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REVIEW ARTICLE

Methylation markers for prostate cancer prognosis: a systematic review

Chun Chao · Margaret Chi · Melissa Preciado · Mary Helen Black

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Abstract
Purpose We conducted a systematic review to summarize current evidence on the prognostic utility of DNA methylation markers in prostate cancer and ascertain knowledge gaps to inform future research.
Methods We identified relevant studies using combined key search against PubMed database. Inclusion criteria were studies of human subjects that examined the association between DNA methylation markers and prostate cancer disease outcomes. The methodological quality of each study was systematically evaluated. Findings were qualitatively summarized. Due to heterogeneity and concerns of internal validity, no meta-analysis was performed.
Results Twenty studies were reviewed; sample size ranged from 35 to 605 men in the prognostic analyses. Sixteen studies examined methylation markers in prostate cancer tissue and four examined circulating DNA methylation markers. Of all genes reviewed, paired-like homeodomain transcription factor 2 (*PITX2*) methylation was examined in two more rigorously designed studies and was found to be associated with biochemical recurrence. Common limitations in current literature included small sample sizes, lack of adequate adjustment for established prognostic factors, and poor reporting quality.
Conclusion Evidence on the prognostic utility of methylation markers in prostate cancer is inconclusive. Future research should ascertain large samples with adequate follow-up and include patients of racial/ethnic minority and those treated with modalities other than prostatectomy (e.g., using prostate cancer diagnostic biopsy as tissue source).

Keywords Prostate cancer · Methylation · Prognosis · Systematic review · Biomarker

C. Chao (✉) · M. Chi · M. Preciado · M. H. Black
Department of Research and Evaluation, Kaiser Permanente Southern California, 100 S Los Robles Ave, 2nd Floor, Pasadena, CA 91101, USA
e-mail: chun.r.chao@kp.org

Introduction

Prostate cancer is the most commonly diagnosed non-cutaneous cancer in men in the USA [1]. As a result of the widely adopted prostate-specific antigen (PSA)-based screening program, the majority of prostate cancer detected in the USA is at a localized stage and often asymptomatic. Treatment decisions for localized prostate cancer, especially for those with an intermediate Gleason grade, are complicated by the fact that a large number of localized prostate cancer tumors are slow growing and will not otherwise cause symptoms even in the absence of treatment [2]. Curative prostate cancer treatments, on the other hand, often result in undesirable side effects such as urinary incontinence and erectile dysfunction [3] and should be considered in light of informed risk and benefit. However, there is currently no established clinical algorithm that can accurately predict risk of progression for these localized, intermediate grade cancers. While nomograms for predicting insignificant prostate cancer have been developed [4–6], studies showed that a considerable proportion of patients remain misclassified by these nomograms [7–10]. These algorithms based on clinical and pathological features also do not give information about biologic targets in novel therapeutic development for aggressive disease.

In addition, prostate cancer recurrence and progression following curative treatment also pose a significant public health challenge. It has been reported that up to 20 % of the

patients receiving radical prostatectomy experienced biochemical recurrence within 5 years of surgery, with many patients subsequently developing metastatic diseases [11]. Given such heterogeneity in the clinical course of prostate cancer, prognostic and predictive biomarkers are urgently needed to inform personalized treatment strategy, disease monitoring, and use of adjuvant therapy. However, despite a large number of studies searching for prognostic/predictive biomarkers for prostate cancer, there is currently no standard biomarker-based clinical test for prostate cancer management. A comprehensive systematic review examining novel prognostic biomarkers for prostate cancer concluded that the majority of these biomarker studies were subjected to weaknesses in study design, limiting the inference of findings in the literature [12]. Reliable prognostic/predictive biomarkers for prostate cancer thus remain to be established.

Aberrant DNA methylation is an early landmark event in carcinogenesis [13]. DNA methylation represents a stable and heritable form of gene silencing and is the most robust and readily measurable epigenetic modification [14]. Hypermethylation of the promoter region of many classic tumor suppressor genes has been found in many cancer types, which suppresses the key cancer-preventing functions such as DNA repair, cell adhesion, cell cycle control, and apoptosis [15]. Several genes, including those that encode glutathione S-transferase pi 1 (*GSTP1*), adenomatous polyposis coli (*APC*), Ras association domain-containing protein 1 (*RASSF1A*), and prostaglandin-endoperoxide synthase 2 (*PTGS2*), are hypermethylated in prostate cancer but not in normal prostate tissue [16–18]. Methylation of these loci may have utility in improving the sensitivity of prostate cancer diagnosis [19–21]. Epigenetic mechanism such as DNA methylation is also found to be involved in the regulation of metastasis development [22, 23]. Therefore, it is reasonable to hypothesize that methylation status of certain genes may serve as useful biomarkers to predict tumor behavior.

