role of *RASSF1A* promoter methylation using multivariate analysis in RCC.

Conclusion

The present findings show that *RASSF1A* promoter methylation correlates with RCC in tissue, blood, and urine samples. *RASSF1A* promoter methylation is not linked to age, gender, lymph node status, distant metastasis, or overall survival, but it is associated with tumor grade, clinical stage, T classification, histologic subtypes, and cancer-specific survival on multivariate analysis. Based on TSA, additional studies with large sample sizes are needed to validate these results

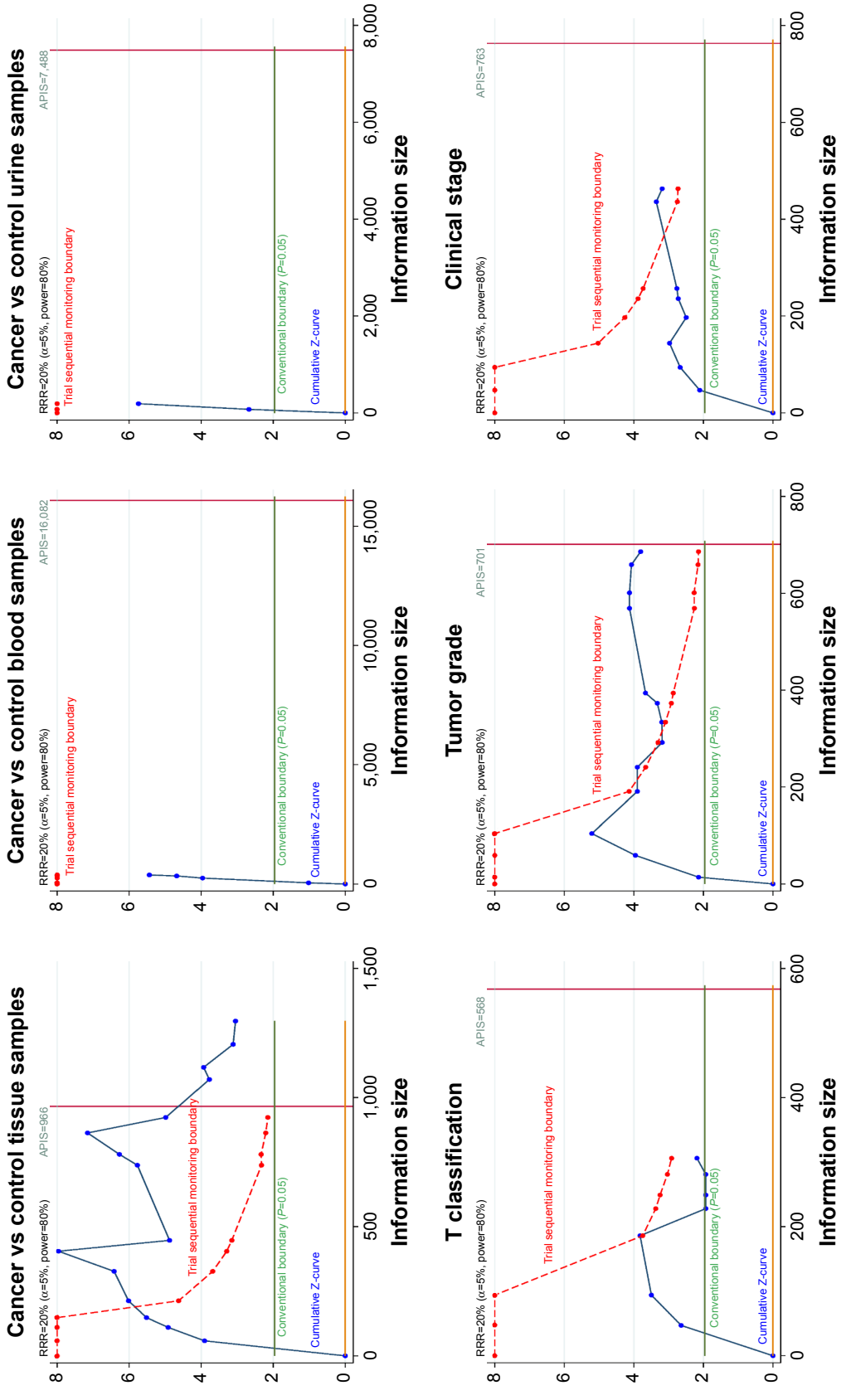

Figure 8 Trial sequential analysis assessing the required sample information in relation to cancer vs control tissue, blood, and urine samples, T classification, tumor grade, and clinical stage.
Abbreviations: APIS, a priori anticipated information size; RRR, relative risk reduction.

