Prognostic DNA methylation markers for renal cell carcinoma: a systematic review

Sophie C Joosten¹, Ivette AG Deckers², Maureen J Aarts¹, Ann Hoebe¹, Joep G van Roermund³, Kim M Smits^{1,2}, Veerle Melotte², Manon van Engeland² & Vivianne C Tjan-Heijnen^{*,1}

¹Division of Medical Oncology, GROW – School for Oncology and Developmental Biology, Maastricht University Medical Center, P.O. Box 5800, 6202 AZ Maastricht, The Netherlands

²Department of Pathology, GROW – School for Oncology and Developmental Biology, Maastricht University Medical Center, P.O. Box 5800, 6202 AZ Maastricht, The Netherlands

³Department of Urology, GROW – School for Oncology and Developmental Biology, Maastricht University Medical Center, P.O. Box 5800, 6202 AZ Maastricht, The Netherlands

* Author for correspondence: Tel.: +31 43 387 7025; vcg.tjan.heijnen@mumc.nl

Aim: Despite numerous published prognostic methylation markers for renal cell carcinoma (RCC), none of these have yet changed patient management. Our aim is to systematically review and evaluate the literature on prognostic DNA methylation markers for RCC. **Materials & methods:** We conducted an exhaustive search of PubMed, EMBASE and MEDLINE up to April 2017 and identified 49 publications. Studies were reviewed according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statement, assessed for their reporting quality using the Reporting Recommendations for Tumor Marker Prognostic Studies (REMARK) criteria, and were graded to determine the level of evidence (LOE) for each biomarker. **Results:** We identified promoter methylation of *BNC1*, *SCUBE3*, *GATA5*, *SFRP1*, *GREM1*, *RASSF1A*, *PCDH8*, *LAD1* and *NEFH* as promising prognostic markers. Extensive methodological heterogeneity across the included studies was observed, which hampers comparability and reproducibility of results, providing a possible explanation why these biomarkers do not reach the clinic. **Conclusion:** Potential prognostic methylation markers for RCC have been identified, but they require further validation in prospective studies to determine their true clinical value.

First draft submitted: 13 March 2017; Accepted for publication: 3 July 2017; Published online: 11 August 2017

Keywords: biomarkers • DNA methylation • kidney cancer • prognosis • promoter CpG island methylation • renal cell carcinoma • survival

Background

Renal cell carcinoma (RCC) is the most common malignancy of the kidney, and its incidence has been rising for the past few decades [1]. Clear cell RCC (ccRCC) is the major histological subtype, accounting for 75–80% of all RCC cases [2]. At time of diagnosis 20–30% of the patients present with distant metastases, and another 30% of all nonmetastatic ccRCC patients recur after (partial) nephrectomy and develop a lethal disease stage [3]. The 5-year survival of distant metastasized ccRCC is as low as 12% [4]. The currently used models for predicting patient outcome, such as the University of California Los Angeles (UCLA) Integrated Staging System (UISS) and the Stage Size Grade Necrosis (SSIGN) Risk Score [5–8], are based on clinicopathological features (Tumor, Node, Metastasis [TNM] stage, tumor size, tumor grade, presence of necrosis and Eastern Cooperative Oncology Group [ECOG] performance status). Despite high predictive capacity of these models (C-statistics of 0.809 [9] and 0.823 [10], respectively), patients with similar clinicopathological features or risk scores can still have divergent outcomes [11]. Therefore, addition of molecular markers to the current prognostic models could improve their prognostic value, which was demonstrated for ClearCode34 [12].

It is becoming increasingly clear that epigenetic alterations, such as promoter methylation, play an important role in renal tumorigenesis by silencing tumor suppressor genes [13]. This is illustrated by the relative high frequency of

promoter methylation as compared to the number of somatic alterations in RCC [14]. Furthermore, in contrast to somatic mutations in RCC, DNA methylation alterations are often shown to be associated with clinicopathological features and patient survival [14,15]. DNA methylation consists of the covalent addition of a methyl group to cytosines that precede a guanosine, the CpG dinucleotide, which are mainly clustered as CpG islands in the promoter region of genes [13]. Promoter CpG island methylation represents a potential class of biomarkers for early detection, prognosis and predicting response to therapy [16]. As promoter CpG island methylation markers reflect tumor biology, they might be able to identify RCC patients with more aggressive tumors who have high risk of metastatic disease and consequently poor prognosis. Identification of these high-risk patients could improve the management of RCC by directing these patients to adjuvant treatment trials, which are now ongoing [17–19], and ultimately improve survival.