To assess current knowledge on the prognostic utility of DNA methylation in prostate cancer, we conducted a systematic review to summarize available evidence and identify gaps in the literature to help guide the direction of future research.

Methods

Study identification

A literature search in PubMed was conducted in June 2011 to identify studies that examined the prognostic utility of DNA methylation markers in prostate cancer using the following keywords in titles and abstracts: the key indexing term "prostate" was combined with search terms "methylation," "methylated," "epigenetic," "epigenetics," "hypermethylation," "hypomethylation," and "unmethylated." The search was not limited to the year of publication, although all articles returned by the search were published after 1990. A preliminary review of abstracts was conducted to determine study relevance. An initial set of eligibility criteria was applied at this stage of the screening: (1) article in English; (2) include human subjects (i.e., not based on in vitro or animal observation only); (3) examined DNA methylation markers from any source of tissue; and (4) examined characteristics associated with disease aggressiveness, including clinical characteristics such as stage, Gleason's score, and clinical outcomes such as recurrence or metastasis. Studies that met these initial eligibility criteria were included for further review of the full-text article. In addition to the electronic search of keywords, we also searched the reference list of all identified relevant review articles on the subject of epigenetic/methylation and prostate cancer.

Study inclusion/exclusion criteria

Upon full-text review, articles that met the initial inclusion criteria and examined the association between DNA methylation markers obtained at the time of diagnosis or treatment (i.e., obtained from tissue prior to the development of study outcomes) and prostate cancer disease outcomes were included in this systematic review. Prostate cancer outcomes were defined as any of the following: (a) biomedical (PSA) recurrence; (b) local recurrence; (c) use of adjuvant therapy; (d) metastasis; (e) disease-free survival; (f) disease-specific survival; and (g) overall survival. In the event that two or more studies examined overlapping study populations, all studies were retained if they reported on different DNA methylation markers. If no additional markers were evaluated, studies of smaller sample size or earlier publication (if equal sample size) were excluded.

Data extraction

For each study, the following information was extracted when possible and applicable, using a standard data collection form: first author, year of publication, country where the study was conducted, study (sample collection) period, study design, subject description, age, race, sample size, prostate cancer treatment, outcome examined, follow-up time, source/type of tissue, genes examined, and method of methylation assay. In addition, information on findings and statistical methods was extracted for genes that were examined by at least three studies, using a data collection form which included study first author and year, statistical method, form of methylation marker modeled, covariate included in the model, definition of outcome evaluated,

survival by marker category, marker effect estimates, and *p* value. Data were extracted by one investigator and checked by another investigator; discrepancy was resolved by consensus.

Study evaluation

The methodological quality of each study was evaluated systematically using the methods proposed by Hayden et al. [24] on appraising the quality of prognosis studies as well as the REMARK reporting guidelines for tumor marker prognostic studies [25]. As recommended by these guidelines, we assessed the quality of each study based on the following six potential sources of bias: (1) study population, (2) sample ascertainment and attrition due to missing data or loss-to-follow-up, (3) prognostic factor measurement, (4) outcome measurement, (5) confounding measurement and account, and (6) statistical analysis. Specific quality assessment items within each of the six areas were then developed, based on the quality assessment algorithms developed by Sutcliffe et al. [12] for prostate cancer prognosis studies.

For each quality assessment item, if a study adequately addressed this item, a "yes" was assigned to that item for that study. If the study provided some, but not all of the critical information that should have been reported, a "partial" was assigned. If the study did not properly address the item or did not provide sufficient information, a "no" or "unsure" was noted in such situations. If the item did not apply to a study, a "N/A" was assigned. The specific quality assessment items and algorithm for scoring are detailed below.

