Clinical Cancer Research

Promoter Methylation of *CDO1* Identifies Clear-Cell Renal Cell Cancer Patients with Poor Survival Outcome

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Abstract

Purpose: In this era of molecular diagnostics, prediction of clear-cell renal cell cancer (ccRCC) survival requires optimization, as current prognostic markers fail to determine individual patient outcome. Epigenetic events are promising molecular markers. Promoter CpG island methylation of *cysteine dioxygenase type 1* (*CDO1*), which was identified as prognostic marker for breast cancer, is studied as a potential marker for ccRCC survival.

Experimental Design: We collected primary tissues of 365 ccRCC cases identified within the prospective Netherlands Cohort Study (NLCS). In this population-based series, *CDO1* promoter methylation was observed in 124 of 324 (38.3%) patients with successful methylation-specific PCR analysis. Kaplan–Meier curves and Wilcoxon tests were used to evaluate 10-year ccRCC-specific survival. Cox regression analysis was used to obtain crude and multivariate HRs and 95% confidence intervals (CI). The relative prognostic value of multivariate models with and without *CDO1* promoter methylation was compared using likelihood-ratio tests.

Results: Patients with *CDO1* promoter methylation have a significantly poorer survival than those without (Wilcoxon $P = 0.006$). Differences in survival were independent of other prognostic factors, including age and sex (HR, 1.66; 95% CI, 1.12–2.45) and TNM stage, tumor size, and Fuhrman grade (HR, 1.89; 95% CI, 1.25–2.85). Multivariate models performed better with than without *CDO1* promoter methylation status (likelihood-ratio $P = 0.003$). Survival curves were validated in an independent series of 280 ccRCC cases from The Cancer Genome Atlas (TCGA; Wilcoxon $P < 0.001$).

Conclusions: *CDO1* promoter methylation may not substitute common prognostic makers to predict ccRCC survival, but offers additional, relevant prognostic information, indicating that it might be a novel molecular marker to determine ccRCC prognosis. *Clin Cancer Res; 21(15); 3492–500. ©2015 AACR.*

Introduction

Major improvements in the understanding of the molecular biology of tumors increased the possibilities for targeted therapies, such as tyrosine kinase inhibitors (TKI) and mTOR inhibitors in clear-cell renal cell cancer (ccRCC) patients. In this era of personalized therapies and molecular diagnostics, current prognostic parameters, such as patient performance status, tumor size, cancer stage, and nuclear grade, fail to determine individual patient outcome and optimization of prognostic parameters is thus necessary.

Promoter CpG island methylation markers are promising tools for early detection of tumors, therapeutic stratification, and to determine clinical prognosis. Promoter CpG island methylation occurs early and frequently in carcinogenesis, providing selective advantage to neoplastic cells and, therefore, contributing to both disease development and progression (1–4). For ccRCC, genetic and epigenetic alterations in the *von Hippel-Lindau* (*VHL*) tumor-suppressor gene appeared to have little value in the prediction of ccRCC prognosis, even though inactivation of the *VHL* gene is an early and key event in the development of ccRCC (5).

Recently, promoter CpG island methylation of *cysteine dioxygenase type 1* (*CDO1*) has been found in primary tissues of multiple tumor types, including colon, breast, esophagus, ovary, lung, bladder, pancreas and stomach tumors (6, 7), cholangiocarcinoma (8), and lung squamous cell carcinoma (9). In addition, it has been identified as prognostic marker for breast cancer (7, 10, 11), but *CDO1* promoter CpG island methylation and its potential association with prognosis has not yet been investigated in ccRCC.

CDO1 is thought to be involved in carcinogenesis, as it plays a role in oxidative stress response of cancer cells by reducing antioxidant capacity (7). Moreover, CDO1 is a key enzyme in the taurine biosynthetic pathway, in which it oxidizes cysteine

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Translational Relevance

Significant heterogeneity in the genetic and epigenetic make-up of clear-cell renal cell cancer (ccRCC) has recently been established. As a result, current prognostic markers fail to accurately determine individual patient outcome. *Cysteine dioxygenase type 1* (*CDO1*) has been identified as a marker of poor breast cancer survival. We show that *CDO1* is also frequently promoter methylated in a population-based series of ccRCC cases, and that *CDO1* promoter methylation is associated with a significantly poorer survival. Differences in survival were independent of other prognostic factors, including age, sex, cancer stage, and nuclear grade. *CDO1* promoter methylation offers additional, relevant prognostic information in addition to these prognostic factors, but may not substitute for these markers. Results were validated in an independent series of ccRCC cases from The Cancer Genome Atlas. Together, data suggest that *CDO1* promoter methylation might be a relevant molecular marker to determine ccRCC prognosis.

into cysteine sulfinic acid (CSA), eventually converting into taurine (2-aminoethanesulfonic acid; refs. 12–14). Taurine is a major intracellular amino acid and is involved in many physiologic and biologic processes in the kidney, including osmoregulation and regulation of renal blood flow and renal cell cycle and apoptosis (15). Thus, epigenetic silencing of *CDO1* through promoter CpG island methylation may be of particular interest for renal carcinogenesis.