In recent years, a substantial amount of studies on the prognostic value of DNA methylation in RCC have been published. Here, we provide a systematic review of the literature on prognostic DNA methylation markers in RCC. We aimed to critically discuss the methodological quality of these biomarker studies and to select the most promising prognostic methylation markers for potential translation into clinic.

Materials & methods

This systematic review was performed according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statement [20,21], where applicable.

Eligibility criteria & study selection

Articles potentially eligible for this review were studies that reported on DNA methylation of one or more genes in RCC, which were assessed for their association with patient survival. The term DNA methylation in this review refers to CpG island methylation located in the promoter region of genes (i.e., around the transcription start site), but also to methylation at other locations in the genome.

The following inclusion criteria were applied: original article (not reviews, editorials, conference abstracts); English language; research was performed on RCC tissue samples or on bodily fluids derived from patients with RCC; and association of the methylation status of candidate genes with patient survival was investigated. Studies were excluded if they did not mention a specific methylation marker or performed clustering analysis based on (epi)genome-wide methylation data and only reported the association of a cluster with patient survival, without reporting methylation of specific genes. We did not restrict the inclusion of articles to specific study designs, to specific patient characteristics such as ethnicity, tumor subtype, disease stage or treatment information, or to the number of included patients.

Search strategy

We performed an electronic search of PubMed, EMBASE (via OvidSP) and MEDLINE (via OvidSP) up to April 2017 using the following search terms: renal cell carcinoma; kidney carcinoma; kidney neoplasms; kidney cancer; DNA methylation; methylation; prognosis; cancer prognosis; cancer survival. No additional limits were used. Furthermore, bibliographies of articles identified by the search were scanned to retrieve additional relevant publications.

Data collection & extraction

Data collection and extraction was performed using a standardized data registration form in which the following items were recorded: research group, year of publication, country, biomarker(s) studied, study design, source of patient material and population characteristics, tumor subtype studied, sample size, methods used to detect DNA methylation and to determine cut-off value, outcome measure(s) studied and statistical methods used, and reported association of the marker with patient outcome.

REMARK reporting & level of evidence assessment

The quality of the reporting of the selected articles was independently assessed by two authors (SC Joosten and IAG Deckers) using the criteria as formulated in the Reporting Recommendations for Tumor Marker Prognostic Studies (REMARK) guidelines [22,23].

The REMARK criteria consist of 20 items for good reporting of prognostic biomarker studies and are presented in Supplementary Table 1 (adapted from McShane *et al.* [22]). Each item of the REMARK criteria can encompass

several aspects, so a study was assigned one point if it stated all aspects of an item, 0.5 points if it mentioned some aspects and no points when an item was not reported on at all. The maximum score was 20 points. Based on the total score, studies were classified into three subgroups: studies with a score of 15 points or more ($\geq 75\%$ of the maximum score) were considered to have a high reporting quality according to REMARK; studies with an average reporting quality had a REMARK score between 5.5 and 14.5 points (25–75% of the maximum score); and ‘low reporting quality’ studies were studies with a REMARK score of 5 points or less ($\leq 25\%$ of the maximum score).

Scoring discrepancies between the two authors that led to changes in subgroup classification, were solved by consensus. This was the case in 6 out of 49 articles. If disagreement in scoring did not lead to differences in subgroup categorization, the score assigned by SC Joosten was considered leading.

Furthermore, studies were classified as category A (prospective randomized controlled trials), B (prospective trials not specifically designed to address biomarkers), C (prospective, observational studies) or D (retrospective, observational studies) and the level of evidence (LOE) for each biomarker was determined as proposed by Hayes *et al.* [24] and Simon *et al.* [25] (see Supplementary Table 2).

Results

Search results

An outline of the selection procedure is shown in Figure 1. Searching the PubMed, EMBASE and MEDLINE databases initially yielded a total of 567 potentially relevant articles. After removal of duplicates, 369 articles remained and were screened for potential eligibility based on their title and abstract. This initial screen led to the exclusion of 221 articles. Of the remaining 148 articles, full text was retrieved. Furthermore, four articles were identified through screening of the reference lists and were also assessed based on full text. After reading the main text of these 152 articles, 103 articles were additionally discarded since they did not meet the predefined inclusion criteria (Figure 1). This selection procedure resulted in 49 articles that were eventually included in this review.