Study population

Two quality assessment items were evaluated: (1) Whether the inclusion and exclusion criteria were adequately described. This should include information on the recruitment period, prostate cancer treatment modality, and use of neoadjuvant therapy. (2) Whether baseline characteristics of the study sample were described. This should include age as well as information on all established prognostic factors such as race, PSA, clinical and/or pathological stage, Gleason grade, and surgical margin status when applicable. For race, we did not require studies conducted in countries other than the USA and Canada to report on race given the relatively limited racial diversity in these countries. As proposed by Sutcliffe et al. [12], rather than defining the degree of representativeness of the study population to the ideal source population of interest, we focused on whether the study had clearly characterized the population to which the study results were applicable.

Study ascertainment and attrition

Three quality assessment items were evaluated: (1) Whether the study reported the number of eligible patients in the pool from which the study subjects were selected (sampling scheme). (2) Whether the study reported the proportion of subjects lost to follow-up at any time after the study baseline. (3) Whether the study addressed the impact of study attrition, including the effect of missing baseline covariate, methylation measurement, or outcome data. If the study appropriately applied multiple imputations for missing values, a "yes" was assigned. If the study applied single or other form of imputation, and compared the results from complete dataset and the imputed datasets, a "partial" was assigned. If the study did not use imputation but discussed the potential impact of missing data in terms of the plausible direction and magnitude of bias, a "partial" was assigned. If the study had no discussion about the impact of missing data, a "no" was assigned. If there was no or minimal (i.e., <10 %) missing data, then this item was considered not applicable (N/A) to that study.

Prognostic marker measurement

Four quality assessment items were evaluated: (1) Whether there was a clear definition of the DNA methylation marker measured, including a description of the gene and the region(s) within the gene. A "yes" was assigned if the PCR primer sequence was provided. (2) Whether there was sufficient information about the laboratory procedures, including the information on source of DNA, storage conditions, sample volume, and specific reagents/kits used for methylation profiling. A "yes" was assigned if all these components were addressed, and a "partial" was assigned if only some were addressed. (3) Whether the measurement method was sufficient to limit misclassification. This should include description of quality control procedures such as the use of positive/negative controls and/or duplicated runs. (4) Whether the DNA methylation level was adequately modeled. If the study used a quantitative methylation assay, and modeled methylation level as a continuous variable, such as the normalized index of methylation (NIM), a "yes" was assigned. A "partial" was assigned if methylation level was only assessed as a binary variable as in non-quantitative methylation PCR or if continuous methylation values were dichotomized using non-outcome-dependent thresholds. If the threshold was outcome-dependent, a "no" was assigned, since this approach is likely to introduce bias. Also, if the study did not standardize for background signals, a "no" was assigned.

Outcome measurement

Three quality assessment items were evaluated: (1) Whether the study outcomes were clearly defined. This should include methods used for assessing the outcome and the length of follow-up. If the outcome was metastasis or prostate cancer-related death, then method of ascertainment should be reported. (2) Whether the definition for biochemical recurrence (when applicable) was based on consensus recommendations, i.e., PSA >0.2 ng/ml after prostatectomy, [26] or for radiotherapy, an increase by >2 ng/ml above the nadir PSA level (2005) [27] or three consecutive PSA rise above the nadir (1997) [28] following radiotherapy. (3) For multicenter studies, we required an explicit statement for whether outcome assessment methods were consistent for all study sites. This should include the use of a standard clinical follow-up protocol for all study subjects. For single center study, we assumed the standard clinical protocol was applied.

Confounding measurement

Confounding measurement and account comprised of one quality assessment item: whether all established prognostic factors were adjusted for, regardless of their crude statistical significance. These factors included race (when applicable), clinical stage, Gleason score, preoperative PSA, and surgical margin status (when applicable) [29]. If only some of the factors listed above were adjusted, a "partial" was assigned. A "no" was assigned if only crude assessment was done.

Statistical analysis

Five quality assessment items were evaluated: (1) Whether there was sufficient presentation of data to assess the quality of the analysis. This should include presentation of crude associations between (a) methylation markers and established prognostic factors, (b) methylation markers and outcomes of interest, and (c) established prognostic factors and outcomes of interest. (2) Multivariable findings were not selectively reported. That is, risk estimates and confidence intervals for all methylation markers included in the multivariable analyses were reported, regardless of statistical significance. If all markers in the multivariable analyses were reported but only p value was reported, a "partial" was assigned. (3) Whether the statistical method was appropriate for the study design. (4) Whether the number of events per variable was adequate. A minimum of 10 was considered acceptable. (5) Whether internal or external validation was performed. An external validation consists of validating the findings in a study sample independently collected, whereas an internal validation may consist of validation in a non-overlapping subset of the original sample or with a bootstrapping technique.