In the present study, we investigated the prognostic utility of *CDO1* promoter CpG island methylation in a population-based series of ccRCC patients embedded within the prospective Netherlands Cohort Study (NLCS) and in an independent series of ccRCC patients from The Cancer Genome Atlas (TCGA).

Materials and Methods

Study population

The population-based series of ccRCC cases is derived from the prospective NLCS, which was initiated in 1986 and included 120,852 men and women in the ages of 55 to 69 years at baseline (16). After 20.3 years of follow-up, 608 incident microscopically confirmed RCC cases (ICD-O: M8010-8119, 8140-8570) were identified within the NLCS using computerized record linkage with the Netherlands Cancer registry and the Dutch Pathology registry PALGA (17–19). Only histologically confirmed RCC cases ($n = 568$) were eligible for the collection of formalin-fixed paraffin-embedded (FFPE) tumor tissues from approximately 50 pathology laboratories throughout the Netherlands. The collection was conducted in two phases; initially only cases from the first 11.3 years of NLCS follow-up were included (20); however, recent efforts were made to expand the series. This study was approved by the review board of Maastricht University (Maastricht, the Netherlands).

Tissue collection

Tumor tissues were available for 79.8% of the RCC cases ($n = 453$). Inclusion ranged over 5-years follow-up periods from 71.3% to 87.7%. For cases identified between 1986 and 1990, retrieval was lowest, because the Dutch pathology registry (PALGA) had incomplete coverage during this period and some pathologic laboratories had problems to locate older tissues in particular (19). In total, the retrieval of tissues was unsuccessful for 115 cases; 46 cases had no surgery, for 24 cases, the availability or location of their tissues was unknown from the Dutch pathology registry (PALGA) and, for 45 cases the pathologic laboratory refused or was unable to send their tissues. Hematoxylin and eosin (H&E) stained slides of tumor tissues were assessed by an experienced genitourinary pathologist to confirm tumor histology based on the WHO classification of tumors and Fuhrman grade (21, 22). The entire series of collected tumor tissues included 80.6% ($n = 365$) tumors with clear-cell histology. Information on patient and tumor characteristics, such as age at diagnosis, gender, TNM stage, tumor size, and initial treatment was available from the pathologic reports and cancer registries. Follow-up was accomplished by record linkage to the municipal population registries and the causes of death registry from Statistics Netherlands. ccRCC cases of which tissues were collected after autopsy were excluded. In total, 356 ccRCC cases were eligible for survival analyses.

DNA isolation and *CDO1* promoter CpG island methylation

Methods used for DNA isolation of material from ccRCC cases included in the initial, 11.3 years follow-up collection have been described previously (20). For recently added cases, vital tumor areas of the FFPE tumor tissues were dissected before DNA isolation. DNA was isolated using the QIAamp DNA Mini Kit for isolation of Genomic DNA from Tissue (Qiagen), according to the manufacturer's instructions.

CDO1 promoter CpG island hypermethylation, in short *CDO1* promoter methylation, was analyzed by nested methylation-specific PCR (MSP), as previously described in detail elsewhere (23–25). All PCRs were done with controls for unmethylated alleles [DNA from normal lymphocytes or DNA from human umbilical vein endothelial cells (HUVEC)], methylated alleles [normal lymphocyte DNA treated *in vitro* with SssI methyltransferase (New England Biolabs)], and a no template control. Reproducibility of MSP analysis in 53 cases was 89%. Primer sequences and MSP conditions are provided in Supplementary Table S1. MSP analyses were performed successfully for 324 of 356 cases (91.0%).

Statistical analyses

Statistical analyses were conducted in STATA version 12 (STATA Corp.). Patient and tumor characteristics of ccRCC cases with and without *CDO1* methylation were compared using the Kruskal–Wallis tests (for continuous variables) or χ^2 tests (for categorical variables). Survival was defined as the time from diagnosis until death or until the end of follow-up. However, for some cases follow-up up to 20 years has been established. As we do not consider such long follow-up relevant for ccRCC-specific survival, follow-up was truncated after 10 years. Kaplan–Meier curves and Wilcoxon tests were used to evaluate 10-year survival of ccRCC cases with and without *CDO1* promoter methylation. In addition, Cox proportional hazards analyses were used to obtain HRs and corresponding 95% confidence intervals (CI) for the association between *CDO1* promoter methylation and 10-year ccRCC-specific survival. HRs were adjusted for age at diagnosis (years) and sex (m/f) and, in a multivariable-adjusted model, additionally for a set of *a priori* selected confounders known for their prognostic value in ccRCC survival; TNM stage (I–II, III, IV; fourth version (1987), tumor size (mm), and Fuhrman grade (I, II, III, IV). Cases with missing data on any of the confounders were excluded for

Cox proportional hazards analyses ($n = 17$). The relative prognostic value of *CDO1* promoter methylation compared with the known prognostic factors was assessed using likelihood-ratio tests for nested models and the Akaike information criterion (AIC) for nonnested models. In general, a lower AIC is considered a better model. In sensitivity analyses, all analyses were repeated after exclusion of patients with distant metastasis (TNM stage IV), patients with (neo)adjuvant treatment and patients with sarcomatoid differentiation. In addition, in subgroup analyses, analyses were conducted by categories of age at diagnosis; ≥55 to <65 years, ≥65 to <75 years, and ≥75 years.