Study characteristics

The characteristics of the included studies are summarized in Supplementary Table 3. The studies were conducted in 13 countries, of which 14 study populations were of Asian origin, 24 series were European, 9 study populations were American and two studies used both an Asian and an American study population. Studies were published between 2004 and 2017. The study sample size ranged from 16 to 993 patients, with a median of 113 patients (in one study the sample size was not provided). Twenty-six articles exclusively studied ccRCC, while one study only used papillary RCC (pRCC) samples and one study only chromophobe RCC tissue. The remaining 21 articles studied various subtypes of RCC. Of the 49 studies that reported on prognostic value of DNA methylation, 48 studies used tissue from the primary tumor and 1 study used cell-free DNA from blood [26]. Only nine studies did not include matched adjacent tissue or normal renal tissue as control samples. In total, 14 different techniques were used to assess DNA methylation. Almost all articles studied methylation located in the promoter region of genes (46 studies), and from three articles it was not clear which genomic location was analyzed.

REMARK reporting assessment

The total REMARK scores ranged from 4.5 to 18 points, with an average of 11.7 points. Eight studies (16%) had a high REMARK score, 38 studies (78%) had an average REMARK score and 3 studies (6%) had a low REMARK score (Supplementary Table 3). In Figure 2, the sum score per item of the REMARK criteria is depicted, which gives an overview of which items of the REMARK criteria are generally adequately or poorly reported among all the included studies.

Overall, most studies adequately specified the examined marker and the objectives and hypothesis of their research. Studies that failed to do so, were mostly studies that used an (epi)genome wide approach to identify methylation markers, or studies in which identifying methylation markers was not the primary objective. The basic demographic characteristics of the included patients, the biological material that was used to study DNA methylation on, including the method of preservation and storage, assay methods, handling of marker values in the analyses (including method of cut point determination) and statistical methods were overall well reported, although methodology greatly varied among the different studies. All studies provided the results of univariate survival analyses, but results of survival analyses corrected for other potentially prognostic variables were often missing or not fully described. Strikingly, information on treatment that patients received lacked in almost all studies.

Figure 1. PRISMA flow diagram showing the study selection process.
PRISMA: Preferred Reporting Items for Systematic Reviews and Meta-Analyses.

Whether case selection and follow-up was performed prospectively or retrospectively was also often not explicitly stated in the articles. We found only eight studies that clearly stated their study design. In order to categorize the studies as proposed by Hayes and Simon *et al.*, we considered studies that reported a time period in the past during which tissue samples were collected or did not report any information on study design, to be retrospective. Consequently, 42 studies were considered as category D studies. There were seven (14%) category C studies, of which four studies retrospectively collected archived tissue from ccRCC cases identified in a prospective cohort study. We found no category A or B studies.

Study findings

Figure 3 & Supplementary Table 4 provide an overview of all the reported methylation markers and their association with RCC survival. For nine genes, that is *SFRP1*, *BNC1*, *GREM1*, *RASSF1A*, *PCDH8*, *SCUBE3*, *GATA5*, *LAD1* and *NEFH*, promoter methylation was found to be associated with patient survival, and their prognostic value was independently validated in other studies (Figure 4 & Supplementary Table 5).

Promoter methylation of *SFRP1* was associated with patient outcome in three different studies [27–29]. Morris *et al.* [27] performed a functional epigenome screen using high-density gene expression microarrays on 11 RCC

Figure 2. Graph showing the sum score of all articles per REMARK item.
REMARK: Reporting Recommendations for Tumor Marker Prognostic Studies.