Each study was independently evaluated by two investigators [CC and MHB]. Discrepancy was resolved by consensus. Results from the evaluation of each quality assessment item across studies were summarized in a bar chart. Given that the sample size in many of the included studies was small, we also discussed findings from studies that included more than 200 subjects, as these represent the most informative studies in the literature to date. Due to the heterogeneity across studies and concerns regarding internal validity, a meta-analysis was not carried out. This review therefore focuses on the assessment of the quality of evidence related to the prognostic utility of methylation markers in prostate cancer, and the identification of methodological and knowledge gaps to inform the direction of future research.

Results

A total of 1,756 articles were retrieved upon the combined key term search. Based on review of the abstracts, 1,507 original articles and eight review articles were excluded for not meeting the initial eligibility criteria. The full text was reviewed for the remaining 214 original articles, and 18 studies were found to meet the final inclusion criteria. Two other studies were identified to meet the inclusion criteria from manual search of the reference list of the 18 included studies. The study population in Bastian et al. [30] overlapped with Bastian et al. [31], Liu et al. (2008 in *The Prostate*) [32] overlapped with Liu et al. (2008 in *Clinical Cancer Research*) [33], and Liu et al. [34] overlapped with Kron et al. [35]. However, since these studies reported on different methylation markers, all of these studies were retained. A total of 20 studies were included in this systematic review. Figure 1 shows the flowchart of the study identification process.

The study design, study population, methylation markers, and outcomes examined in these 20 studies are summarized in Table 1. Sixteen studies were based on a retrospective cohort design, and four studies used a case–control design. Sixteen studies focused on subjects who underwent radical prostatectomy. Of these, subjects from 10 studies were free of neoadjuvant treatment, while six studies did not describe whether there was use of neoadjuvant therapy. One study examined hormone-refractory prostate cancer in which all patients were initially treated with maximum androgen blockage. The remaining three studies did not include treatment modality as a selection criterion. Most studies included subjects diagnosed in the PSA era (after 1986) [2], although one study had included subjects diagnosed in the pre-PSA era, and six studies did not report the calendar time period from which their subjects were included. Most studies