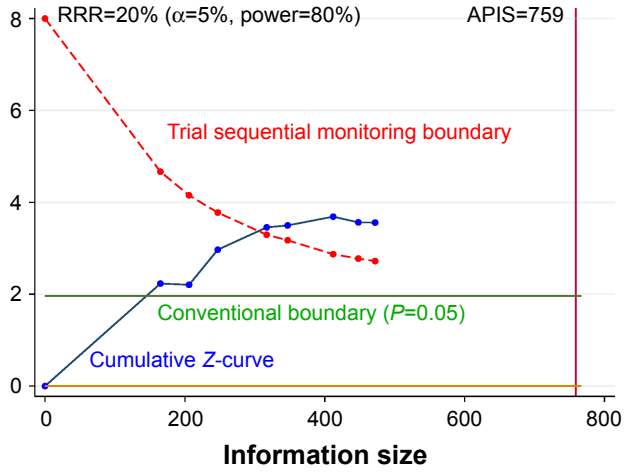

Figure 9 Trial sequential analysis assessing the required sample information in relation to tumor histology.
Abbreviations: APIS, a priori anticipated information size; RRR, relative risk reduction.

Table 3 Association between *RASSF1A* methylation and clinical pathological characteristics from TCGA dataset

Clinical characteristics	Total (n)	OR with 95% CI	*P*-value
Gender (male vs female)	594	1.35 (0.95–1.91)	0.094
Tumor stage (stage 3–4 vs stage 1–2)	594	0.67 (0.47–0.94)	0.023
Tumor histology (papillary RCC vs clear cell RCC)	568	23.19 (15.07–35.7)	<0.001

Note: n, the number of the study population.
Abbreviations: RCC, renal cell cancer; TCGA, The Cancer Genome Atlas.

in cancer vs control blood and urine samples and to confirm the findings regarding T classification and prognosis.

Data sharing statement

All relevant data are included in the paper.

Ethics approval

Although this study was not primary research involving human samples, our study was a secondary analysis regarding human subject data published in the public domain. For this type of study, formal consent is not required.

Acknowledgment

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Disclosure

The authors report no conflicts of interest in this work.

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Supplementary material

Table S1 Baseline characteristics of the included publications with clinicopathologic features