cell lines. Of the identified genes, eight genes, including *SFRP1*, *GREM1* and *BNC1*, were also frequently methylated in primary RCC samples. Promoter methylation of *SFRP1* was associated with poorer overall survival (OS) (hazard ratio [HR]: 4.88; 95% confidence interval [CI]: 2.76–87.95; $p = 0.002$). These findings were confirmed by Ricketts *et al.* [29] in two large cohorts derived from The Cancer Genome Atlas (TCGA) Kidney Renal Clear Cell Carcinoma (KIRC) project, in which they showed that promoter methylation of *SFRP1* was an independent predictor of OS in both cohorts (HR: 2.40; 95% CI: 1.25–4.64 and HR: 1.92; 95% CI: 1.05–3.50). In contrast, a third study found that methylation of *SFRP1* was associated with prolonged recurrence-free survival (RFS) (HR: 0.13; 95% CI: 0.03–0.58; $p = 0.007$) [28] (Supplementary Table 5 & Figure 4A). *BNC1* was also identified as a gene that is frequently methylated in RCC and was associated with worse OS (HR: 4.87; 95% CI: 1.14–20.88; $p = 0.033$) [27]. Promoter methylation of *BNC1* was found to be an independent prognostic factor in the TCGA KIRC project [29] (Supplementary Table 5 & Figure 4B). Although *GREM1* was frequently methylated in RCC, Morris and colleagues [27] could not find an association with patient survival. In contrast, our group and Ricketts' group [29–31] identified promoter CpG island methylation of *GREM1* as predictor of worse survival, even when corrected for other prognostic factors (HR: 3.05; 95% CI: 1.36–6.86 and HR: 2.27; 95% CI: 1.44–3.59, respectively) (Supplementary Table 5 & Figure 4C). In a subsequent study of Morris' group [32], nine genes were identified to be frequently methylated in primary RCC tumor samples. Only methylation of *SCUBE3* was found to be associated with increased risk of cancer death and relapse ($p = 0.0046$). The prognostic value of *SCUBE3* was confirmed using data derived from the TCGA KIRC project [29] (Supplementary Table 5 & Figure 4D). In 2012, Peters *et al.* [33] showed that promoter methylation of *GATA5* is common in RCC and was associated with shorter progression-free survival (PFS), both in the overall RCC population (HR: 3.48; 95% CI: 1.38–8.83; $p = 0.008$)

Figure 3. Scatterplot of all reported prognostic methylation markers for renal cell carcinoma and their association with patient survival. Each dot represents one methylation marker. Large dots indicate methylation markers that have been independently validated in different studies, medium dots indicate that a methylation marker has been reported in one study but validated within this study in an additional patient cohort (internal validation) and small dots represent methylation markers that are reported by a single study in a single patient cohort.

Top left panel: Methylation markers with a statistically significant positive association with patient survival; Top right panel: Methylation markers with a nonsignificant positive association with patient survival; Bottom left panel: Methylation markers with a statistically significant negative association with patient survival; Bottom right panel: Methylation markers with a nonsignificant negative association with patient survival.

^aMethylation marker panel: Three or more of the following genes methylated: *p16*, *hMLH1*, *VHL*, *THBS-1*, *MINT1*, *MINT2*, *MINT12*, *MINT25* and *MINT31*.

^bRisk score: Risk score based on a multivariate LASSO Cox regression model: Risk score = $(0.0066 \times \text{PITX1}) + (0.0034 \times \text{FOX3}) - (0.027 \times \text{TWF2}) - (0.018 \times \text{EHBP1L1}) - (0.03 \times \text{RIN1})$.

^cMethylation marker panel: $\geq 16/23$ CpG units methylated (encompassing seven genes, i.e., *TRH*, *FAM150A*, *PRAC*, *SLC13A5*, *ZNF540*, *ZNF154* and *ZNF671*).

and in the ccRCC subgroup (HR: 4.59; 95% CI: 1.57–13.40; $p = 0.005$), which was confirmed in an independent patient cohort [34]. Moreover, Ricketts and colleagues confirmed these results using data from the TCGA KIRC project [29] (Supplementary Table 5 & Figure 4E).

Besides for *GREM1* and *SFRP1*, inconsistent results were also found for methylation of *RASSF1A*, *PCDH8*, *LAD1* and *NEFH* and their association with patient outcome. Whereas Ellinger and colleagues [35] and Kawai and colleagues [36] were able to link promoter methylation of *RASSF1A* to shorter PFS in papillary RCC ($p = 0.046$) and shorter disease-specific survival (DSS) in ccRCC (multivariate HR: 1.78, 95% CI: 1.18–2.78, $p = 0.0053$), respectively, de Martino *et al.* [26] could not find such an association when studying promoter methylation in cell-free DNA derived from blood samples from patients with various subtypes of RCC ($p = 0.232$). Recently, Klacz *et al.* [37] found that promoter methylation of *RASSF1A* was associated with worse OS in patients with ccRCC

Figure 4. Forest plots of prognostic methylation markers that are independently validated in different studies.