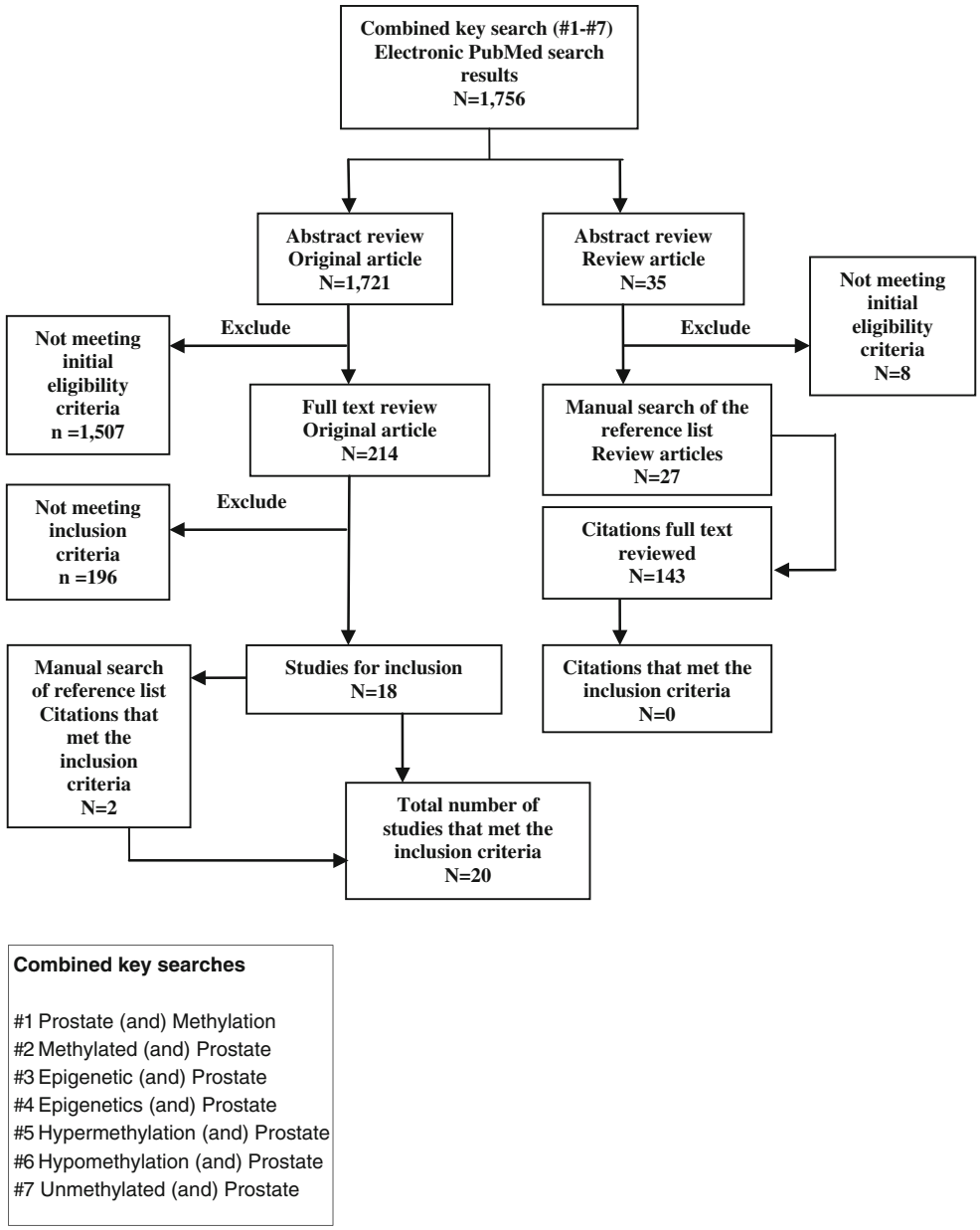

Fig. 1 Study identification flowchart

($n = 16$) examined methylation markers in prostate tissues, while four studies examined the methylation in serum markers. Most studies ($n = 18$) used bisulfite conversion and methylation-specific PCR for the methylation sequencing. Twelve studies examined the outcome of biochemical recurrence only.

Findings of study quality evaluation

Summary of findings

Overall, none of the studies examined in this review fulfilled all evaluation criteria. In general, most studies either fully or partially fulfilled requirements for characterization of the inclusion/exclusion criteria and baseline study population. All studies provided a clear description of the prognostic factors evaluated, and most studies appeared to employ the standard methylation assays with quality control procedures to limit assay error. In terms of outcome assessment, most studies either fully or partially described the outcome definition. For studies that examined the biochemical recurrence outcome, most studies used the standard definition. Studies largely varied on methods and reporting quality in the area of statistical analysis. That said, most studies employed an adequate statistical modeling approach and did not selectively report multivariable

Table 1 Descriptions of study setting, population, and methylation markers examined of the 20 studies reviewed

First author/year	Country	Sample collection period	Study design	Race	Cohort description[a]/ sample size	Prostate cancer treatment	Clinical outcome examined	Follow-up time	Sample size for the prognosis analysis	Source/type of tissue	Genes examined	Method of methylation profiling
Yegnasubramanian/ 2004 [44]	USA	1988–1995	Cohort study	NR	73 Men undergoing radical prostatectomy for clinically localized PCa at Johns Hopkins Hospital Median age (range) 61 (38–73) years	Radical prostatectomy None of the men received androgen deprivation therapy	Biochemical recurrence: PSA post-prostatectomy >0.2 ng/ml n = NR	8–13 Years	36 Men with recurrence data and reach undetectable PSA post-surgery	Surgical specimens snap-frozen at −80 °C	MYOD1, GSTP1, APC, RASSF1A, PTGS2 MDR1, HIC1, EDNRB, ESR1, CDKN2A, CDKN2b, p14/ ARF, MGMT, hMLH1, TIMP3, DAPK, CDH1	Bisulfite conversion and real-time methylation-specific PCR for determining copies of fully methylated promoter site
Bastian/2005 [31]	USA	1999	Cohort study (initial cohort) Case–control (validation)	NR	85 Consecutive men who underwent radical prostatectomy in 1999 at Johns Hopkins Hospital for localized prostate cancer constituted the initial cohort Mean age (range) 59 (40–71) years Additional 55 matched pairs of men with clinically localized PCa treated with radical prostatectomy constituted the validation case–control set Mean age (range) 59 (43–71) years	Radical prostatectomy Info on neoadjuvant or adjuvant therapy NR	Biochemical recurrence: PSA post-prostatectomy >0.2 ng/ml N = 4 for initial cohort N = 55 for validation case–control	Initial cohort: 1–5 years Validation set: Median follow-up time (range) 4 (1–14) years and 3 (1–7) years for those who with and without recurrence	192 (combined analysis of initial cohort and validation set)	Serum sample collected prior to RP, stored at −80 °C	GSTP1	Methylation-specific restriction enzyme quantitative PCR
Rosenbaum/2005 [42]	USA	NR	Cohort design	NR	95 Patients with Gleason 3 + 4=7, underwent radical prostatectomy at Johns Hopkins, for whom both tissue sample and >7 years of follow-up were available	Radical prostatectomy None of the patients received any adjuvant treatment before recurrence	Biochemical recurrence: PSA of 0.2 mg/ml and increasing n = 14 Metastatic disease and/or death from clinical data n = 23	Median follow-up time: Patients without progression: 9 years Patients with progression: 8 years	74 Patients with adequate tumor presented in the surgical specimens	Surgical specimens, FFPE	GSTP1, APC, Cyclin D2, RARβ2, TIG1, RASSF1A	Bisulfite conversion, quantitative methylation-specific PCR