To independently validate the findings obtained in the population-based ccRCC series, all Kaplan–Meier curves and Wilcoxon tests were reevaluated in 280 ccRCC cases from the TCGA series with full data available on patient and tumor characteristics and survival, only then using a maximum of 5-year survival, as longer follow-up was not available. In the TCGA data, promoter methylation was measured on the Illumina Human Methylation 450 K platform as a quantitative treat. We identified three probes near our primer region and determined a composite *CDO1* methylation level by taking the maximum beta value of those three probes. We defined methylated samples as those with a composite methylation level of at least 30%. A two-sided *P* value of 0.05 or less was considered statistically significant.

Results

The median ccRCC-specific survival in the population-based series was 4.5 years. For 13.7% of the patients a distant metastasis was reported (TNM stage IV, Table 1). The majority of patients (94.1%) had no (neo)adjuvant treatment. *CDO1* promoter methylation was observed in 124 of 324 patients (38.3%). *CDO1* promoter methylation was more frequent in male patients ($P = 0.001$) and in those with a longer duration and a higher intensity of cigarette smoking ($P = 0.002$ and 0.002). However, age at diagnosis and other lifestyle indicators, including body mass index, hypertension, and alcohol intake, did not differ by *CDO1* promoter methylation. Tumors with *CDO1* promoter methylation were similar in TNM stage and tumor size compared with those without, yet they showed a significantly higher Fuhrman grade ($P < 0.001$). Interestingly, for none of the tumors without *CDO1* promoter methylation sarcomatoid differentiation was observed, whereas 6.5% of tumors with *CDO1* promoter methylation had signs of such sarcomatoid differentiation.

To independently validate our findings from the population-based series of ccRCC patients, we used data of ccRCC patients from the TCGA (Table 2). Compared with the population-based series, ccRCC patients in the TCGA series had a lower mean age at diagnosis, but with a considerably larger SD (70.6 ± 5.9 and 61.5 ± 12.0 years, respectively), and a shorter median survival time (4.5 and 2.8 years). In the TCGA series, 54 of 280 patients (19.3%) showed a distant metastasis (TNM stage IV) and 7 of 280 patients (2.5%) had a history of (neo)adjuvant treatment. *CDO1* promoter methylation was present in 122 of 280 ccRCC patients (43.6%). Patients with *CDO1* promoter methylation were significantly older ($P < 0.001$), more frequently male ($P = 0.03$) and their tumor characteristics, that is, TNM stage and Fuhrman grade were less favorable compared with those without ($P < 0.001$ and <0.001, respectively).

Figure 1 shows the Kaplan–Meier curve for *CDO1* promoter methylation and 10-year ccRCC-specific survival in our population-based series of ccRCC patients. We observed a significantly poorer survival for patients with *CDO1* promoter methylation

Table 1. Characteristics of ccRCC patients overall and by promoter methylation of *CDO1*—the NLCS, 1986–2006

Characteristics (mean; SD)	Total ccRCC ($n = 324$)	*CDO1* promoter methylation		
		No ($n = 200$)	Yes ($n = 124$)	P[a]
Patient				
Age at diagnosis (y)	70.6 (5.9)	70.6 (5.9)	70.6 (6.1)	0.78
Sex, men (%)	194 (59.9)	105 (52.5)	89 (71.8)	0.001
ccRCC-specific survival (y)	5.9 (5.0)	6.43 (5.1)	5.0 (4.8)	0.005
Tumor				
TNM stage[b] (%, $n = 321$)				
Stages 1 and 2	189 (58.9)	124 (62.0)	65 (53.7)	
Stage 3	88 (27.4)	50 (25.0)	38 (31.4)	
Stage 4	44 (13.7)	26 (13.0)	18 (14.9)	0.33
Tumor size (mm, $n = 309$)	67.6 (32.9)	66.5 (32.2)	69.4 (34.0)	0.48
Fuhrman grade (%)				
Grade 1	44 (13.9)	39 (19.5)	5 (4.0)	
Grade 2	129 (39.8)	85 (42.5)	44 (35.5)	
Grade 3	103 (31.8)	55 (27.5)	48 (38.7)	
Grade 4	48 (14.8)	21 (10.5)	27 (21.8)	<0.001
CDO1 methylation (%)	124 (38.3)	0 (0.0)	124 (100.0)	—
Sarcomatoid differentiation (%)	8 (2.5)	0 (0.0)	8 (6.5)	<0.001
Eosinophilic variant (%, $n = 155$)	2 (1.3)	1 (1.0)	1 (1.8)	1.0
No (neo)adjuvant treatment (%, $n = 322$)	303 (94.1)	185 (93.4)	118 (95.2)	0.63
Lifestyle				
Body mass index (kg/m^2, $n = 309$)	25.5 (2.9)	25.4 (2.9)	25.7 (16.2)	0.30
Hypertension (%)	110 (34.0)	71 (35.5)	39 (31.5)	0.45
Cigarette smoking				
Status—current (%)	105 (32.6)	62 (31.2)	43 (35.0)	0.48
Duration (y, $n = 318$)	23.3 (17.6)	20.8 (18.0)	27.4 (16.2)	0.002
Intensity (cig/d, $n = 306$)	11.9 (12.3)	10.0 (10.3)	14.9 (14.4)	0.002
Alcohol intake (g ethanol/d, $n = 307$)	11.4 (15.1)	10.5 (15.4)	12.7 (14.6)	0.22