Forest plot of hazard ratios of patient survival associated with methylation of *SFRP1* (A), *BNC1* (B), *GREM1* (C), *SCUBE3* (D), *GATA5* (E), *RASSF1A* (F), *PCDH8* (G), *LAD1* (H) and *NEFH* (I).

The x-axis shows the HR; the diamonds denote the HR and the horizontal lines represent 95% confidence intervals (CIs). The level of evidence for each marker is indicated in the lower right-hand corner.

^aMorris 2010; ^bRicketts 2014, Infinium 27K cohort; ^cRicketts 2014, Infinium 450K cohort; ^dvan Vlodrop 2017, TCGA series; ^evan Vlodrop 2010, population-based series; ^fvan Vlodrop 2010, hospital-based series; ^gMorris, 2011; ^hPeters, 2014; ⁱPeters 2012; ^jPeters, 2014; ^kvan Vlodrop 2017, population-based series; ^lvan Vlodrop 2017, hospital-based series.

95% CI: 95% confidence interval; DSS: Disease-specific survival; HR: Hazard ratio; KM: Kaplan–Meier curve; LOE: Level of evidence; Multi Cox: Multivariate Cox regression analysis; NA: Not available; OS: Overall survival; PFS: Progression-free survival; RFS: Recurrence-free survival; S/A Cox: Sex and age adjusted Cox regression analysis; Uni Cox: Univariate Cox regression analysis.

in univariate analyses (HR: 3.00; 95% CI: 1.18–7.65; $p = 0.02$), however, this association was lost when correcting for tumor stage and grade in multivariate analyses (Supplementary Table 5 & Figure 4F).

Methylation of *PCDH8* was identified as an independent prognostic factor for PFS and OS by Lin *et al.* [38] and Ricketts *et al.* [29] (HR: 2.35; 95% CI: 1.54–6.03 and HR: 2.13; 95% CI: 1.18–3.82, respectively), but Morris' group [32] did not find any association with patient survival (Supplementary Table 5 & Figure 4G).

In 2014, Peters *et al.* [39] reported that methylation of *LAD1* was associated with both shortened PFS and OS (HR: 6.4, 95% CI: 1.6–26.0 and HR: 2.9, 95% CI: 1.0–8.6, respectively) in RCC patients treated with antiangiogenic agents such as sunitinib, sorafenib, axitinib and bevacizumab. Recently, our group also demonstrated the prognostic value of methylation of *LAD1* for patients with ccRCC [31]. Promoter methylation of *LAD1* was associated with poorer OS in a hospital-, population- and TCGA-based series (HR: 2.3; 95% CI: 1.2–4.3; HR: 2.3; 95% CI: 1.5–3.6 and HR: 2.4, 95% CI: 1.6–3.6, respectively), however, this association was lost in the hospital-based series when corrected for other prognostic variables (Supplementary Table 5 & Figure 4H). These results indicate that methylation of *LAD1* might be of potential clinical relevance for both prediction of prognosis as well as response

to therapy. The same holds true for methylation of *NEFH*. Dubrowskaja *et al.* [40] found that methylation of *NEFH* not only predicted poorer PFS in patients with RCC (HR: 8.6, 95% CI: 3.0–24.5), but could also predict response to antiangiogenic therapy. The prognostic value of *NEFH* was confirmed by our group [31] in the population- and TCGA-based series (multivariate HR: 2.6; 95% CI: 1.5–4.6 and sex/age adjusted HR: 1.7; 95% CI: 1.1–2.6, respectively), although again not in the hospital-based series (multivariate HR: 0.6; 95% CI: 0.3–1.2) (Supplementary Table 5 & Figure 4I).