Table 1 continued

First author/ year	Country	Sample collection period	Study design	Race	Cohort description[a]/ sample size	Prostate cancer treatment	Clinical outcome examined	Follow-up time	Sample size for the prognosis analysis	Source/type of tissue	Genes examined	Method of methylation profiling
Woodson/ 2006 [40]	USA	1992–1998	Cohort design	82 % White 13 % black 5 % Asian	60 Men with node-negative clinically localized prostate cancer who were treated with radical prostatectomy at Madigan Army Medical Center	Radical prostatectomy Pts who received any adjuvant or hormone therapy pre- or post-surgery were excluded	Biochemical recurrence: PSA ≥0.2 mg/ml for at least 2 consecutive visits $n = 11$	Mean follow-up time: Patients without recurrence: 78 months Patients with recurrence: 56 months	All 60	Surgical specimens, FFPE	GSTP1, RARβ2, CD44, PTGS2	Bisulfite conversion, quantitative methylation-specific PCR
Cottrell/ 2007 [63]	USA, Germany	1990–2002	Case–control design	NR	Patients who underwent RP at Baylor College of Medicine, Charite Hospital Berlin, U of Muenster, Virginia Mason Medical Center and Ardais Corporation Median age: 60 years Standfor U, U of Muenster, and U of Ragensburg 304 initial set and 223 independent validation set Median age: 66 years	Radical prostatectomy None of the patients received any neoadjuvant or adjuvant therapy	PSA recurrence, defined based on the clinical criteria at each institution. PSA recurrence within 2 years was counted as early recurrence, and no PSA recurrence within 4 years was counted as no early recurrence $n = 59$ (in final multivariable analysis)	2–4 years	193 from an independent validation set in the multivariable analysis	Surgical samples, frozen as well as FFPE	Initial analysis: whole genome search for prognostic markers Validation analysis: Mainly evaluated ABHD9 and Chr3-EST	Bisulfite conversion, methylation microarray and methylation-specific quantitative PCR
Henrique/ 2007 [38]	Portugal	NR	Cohort design	NR	83 Consecutive prostate biopsy specimens collected at the Portuguese Oncology Institute—Porto for individuals referred to for elevated PSA levels Age (range) 51–85 years	Hormone, RP and radiation therapy (treatment was not a selection criteria) Info on neoadjuvant or adjuvant therapy NR	Primary: disease-specific survival $n = 15$ (die from PCa) Secondary: Biochemical recurrence: 2 consecutive PSA >0.4 ng/ml ($n = 37$)	Median (range) 45 (6–60) months	All 83	Biopsy core, FFPE	APC, CCND2, GSTP1, RARB2, RASSF1A	Bisulfite conversion, quantitative methylation-specific PCR
Alumkal/ 2008 [36]	USA	NR	Case–control design	93 % White 7 % non-white	151 Patients underwent RP at Johns Hopkins Hospital for whom tissue specimens and at least 5 years of follow-up were available Median age (range) 59 (41–71)	Radical prostatectomy None of the patients received adjuvant therapy Use of neoadjuvant therapy NR	Biochemical recurrence: PSA ≥0.2 $n = 47$	5 years of follow-up after prostatectomy	All 151	Surgical specimens, FFPE	CDKN2A, CD44, WIF1, GSTP1, EDNRB, ASC, CDH13, RUNX3, MGMT, TIMP3, APC	Bisulfite conversion, Nested methylation-specific PCR