Author, year	Country	Ethnicity	Method	Histology	Sample	Case		≥50 years		<50 years	
						M+	Total	M+	Total	M+	Total
Battagli et al, 2003[13]	USA	Caucasians	MSP	RCC	Tissue	26	50	22	42	4	8
Battagli et al, 2003[13]	USA	Caucasians	MSP	RCC	Urine	25	50	21	42	4	8
Dulaimi et al, 2004[12]	USA	Caucasians	MSP	RCC	Tissue	45	99	40	86	5	13
Hori et al, 2007[8]	Japan	Asians	MSP	RCC	Tissue	41	42	33	34	8	8
Onay et al, 2009[6]	Turkey	Caucasians	MSP	RCC	Tissue	11	21	9	18	2	3
Ellinger et al, 2011[3]	Germany	Caucasians	QMSP	pRCC	Tissue	32	32	26	26	6	6
Skrypkina et al, 2016[1]	Ukraine	Caucasians	QMSP	RCC	Blood	17	27	13	22	4	5
Author, year	**Country**	**Ethnicity**	**Method**	**Histology**	**Sample**	**Case**		**Male**		**Female**	
						M+	Total	M+	Total	M+	Total
Battagli et al, 2003[13]	USA	Caucasians	MSP	RCC	Tissue	26	50	16	34	10	16
Battagli et al, 2003[13]	USA	Caucasians	MSP	RCC	Urine	25	50	15	34	10	16
Dulaimi et al, 2004[12]	USA	Caucasians	MSP	RCC	Tissue	45	99	29	65	16	34
Hori et al, 2007[8]	Japan	Asians	MSP	RCC	Tissue	41	42	32	33	9	9
Kawai et al, 2010[5]	Japan	Asians	COBRA	ccRCC	Tissue	89	179	69	129	20	50
Ellinger et al, 2011[3]	Germany	Caucasians	QMSP	pRCC	Tissue	32	32	26	26	6	6
Klacz et al, 2016[2]	Poland	Caucasians	MSHRM	ccRCC	Tissue	23	58	9	30	14	28
Skrypkina et al, 2016[1]	Ukraine	Caucasians	QMSP	RCC	Blood	17	27	11	13	6	14
Author, year	**Country**	**Ethnicity**	**Method**	**Histology**	**Sample**	**Case**		**Grade 3–4**		**Grade 1–2**	
						M+	Total	M+	Total	M+	Total
Yoon et al, 2001[14]	USA	Caucasians	BSQ	RCC	Tissue	18	32	6	8	0	6
Battagli et al, 2003[13]	USA	Caucasians	MSP	RCC	Tissue	26	50	18	19	8	26
Battagli et al, 2003[13]	USA	Caucasians	MSP	RCC	Urine	25	50	17	19	8	26
Dulaimi et al, 2004[12]	USA	Caucasians	MSP	RCC	Tissue	45	99	24	36	17	51
Tokinaga et al, 2004[11]	Japan	Asians	COBRA	ccRCC	Tissue	39	50	0	0	20	50
Loginov et al, 2004[10]	Russia	Caucasians	MSRA	ccRCC	Tissue	50	53	12	13	36	38
Hori et al, 2007[8]	Japan	Asians	MSP	RCC	Tissue	41	42	13	13	28	29
Loginov et al, 2009[4]	Russia	Caucasians	MSP	RCC	Tissue	29	39	10	12	19	27
Onay et al, 2009[6]	Turkey	Caucasians	MSP	RCC	Tissue	11	21	2	2	9	19
Kawai et al, 2010[5]	Japan	Asians	COBRA	ccRCC	Tissue	89	179	17	24	69	151
Ellinger et al, 2011[3]	Germany	Caucasians	QMSP	pRCC	Tissue	32	32	5	5	27	27
Klacz et al, 2016[2]	Poland	Caucasians	MSHRM	ccRCC	Tissue	23	58	15	33	8	25
Skrypkina et al, 2016[1]	Ukraine	Caucasians	QMSP	RCC	Blood	17	27	5	8	12	19
Author, year	**Country**	**Ethnicity**	**Method**	**Histology**	**Sample**	**Case**		**Stage 3–4**		**Stage 1–2**	
						M+	Total	M+	Total	M+	Total
Battagli et al, 2003[13]	USA	Caucasians	MSP	RCC	Tissue	26	50	10	12	16	35
Battagli et al, 2003[13]	USA	Caucasians	MSP	RCC	Urine	25	50	9	12	16	35
Tokinaga et al, 2004[11]	Japan	Asians	COBRA	ccRCC	Tissue	39	50	5	8	15	42
Loginov et al, 2004[10]	Russia	Caucasians	MSRA	ccRCC	Tissue	50	53	23	25	27	28
Loginov et al, 2009[4]	Russia	Caucasians	MSP	RCC	Tissue	29	39	17	21	12	18
Onay et al, 2009[6]	Turkey	Caucasians	MSP	RCC	Tissue	11	21	2	3	9	18
Kawai et al, 2010[5]	Japan	Asians	COBRA	ccRCC	Tissue	89	179	32	52	57	127
Skrypkina et al, 2016[1]	Ukraine	Caucasians	QMSP	RCC	Blood	17	27	14	23	3	4
Author, year	**Country**	**Ethnicity**	**Method**	**Histology**	**Sample**	**Case**		**pT2–4**		**pT1**	
						M+	Total		Total	M+	Total
Battagli et al, 2003[13]	USA	Caucasians	MSP	RCC	Tissue	26	50	14	17	12	30
Battagli et al, 2003[13]	USA	Caucasians	MSP	RCC	Urine	25	50	13	17	12	30
Dulaimi et al, 2004[12]	USA	Caucasians	MSP	RCC	Tissue	45	99	24	40	20	52
Hori et al, 2007[8]	Japan	Asians	MSP	RCC	Tissue	41	42	8	9	33	33
Onay et al, 2009[6]	Turkey	Caucasians	MSP	RCC	Tissue	11	21	11	21	0	0
Ellinger et al, 2011[3]	Germany	Caucasians	QMSP	pRCC	Tissue	32	32	13	13	19	19

(*Continued*)

Table S1 (*Continued*)