Furthermore, combination of methylation of *GREM1*, *LAD1*, *NEFH* and *NEURL* in a four-marker panel showed superior prognostic value as compared to the markers alone (Supplementary Table 4), indicating that the combination of several markers can improve predictive capacity. This was also shown by Wei *et al.* [11] who developed a prognostic risk score based on a five-CpG-based-classifier encompassing methylation of *PITX1*, *FOXE3*, *TWF2*, *RINI* and *EHPIL1*. Patients in the high-risk group had poorer OS as compared with patients in the low-risk group (HR: 4.27, 95% CI: 2.18–8.37), also when corrected for age, TNM stage, tumor grade and tumor necrosis (HR: 4.10; 95% CI: 2.05–8.19). These results were validated in three independent patient series, including in total up to almost 1000 patients, with comparable HRs of 1.80 (95% CI: 1.11–2.93, TCGA set), 3.36 (95% CI: 1.78–6.34, UTSW set) and 3.73 (95% CI: 2.28–6.09, MCHC set) (Supplementary Table 4). Furthermore, based on this five-CpG based classifier, patients within the same clinical stage could be successfully separated into subgroups with better and worse prognosis, providing prognostic information that complements the current staging system.

For several other genes statistically significant associations between methylation and patient survival were found, but were only reported in a single study [27,31,39,41–66], and for some other genes null associations were reported [26–27,32,34–35,39,42,67–70] (Figure 3 & Supplementary Table 4).

Furthermore, it was demonstrated in several studies that promoter methylation of *VHL* [26,71–72] and *p16* [26,73] has no impact on patient survival (Figure 3 & Supplementary Table 4).

Discussion

In this systematic review, we performed an extensive search of the literature on prognostic methylation markers in RCC and we identified *BNC1*, *SCUBE3*, *GATA5*, *SFRP1*, *GREM1*, *RASSF1A*, *PCDH8*, *LAD1* and *NEFH* as individual methylation markers that warrant further investigation of their prognostic value in patients with RCC. Promoter methylation of *BNC1* [27,29] and *SCUBE3* [29,32] was shown to be associated with poorer OS in two studies. Promoter CpG island methylation of *GATA5* [29,33–34] was associated with poorer prognosis in three studies, although different end points were used. Promoter methylation of *LAD1* [31,39] and *NEFH* [31,40] not only predicted patient survival, but also response to treatment with antiangiogenic agents. Conflicting results regarding the prognostic value of promoter methylation of *SFRP1* [27–29], *PCDH8* [29,32,38], *GREM1* [27,29–30] and *RASSF1A* [26,35–37] were found. Several reasons for these inconsistent results can be adduced. The overarching issue is the observed methodological, clinical and statistical heterogeneity across the included studies.

The methodological heterogeneity is illustrated by the large variety in assay methods, with a total of 14 different techniques and also different thresholds used to assess DNA methylation. Moreover, also nonquantitative methods to detect DNA methylation were used, precluding the need for cut point determination. Besides choice of method and cut-off value, also the exact genomic location of the analyzed CpG dinucleotides is important [74]. This was illustrated by van Vlodrop and colleagues [30], who showed that *GREM1* methylation varies within the promoter CpG island, with also methylation-region-specific effects on patient outcome.

In terms of the clinical heterogeneity, a lot of studies included various subtypes of RCC and often no subgroup analysis per tumor type was performed. This is important as the prognostic value of the studied methylation marker can vary according to the specific tumor subtype, which was demonstrated for methylation of *RASSF1A* [26,35–37]. Furthermore, only 30% of the studies specified whether patients had received systemic treatment. As this can influence disease outcome, it should be clearly stated which treatments patients received, and ideally, subgroup analyses of patients treated without systemic therapy (e.g., with assessing DFS) and of patients treated with the same systemic therapy should be performed [23], to avoid confounding of the results. This is especially important in order to discriminate between the prognostic and predictive value of the investigated markers, as markers with predictive value have a different outcome related to a specific therapy. Moreover, it could be that methylation markers have combined prognostic and predictive value, as was demonstrated for methylation of *NEFH* [31,39] and *LAD1* [31,40]. Additionally, most studies included in this review were retrospective and conducted in a small number of patients, with 21 out of 49 studies (43%) including <100 patients. Selecting patients based on the availability of tissue or

patient data instead of on predefined in- and exclusion criteria, could lead to selection bias and thus to a study population that is not representative for the patient population [23].