Table 1 continued

First author/ year	Country	Sample collection period	Study design	Race	Cohort description[a]/ sample size	Prostate cancer treatment	Clinical outcome examined	Follow-up time	Sample size for the prognosis analysis	Source/type of tissue	Genes examined	Method of methylation profiling
Bastian/2008 [30]	USA	NR	Mixed cohort design + case control design	NR	Radical prostatectomy at Johns Hopkins Hospital for localized PCa Mean age (range) 59 (40–71) years (same population as in Bastian 2005)	Radical prostatectomy Info on neoadjuvant or adjuvant therapy NR	Biochemical recurrence: PSA post-RP >0.2 ng/ml $n = 4$ for initial cohort $n = 55$ for validation case–control	Initial cohort: 1–5 years Mean follow-up (range) 4 (1–14) and 3 (1–7) for those without and with recurrence	192 (combined analysis of initial cohort and validation set)	Serum sample collected prior to RP, stored at −80 °C	MDR1, EDNRB, CD44, NEP, PTGS2, RASSF1A, RAR-beta, ESR1	Methylation-specific restriction enzyme quantitative PCR
Ellinger/ 2008 [37]	Germany	1995–1999	Cohort design	NR	80 Men with clinically localized PCa who underwent RP Median age (range) 64 (51–75)	Radical prostatectomy Info on neoadjuvant or adjuvant therapy NR	Biochemical recurrence: PSA post-RP >0.2 ng/ml $n = 13$	Median (range) 20.2 (6.5–72.3) months	41 Men had follow-up information	Surgical specimens, FFPE	ACTB, Annexin2, APC, EDNRB, GSTP1, MDR1, PTGS2, RARbeta, Reprimo, TIG1	Bisulfite conversion, quantitative methylation-specific PCR
Liu (1)/2008 [32]	Portugal	NR	Cohort design	NR	88 Prostate adenocarcinomas with no metastatic lesion and treated with RP at Portuguese Oncology Institute-Porto Age median (range) 64 (40–76)	Radical prostatectomy Info on neoadjuvant or adjuvant therapy NR	Relapse: not defined $n = 18$	NR	All 88	Surgical specimens, stored at −80 °C	36 Candidate genes, results for MCAM presented	Bisulfite conversion, quantitative methylation-specific PCR
Liu (2)/2008 [33] (same population as in Liu (1) 2008)	Portugal	NR	Cohort design	NR	88 Prostate adenocarcinomas with no metastatic lesion and consecutively treated with RP at Portuguese Oncology Institute-Porto Age median (range) 64 (40–76)	Radical prostatectomy Info on neoadjuvant or adjuvant therapy NR	Relapse: not defined $n = 19$	NR	All 88	Surgical specimens, stored at −80 °C	SSBP2	Bisulfite conversion, quantitative methylation-specific PCR
Roupret/ 2008 [66]	UK	2000–NR	Case–control design	White	20 PCa cases with relapse and 22 PCa cases with no relapse during follow-up (matched on follow-up time), prospectively Enrolled in study at University of Sheffield Age median (range) 73–75 (58–81) years	Selection does not depend on treatment: 17 % RP, 12 % radiotherapy, 71 % hormone therapy Info on neoadjuvant or adjuvant therapy NR	Biochemical recurrence: PSA post-RP >0.2 ng/ml $n = 20$	Time to relapse median (range) 15 (9–32) months	All 42	Serum samples at diagnosis collected at diagnosis, stored at −80 °C	RASSF1A, CDH1, APC, DAPK, MGMT, p16, p14, GSTP1, RARbeta2, TIMP3	Bisulfite conversion, quantitative methylation-specific PCR