Author, year	Country	Ethnicity	Method	Histology	Sample	Case		pT2–4		pT1	
						M+	Total	M+	Total	M+	Total
Skrypkina et al, 2016[1]	Ukraine	Caucasians	QMSP	RCC	Blood	17	27	7	10	9	15
Author, year	**Country**	**Ethnicity**	**Method**	**Histology**	**Sample**	**Case**		**Node+**		**Node–**	
						M+	Total	M+	Total	M+	Total
Battagli et al, 2003[13]	USA	Caucasians	MSP	RCC	Tissue	26	50	2	3	24	44
Battagli et al, 2003[13]	USA	Caucasians	MSP	RCC	Urine	25	50	2	3	23	44
Dulaimi et al, 2004[12]	USA	Caucasians	MSP	RCC	Tissue	45	99	5	8	36	79
Onay et al, 2009[6]	Turkey	Caucasians	MSP	RCC	Tissue	11	21	0	0	11	21
Kawai et al, 2010[5]	Japan	Asians	COBRA	ccRCC	Tissue	89	179	8	12	81	167
Ellinger et al, 2011[3]	Germany	Caucasians	QMSP	pRCC	Tissue	32	32	3	3	29	29
Skrypkina et al, 2016[1]	Ukraine	Caucasians	QMSP	RCC	Blood	17	27	0	1	16	24
Author, year	**Country**	**Ethnicity**	**Method**	**Histology**	**Sample**	**Case**		**Distant metastasis+**		**Distant metastasis–**	
						M+	Total	M+	Total	M+	Total
Onay et al, 2009[6]	Turkey	Caucasians	MSP	RCC	Tissue	11	21	2	3	9	18
Kawai et al, 2010[5]	Japan	Asians	COBRA	ccRCC	Tissue	89	179	14	24	75	155
Ellinger et al, 2011[3]	Germany	Caucasians	QMSP	pRCC	Tissue	32	32	1	1	31	31
Skrypkina et al, 2016[1]	Ukraine	Caucasians	QMSP	RCC	Blood	17	27	3	3	13	22
Author, year	**Country**	**Ethnicity**	**Method**	**Histology**	**Sample**	**Case**		**ccRCC**		**pRCC**	
						M+	Total	M+	Total	M+	Total
Morrissey et al, 2001[15]	UK	Caucasians	#	RCC	Tissue	59	211	32	138	12	27
Battagli et al, 2003[13]	USA	Caucasians	MSP	RCC	Tissue	26	50	17	35	6	6
Battagli et al, 2003[13]	USA	Caucasians	MSP	RCC	Urine	25	50	17	35	5	6
Dulaimi et al, 2004[12]	USA	Caucasians	MSP	RCC	Tissue	45	99	23	50	14	20
Gonzalgo et al, 2004[9]	USA	Caucasians	QMSP	RCC	Tissue	30	38	19	21	9	9
Costa et al, 2007[7]	Portugal	Caucasians	QMSP	RCC	Tissue	68	85	42	52	13	13
Hori et al, 2007[8]	Japan	Asians	MSP	RCC	Tissue	41	42	30	31	5	5
Skrypkina et al, 2016[1]	Ukraine	Caucasians	QMSP	RCC	Blood	17	27	15	23	1	1

Notes: "#"stands for bisulfite modification, direct sequencing, and restriction enzyme digestion. T classification, pT; node, lymph node status.

Abbreviations: BSQ, bisulfite sequencing; ccRCC, clear cell RCC; COBRA, combined bisulfite restriction analysis; M, methylation-positive status; MSHRM, methylation-sensitive high-resolution melting analysis; MSP, methylation-specific polymerase chain reaction; MSRA, methylation-sensitive restriction enzyme analysis; pRCC, papillary RCC; QMSP, quantitative methylation-specific polymerase chain reaction; RCC, renal cell carcinoma.

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3. Ellinger J, Holl D, Nuhn P, et al. DNA hypermethylation in papillary renal cell carcinoma. *BJU Int*. 2011;107(4):664–669.
4. Loginov VI, Khodyrev DS, Pronina IV, et al. Methylation of promoter region of RASSF1A gene and frequencies of allelic imbalances in chromosome 3 critical regions are correlated with progression of clear cell renal cell carcinoma. *Mol Biol (Mosk)*. 2009;43(3):429–438.
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9. Gonzalgo ML, Yegnasubramanian S, Yan G, et al. Molecular profiling and classification of sporadic renal cell carcinoma by quantitative methylation analysis. *Clin Cancer Res*. 2004;10(21):7276–7283.
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