Another source of heterogeneity lies within statistical methodology. Besides great diversity in study end points used, we also observed lack of exact definition of these outcome measures in more than half of the studies. Absence of an uniform definition of clinical end points prevents results to be readily compared between studies [23]. Effort to standardize time-to-event end points in RCC has already been made in the DATECAN project [75], but needs to be implemented on a larger scale. Furthermore, the confounders assessed and corrected for differed from study to study. The prognostic value of a potential marker should always be adjusted for known prognostic variables, such as tumor stage, tumor size, Fuhrman grade and presence of necrosis, and preferably, evaluated in a multivariable prediction model to assess its incremental prognostic value compared to the current reference standard.

This multilevel heterogeneity influences the comparability and reproducibility of results, and hampers the validation of the biomarkers. The highest LOE found in this review was therefore only III (for *GREM1*, *RASSF1A*, *LAD1* and *NEFH*). However, the fact that prognostic significance for these DNA methylation alterations is found in several studies despite the methodological, clinical and statistical heterogeneity, only further endorses the biological importance and the robustness of these markers. For *SFRP1* [27,76], *BNC1* [27], *GREM1*, *LAD1* and *NEFH* (unpublished data), *SCUBE3* [32] and *RASSF1A* [77] tumor suppressor characteristics have been shown in RCC *in vitro*, which also further supports their value as possible prognostic markers.

The field of prognostic methylation marker research could greatly benefit from standardization of methylation analyses techniques, universal definition and use of clinical end points and collaboration between research groups to obtain larger patient cohorts. Moreover, despite the fact that criteria according to which prognostic biomarker studies have to report their results (i.e., the REMARK criteria) have already been installed, they have to be adopted progressively by the field. To this end, more and more journals include reporting according to REMARK as a prerequisite for publication of prognostic biomarker studies.

This systematic review has some limitations. First, we used the REMARK criteria to assess the quality of the reporting of the included articles. Because these criteria only say something about the reporting aspect of a study, they are not a measure of study quality as such, as reporting quality and study quality are not necessarily interchangeable. Furthermore, it should be noted here that the REMARK criteria tell us something about the quality of publications, but not about the potential quality of the biomarker *per se*. If a marker is described in a high-scoring study, but it cannot be validated by others, than implementation in the clinic will never be achieved. The question then rises whether the high-scoring studies indeed identified more methylation markers that were reproduced and validated. This does not appear to be the case since the average REMARK scores of the studies that reported on prognostic value of methylation of *SFRP1*, *BNC1*, *GREM1*, *RASSF1A*, *PCDH8*, *SCUBE3*, *LAD1*, *NEFH* and *GATA5* is 11 (see also Figure 5 for average REMARK scores per marker). In this respect it should also be mentioned that assessment of reporting quality according to the REMARK criteria of studies that used a (epi)genome wide approach to identify methylation markers, or studies that used a large amount of candidate markers, was found to be troublesome as the REMARK criteria do not deal with the specific study design and statistical analyses that are required in these studies, and generally these studies therefore had lower REMARK scores. Furthermore, we selected markers to be 'promising', if their prognostic value was independently validated in other publications. It could be that in this way we missed methylation markers that are very promising, but are not yet reproduced by others. Finally, due to the extensive heterogeneity we were not able to perform meta-analyses to quantify the results, which makes interpretation of the prognostic value of each marker more difficult.

Conclusion & future perspective

In conclusion, *BNC1*, *SCUBE3*, *GATA5*, *SFRP1*, *GREM1*, *RASSF1A*, *NEFH*, *LAD1* and *PCDH8* have been identified as promising prognostic methylation markers for RCC in this systematic review, but they require further prospective validation and evaluation to assess their true biomarker potential before implementation in clinical practice can be considered.

This systematic review revealed some of the reasons why none of the so far published prognostic methylation markers for RCC are clinically successful, with extensive heterogeneity on several levels as overarching issue. These problems are not unique to the field of prognostic methylation marker research in RCC, but apply to the whole biomarker field in general. One step toward overcoming these problems is the establishment of the REMARK guidelines. However, we believe that in order to develop clinically successful prognostic methylation markers in the future, more rigorous changes are required. Standardization of methylation analyses techniques and marker

Figure 5. Graph showing average REMARK score per promising prognostic methylation marker.

values, and universal definition and use of clinical end points would be of great benefit. Also registration of planned (prognostic) biomarker studies in a database, accompanied by a prespecified protocol including a detailed description of the analysis methods, could help to reduce the number of overanalyzed studies that report specious findings in the future [78]. Moreover, we think that more studies and research groups should focus on further validation of potential promising methylation markers, instead on further increasing the pool of methylation markers that have been reported in a single, underpowered study without any validation. In Box 1, we provide some recommendations for future studies on prognostic methylation markers. These recommendations are not exhaustive and should be used in addition to existing recommendations and guidelines such as REMARK. However, in our opinion, these recommendations represent the most important aspects specific for prognostic methylation marker studies.