[a]A *P* value tested with the Kruskal–Wallis test (for continuous variables) or the χ^2 test (for categorical variables).
[b]TNM stage as defined in 1987.

Table 2. Characteristics of ccRCC patients overall and by promoter methylation of *CDO1*—TCGA

Characteristics (mean; SD)	Total ccRCC ($n = 280$)	*CDO1* promoter methylation		
		No ($n = 158$)	Yes ($n = 122$)	P[a]
Patient				
Age at diagnosis (y)	61.5 (12.0)	59.3 (12.6)	64.3 (10.6)	<0.001
Sex, men (%)	185 (66.1)	96 (60.8)	89 (73.0)	0.033
Overall survival (y)	2.8 (2.3)	2.7 (2.3)	2.9 (2.2)	0.38
Tumor				
TNM stage (%)				
Stages 1 and 2	153 (54.6)	115 (72.8)	38 (31.2)	
Stage 3	73 (26.1)	25 (15.8)	48 (39.3)	
Stage 4	54 (19.3)	18 (11.4)	36 (29.5)	<0.001
Tumor size (mm)	—	—	—	—
Fuhrman grade (%, $n = 277$)				
Grade 1	5 (1.8)	5 (3.2)	0 (0.0)	
Grade 2	113 (40.8)	82 (52.6)	31 (25.6)	
Grade 3	111 (40.1)	58 (37.2)	53 (43.8)	
Grade 4	48 (17.3)	11 (7.1)	37 (30.6)	<0.001
CDO1 methylation (%)	122 (43.6)	0 (0.0)	122 (100.0)	—
Sarcomatoid differentiation (%)	—	0.0	6.5	—
Eosinophilic variant (%)	—	1.0	1.8	0.67
No neoadjuvant treatment (%)	273 (97.5)	152 (96.2)	121 (99.2)	0.14

[a]A P value tested with the Kruskal–Wallis test (for continuous variables) or the χ^2 test (for categorical variables).

compared with patients without *CDO1* promoter methylation (Wilcoxon $P = 0.006$). In Cox proportional hazards analyses, *CDO1* promoter methylation was also associated with unfavorable patient outcome (HR, 1.66; 95% CI, 1.13–2.44), Table 3). HRs did not substantially change after adjustment for age at diagnosis and sex (HR, 1.66; 95% CI, 1.12–2.45) and after additional adjustment for relevant clinicopathologic parameters, including TNM stage, tumor size, and Fuhrman grade (HR, 1.89; 95% CI, 1.25–2.85). In sensitivity analyses, both Kaplan–Meier curves (Fig. 2) and multivariable-adjusted Cox proportional hazards analyses (Table 3) showed that the exclusion of TNM stage IV patients resulted in a slight attenuation of the association between *CDO1* promoter methylation and ccRCC-specific 10-year survival (Wilcoxon $P = 0.09$; HR, 1.44; 95% CI, 0.90–2.30), and that the restriction to patients without (neo)adjuvant treatment did not substantially change survival (Wilcoxon $P = 0.007$; HR, 1.86; 95% CI, 1.22–2.86). However, after excluding patients with sarcomatoid differentiation, the association between *CDO1* promoter methylation and ccRCC survival was in the multivariable-adjusted model stronger than in the total population (HR, 1.94; 95% CI, 1.27–2.97).

In line with the results from the population-based series, Kaplan–Meier curves in the TCGA series showed a significantly poorer 5-year survival for patients with *CDO1* promoter methylation compared with those without (Wilcoxon $P < 0.001$, Fig. 2A). In sensitivity analyses, the 5-year survival attenuated after the exclusion of TNM stage IV patients, but was still significantly different by *CDO1* promoter methylation status (Wilcoxon $P = 0.047$, Fig. 2B). In addition, the 5-year survival did not change after restricting the population to patients without (neo)adjuvant treatment (Wilcoxon $P < 0.001$, Fig. 2C).