Box 1. Recommendations for design and reporting of prognostic methylation marker studies.

Study design

– Study population

- Include samples from one tumor subtype and TNM stage OR perform subgroup analysis for the different subtypes and stages.
- Always include control samples (normal or normal adjacent tissue).
- Specify whether patients received any (neo)adjuvant systemic treatment, if so, correct for these treatments in a multivariable analysis OR perform subgroup analysis per systemic agent.
- Use *a priori* power analysis to calculate the minimum required sample size OR use alternative (*post hoc*) measures such as the minimal detectable hazard ratio (MDHR).
- Use an independent study population to validate the results.

– Methylation analysis technique

- If possible, use publicly available methylation data from The Cancer Genome Atlas to gain insight into the methylation pattern of the specific gene and to assess the best genomic location for designing the methylation marker assay.
- When using primer-based techniques, be sure that the primers specifically amplify the region of interest and test this using tools such as BiSearch.

– Definition of end points

- Use the end points and their definitions as they are established by the DATECAN Renal Cancer group.
- **Statistical analysis**
- Correct for known prognostic variables such as tumor stage, tumor size, Fuhrman nuclear grade and the presence of necrosis, by performing multivariable analysis.
- Evaluate the prognostic value of the methylation marker in a multivariable prediction model and assess its incremental prognostic value compared to the current reference standard (such as the Stage Size Grade Necrosis [SSIGN] score and the UCLA Integrated Staging System [UISS]).
- Study reporting**
- Adhere to existing guidelines and checklists such as REMARK, CONSORT, STROBE, TRIPOD.
- General recommendations**
- Check the literature for previous studies reporting the prognostic value of a specific methylation marker and establish its current level of evidence (LOE). Check whether your planned study will indeed contribute to the current LOE, if not, consider changing the study design to increase the LOE for that specific marker.

If joint efforts are made to increase the quality of methylation biomarker research, we believe that methylation markers can become powerful tools in the management of several cancer types, including RCC.

Financial & competing interests disclosure

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

No writing assistance was utilized in the production of this manuscript.

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Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.futuremedicine.com/doi/full/10.2217/epi-2017-0040

Executive summary

Systematic review of the literature on prognostic methylation markers for renal cell carcinoma

- Forty-nine articles that studied the prognostic value of DNA methylation of one or more genes in renal cell carcinoma (RCC) were included.
- In total, these studies reported over 70 potential prognostic methylation markers for RCC.

REMARK scoring

- Scoring of the articles included in the systematic review according to the REMARK guidelines reveals incomplete reporting in almost all studies.
- REMARK scoring also reveals substantial methodological heterogeneity among the studies, including characteristics of patient series, methylation detection techniques, determination of marker cut-off values, use of clinical end points and method of statistical analysis.

Promising prognostic methylation markers for RCC

- Despite this extensive heterogeneity, *BNC1*, *SCUBE3*, *GATA5*, *SFRP1*, *GREM1*, *RASSF1A*, *NEFH*, *LAD1* and *PCDH8* are identified as promising prognostic methylation markers for RCC, since their prognostic value was independently validated in different studies.

Conclusion

- *BNC1*, *SCUBE3*, *GATA5*, *SFRP1*, *GREM1*, *RASSF1A*, *NEFH*, *LAD1* and *PCDH8* are prognostic methylation markers for RCC that hold promise for the future, but they require further prospective validation.
- Extensive methodological, clinical and statistical heterogeneity among studies on prognostic methylation markers for RCC, prevents translation of these markers to the clinic.
- Rigorous changes in the (methylation) biomarker research field, such as standardization of methodology, is needed to bring more markers to the clinic.

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- Critical editorial on why clinical translation of biomarkers is failing and the problems this research field has to face and tackle.