In Table 4, we show the goodness-of-fit of five relevant models, to indicate each model's prognostic value. In the population-based series, *CDO1* methylation status alone (Model 1) is better in predicting prognosis than the models, including age at diagnosis and sex (Model 2 and 3). In fact, all models with *CDO1* methylation status performed significantly better in predicting survival than the same models without *CDO1* methylation status (e.g., Model 4 vs. 5, $P = 0.003$). In the TCGA series, *CDO1* promoter methylation only improved the model, including age at diagnosis and sex (Model 2 vs. 3, $P = 0.001$), and not the model, including all other prognostic variables (Model 4 vs. 5, $P = 0.40$).

In our population-based series of ccRCC patients, subgroup analyses by categories of age at diagnosis showed substantially different Kaplan–Meier curves; a poorer survival by *CDO1* promoter methylation was observed for patients ages ≥55 to <65 years at diagnosis (Wilcoxon $P = 0.009$), but not for patients ages ≥65 to <75 or >75 years at diagnosis (Wilcoxon $P = 0.20$ and 0.20, respectively; Supplementary Fig. S1). Although there is no significant interaction between age at diagnosis and *CDO1* promoter methylation in relation to survival in multivariable-adjusted analyses ($P_{\text{interaction}} = 0.68$), risk estimates of unfavorable patient outcome were also substantially different by categories of age at diagnosis. For ccRCC patients ages ≥55 to <65 years at diagnosis the HR, 3.21 (95% CI, 1.25–8.23) in the age- and sex-adjusted model and, as a result of limited statistical power, HR, 2.81 (95% CI, 0.67–11.71) in the multivariable-adjusted model. In older patients, none of risk estimates were statistically significant. In the validation series, subgroup analyses by categories of age at diagnosis showed the strongest association between *CDO1* promoter methylation and 5-year survival in the youngest patients (<55 years at diagnosis, Wilcoxon $P < 0.013$, Supplementary Fig. S2). In all other age categories, the observed difference in Kaplan–Meier curves was not significant.

Discussion

In the present study, we reported for the first time that *CDO1* promoter CpG island methylation is associated with poorer survival in a population-based series of ccRCC patients and validated these finding in an independent series of ccRCC patients from the TCGA. Therefore, these data suggest that *CDO1* promoter methylation may be a relevant marker for ccRCC prognosis.

The investigated association between *CDO1* promoter methylation and ccRCC survival remained significant after multivariable adjustment for age at diagnosis, sex, and other clinicopathologic parameters, including TNM stage, tumor size, and Fuhrman grade. Thus, the association appeared to be independent of

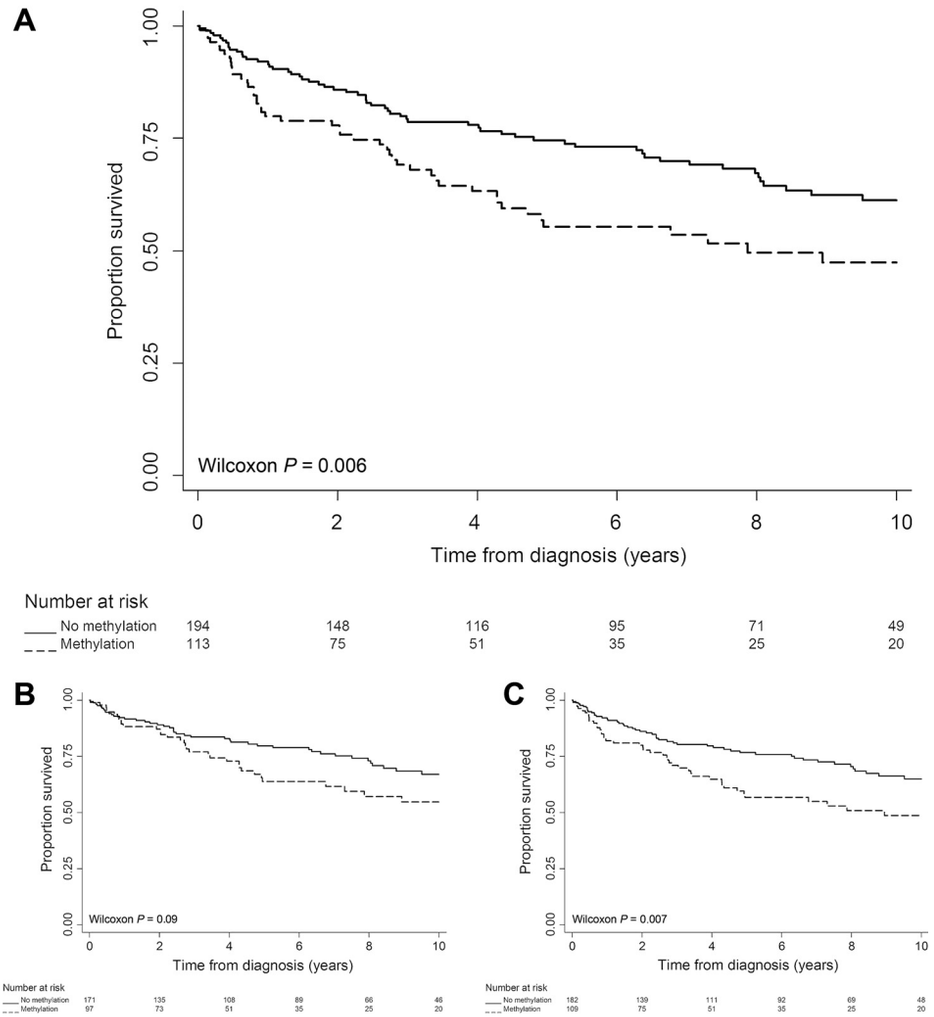

Figure 1.
Ten-year survival of ccRCC patients from a population-based case series according to *CDO1* methylation in the total population (A), in patients with TNM stage I–III (B), and in patients without (neo)adjuvant treatment (C).

current prognostic factors. Moreover, based on results from the population-based series, *CDO1* promoter methylation status may be included in the model as prognostic marker to assess survival, in addition to common clinicopathologic parameters. The additional value of *CDO1* promoter methylation in the model, including sex and age, but not in the extended model, was confirmed in ccRCC patients from the TCGA. Thus, although *CDO1* promoter methylation status by itself is not suitable to substitute common clinicopathologic parameters in the prediction of ccRCC survival, it offers additional, relevant prognostic information. It has been suggested that prognostic risk models, such as the University of California Los Angeles integrated staging system (UISS) and the Stage Size Grade Necrosis (SSIGN) risk score, which combine independent prognostic factors, may be more accurate in predicting survival than TNM stage and Fuhrman grade alone (26). However, the UISS requires information on patient performance stage and the SSIGN risk score requires information on necrosis, which are both not available in the NLCS.

Several conditions can be hypothesized that might influence the association between *CDO1* promoter methylation and ccRCC survival. First, it can be argued that in patients with distant metastases, that is, TNM stage IV, poor prognosis may be predominantly determined by the presence of the distant metastasis rather than by their *CDO1* promoter methylation status. We observed a slight attenuation in HRs in both series when excluding these patients.

Second, the choice of treatment may influence a patient's survival regardless of the *CDO1* promoter methylation status, and thus conceal the potential effect of *CDO1* promoter methylation on ccRCC survival. Moreover, the choice of treatment may also be based on the expected patient's survival (e.g., adjuvant treatment for patients with the worst prognostic profile) and bias the association under study. No substantial changes in the results were observed after restricting to patients without (neo)adjuvant treatment, partly because surgery has been the primary and only treatment with curative intent for long and, consequently, the proportion of patients with (neo) adjuvant treatment in our population is very low. In addition, the inclusion of patients in our series depended on the availability of tissues, and we observed that the proportion of patients with TNM stage IV or with (neo)adjuvant treatment was lower for the ccRCC cases with tissues, compared with those without.

Table 3. HRs and 95% CIs of 10-year ccRCC survival for promoter methylation of *CDO1*—the NLCS, 1986–2006

Population	Crude model HR (95% CI)	Age- and sex-adjusted model[a] HR (95% CI)	Multivariable adjusted model[b] HR (95% CI)
Total population ($n = 307$)	1.66 (1.13–2.44)	1.66 (1.12–2.45)	1.89 (1.25–2.85)
TNM stage I–III ($n = 268$)	1.55 (0.98–2.44)	1.56 (0.98–2.48)	1.44 (0.90–2.30)
No (neo)adjuvant treatment ($n = 291$)	1.76 (1.17–2.64)	1.76 (1.16–2.66)	1.86 (1.22–2.86)
No sarcomatoid differentiation ($n = 300$)	1.61 (1.09–2.39)	1.61 (1.08–2.41)	1.94 (1.27–2.97)
Total population ($n = 307$)[c]			
Age at diagnosis >55, ≤65 ($n = 40$)	3.10 (1.24–7.77)	3.21 (1.25–8.23)	2.81 (0.67–11.71)
Age at diagnosis >65, ≤75 ($n = 183$)	1.34 (0.81–2.23)	1.34 (0.80–2.25)	1.43 (0.81–2.54)
Age at diagnosis >75 ($n = 84$)	1.73 (0.77–3.85)	1.62 (0.72–3.66)	2.01 (0.81–5.01)

[a]Adjusted for age at diagnosis (y) and sex (m/f).
[b]Adjusted for age at diagnosis (y), sex (m/f), TNM stage (I–II, III, IV; 1987), tumor size (mm), and Fuhrman grade (I–IV).
[c]$P_{interaction} = 0.68$ in multivariable adjusted model.

Third, the presence of a sarcomatoid differentiation in the ccRCC tumor portends a worse prognosis (27). Information on sarcomatoid differentiation was only available in the population-based series. As we observed that for all ccRCC tumors with a sarcomatoid component *CDO1* promoter methylation was present, poor prognosis may be predominantly determined by the presence of the sarcomatoid differentiation rather than by the *CDO1* promoter methylation itself. However, in contrast with this hypothesis, the association between *CDO1* promoter methylation and ccRCC-specific survival was stronger when excluding cases with sarcomatoid differentiation. Excluding these cases did not substantially change Kaplan–Meier curves (Wilcoxon $P = 0.01$).

Finally, it has been hypothesized that global promoter methylation is associated with age (28). Generally, the association

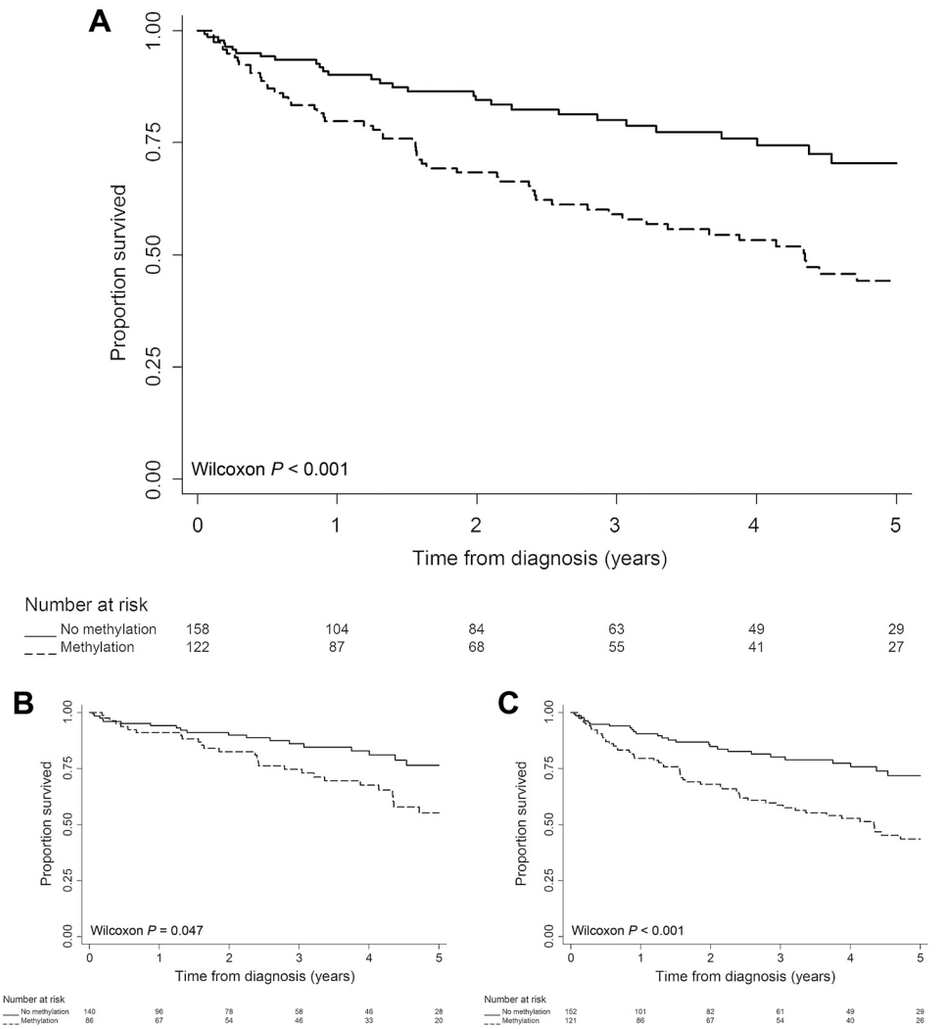

Figure 2.
Five-year survival of ccRCC patients from the TCGA according to *CDO1* methylation in the total population (A), in patients with TNM stage I–III (B), and in patients without (neo) adjuvant treatment (C).

Table 4. Model fit among five models, including prognostic factors in relation to survival of ccRCC patients

	NLCS				TCGA			
Models	**N**	**Df**	**AIC**	***P*[a]**	**N**	**Df**	**AIC**	***P*[a]**
Model 1[b]	307	1	1,117.76		277	1	832.65	
Model 2[c]	307	2	1,126.05		277	2	842.77	
Model 3[d]	307	3	1,121.76	0.012	277	3	834.24	0.001
Model 4[e]	307	8	1,073.19		277	6	775.80	
Model 5[f]	307	9	1,066.26	0.003	277	7	777.40	0.40

Abbreviation: Df, degrees of freedom.

[a]A *P* value for the likelihood-ratio test, current model compared with the model above.

[b]Model including *CDO1* methylation (n/y).

[c]Model including age at diagnosis (y) and sex (m/f).

[d]Model including age at diagnosis (y), sex (m/f), and *CDO1* methylation (n/y).

[e]Model including age at diagnosis (y), sex (m/f), TNM stage (I–II, III, IV), and Fuhrman grade (I–IV). The NLCS uses the 1987 version of the TNM classification and also includes tumor size (mm).

[f]Model including age at diagnosis (y), sex (m/f), TNM stage (I–II, III, IV), Fuhrman grade (I–IV), and *CDO1* methylation (n/y). The NLCS uses the 1987 version of the TNM classification and also includes tumor size (mm).

between *CDO1* promoter methylation and ccRCC survival was stronger in the lowest age category. For the TCGA data, it is plausible that smaller effects of *CDO1* methylation may be observed in older patients, because in this series survival was defined as overall survival rather than RCC-specific survival and RCC is likely a smaller contributor to total deaths in the elderly population than in the younger one. However, in the population-based series, survival was RCC specific. For this series, we confounding may have influenced differential observations by categories of age at baseline in unadjusted Kaplan–Meier curves, as the multivariable-adjusted interaction between *CDO1* promoter methylation and age was not statistically significant.

Although both series of ccRCC patients included in this study generally showed the same results, some methodologic differences are of particular interest. The population-based series of ccRCC patients, which is derived from the NLCS, has the advantage of, including approximately 80% of all incident cases from a representative base population, hereby limiting the likelihood of selection bias. Any selection criterion for the inclusion of patients runs the risk of overestimating the effect of a particular marker, in this case *CDO1* promoter methylation. Indeed, associations between *CDO1* promoter methylation and ccRCC survival were stronger in the hospital-based TCGA series compared with the population-based series. In addition, in the population-based series, cancer stage was classified according to the 1987 version of the TNM classification (29). A uniform cancer stage increases consistency within the series over time, as the TNM classification regularly changes. However, old TNM classifications lack important information that allows recoding into more recent versions. The difference in tumor size between the 1987 version and the more recent versions of the TNM classification was addressed by the inclusion of the tumor size as covariate with TNM stage in the statistical models.

Promoter methylation of the *CDO1* gene has previously been associated with downregulation of *CDO1* mRNA expression (7). Moreover, in the TCGA dataset a negative correlation was observed for all three individual CpG's present at the Infinium human methylation 450 k platform, with only one CpG being significantly correlated (Pearson Rho = 0.12; $P = 0.036$). Interestingly, the expression of exon 1 was significantly lower in the methylated than unmethylated samples when adhering to the binary classification integrating the β values of all three CpG's, as applied herein ($P = 0.020$).

CDO1 catalyzes the oxidation of cysteine to CSA (30), and is, thus, crucial for the removal of excess cysteine in the body. Deficient CDO1 activity and subsequent high cysteine levels may be cytotoxic and have been associated with multiple human diseases, such as Parkinson's and Alzheimer's disease (31) and rheumatoid arthritis (32). Importantly, high cysteine levels may also be related to cancer, as higher cysteine levels have been found in prostate cancer tissues compared with benign prostate tissues (33). Furthermore, CDO1 may indirectly regulate intracellular redox homeostasis (14). Dominy and colleagues (30) demonstrated that glutathione levels, a major antioxidant molecule, and its major substrate, intracellular cysteine, were lowered in presence of CDO1, suggesting that CDO1 may be able to diminish antioxidant capacity. Loss of CDO1 function in cancer cells may, thus, help these cells to escape oxidative damage and cell death due to enhanced antioxidant capacity, as cancer cells tend to develop enhanced antioxidant capacity under excessive and toxic amounts of reactive oxygen species (ROS; 34). The principle of increased ROS detoxification capacity and its advantage for cancer cell survival as a result of DNA methylation of the *CDO1* gene was demonstrated by Jeschke and colleagues (7) in breast cancer cells. Whether this mechanism may also apply for ccRCC cells has not been elucidated.

CDO1 promoter methylation status may, in addition to prognostic value, also have predictive value and have clinical implications for cancer therapy. In breast cancer, *CDO1* methylation is suggested to be a useful marker for prediction of resistance to the commonly used treatment with anthracyclines (7). Interestingly, cytotoxic chemotherapy, including anthracyclines, was never found to be effective in ccRCC (35). It has been suggested that the resistance of ccRCC to chemotherapeutic agents may partly be due to increased levels of the transcription factor NF-κB (36, 37), yet *CDO1* promoter methylation may play a role too.

In conclusion, *CDO1* promoter methylation could be a relevant marker for ccRCC prognosis as it is a significant predictor of poor ccRCC-related survival in two independent series of ccRCC patients. However, further research needs to evaluate potential age-related differences in this association and the potential use of *CDO1* promoter methylation in therapeutic stratification.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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