Table 1 continued

First author/ year	Country	Sample collection period	Study design	Race	Cohort description[a]/ sample size	Prostate cancer treatment	Clinical outcome examined	Follow-up time	Sample size for the prognosis analysis	Source/type of tissue	Genes examined	Method of methylation profiling
Richiardi/ 2009 [47]	Italy	1980 Cohort: 1982–1988 1990 cohort: 1993–1996	Cohort design	NR	Consecutive PCa patients for biopsy, transurethral resection or RP in pathology ward of San Giovanni Battista Hospital 1980 cohort $n = 298$ Age: mean 72 years Patients diagnosed at the hospital: 1990 cohort $n = 280$ Age: mean 70 years	Selection does not depend on treatment modality Info on neoadjuvant or adjuvant therapy NR	PCa mortality $n = 121$ in 1980 cohort $n = 76$ in 1990 cohort	14 years	Those with successful DNA extraction 1980 cohort $n = 228$ 1990 cohort $n = 253$	Biopsy and surgical specimens, FFPE	GSTP1, APC, RUNX3	Bisulfite conversion, methylation-specific PCR
Vanaja/ 2009 [43]	USA	1999–2003	Case–control design	NR	Patients undergone RP at Mayo Clinic, including 32 non-recurrent, free of disease >5 years after RP, and 32 with recurrence in 5 years Age: NR	RP with no prior therapy Info on adjuvant therapy NR	Biochemical recurrence: PSA $\geq$0.2 ng/ml, followed by a value higher than the first ($n = 10$) + local recurrence: positive biopsy or salvage radiation therapy ($n = 10$) + systemic progression: positive bone scan or positive biopsy at other than the prostate bed ($n = 12$)	5 years	All 64	Surgical specimens, fresh frozen	FLNC, EFS, ECRG4, KCNMA1, GSTP1, PDLIM4, RARB2, PITX2	Bisulfite conversion, quantitative Methylation-specific PCR
Weiss/ 2009 [58]	USA	1993–2000	Cohort design	NR	All 605 patients aged 40–80 treated with RP at Baylor College of Medicine SPORE, Standard University and Virginia Mason Medical Center Age (range) 40–80 years	RP Pts who received neoadjuvant or adjuvant therapy were excluded	Biochemical recurrence: PSA >0.2 ng/ml on 2 consecutive tests $n = 65$	Median: 66 months	All 605	Surgical specimens, FFPE	ABHD9, CCND2, Chr3-EST, GPR7, HIST2H2BF, PITX2	Bisulfite conversion, quantitative methylation-specific PCR
Banez/ 2010 [45]	USA and Netherland, Germany	1995–2001	Cohort design	56 % White 19 % black 25 % other/ unknown	548 Men with stage pT2 or pT3, localized PCa who underwent RP at Baylor College of Medicine, Duke University, Durham Veterans Affairs Medical Center, Erasmus Medical Center Age (range) 40–79 years	RP Pts who received any therapy before RP were excluded	Biochemical recurrence: PSA >0.2 ng/ml on 2 consecutive tests $n = 106$	NR	476 with sufficient DNA sample and successful marker data	Surgical specimens, FFPE	PITX2	Bisulfite conversion, quantitative methylation-specific PCR (customized PIX2 microarray)

Table 1 continued

First author/ year	Country	Sample collection period	Study design	Race	Cohort description[a]/ sample size	Prostate cancer treatment	Clinical outcome examined	Follow-up time	Sample size for the prognosis analysis	Source/type of tissue	Genes examined	Method of methylation profiling
Kron/2010 [35]	Canada	1998–2001	Cohort design	NR	243 Pts diagnosed with PCa at the University Health Network in Toronto Mean age (range) 62 (41–75) years	RP Pts who received neoadjuvant therapy before RP were excluded	Biochemical recurrence: not defined $n = 85$	Mean (range) 1,600 (63–3,460) days	232 Pts with no lymph node metastasis	Surgical specimens, FFPE	HOXD3	Bisulfite conversion, quantitative methylation-specific PCR (MethyLight)
Okegawa/ 2010 [39]	Japan	2005–2009	Cohort design	NR	76 Pts with bone metastatic hormone-refractory PCa who were treated at Kyorin University Hospital Age mean (range) 72 (57–83)	All pts initially treated with maximum androgen blockage (MAB). Second line therapy was also MAB 16 pts received taxane-based chemotherapy	Overall mortality $n = 38$	Time to death: Median (range) 21 (3–48) months	All 76	Serum collected 3.0–4.8 months after initiation of therapy, stored at −80 °C	GSTP1, APC, PTGS2, MDR1, RASSF1A	Bisulfite conversion, quantitative methylation-specific PCR
Liu/2011 [34] (study population overlap with Kron 2010)	Canada	1998–2001	Cohort design	NR	219 Pts who underwent RP at the University Health Network in Toronto Age mean (range) 61 (42–76) years	RP Pts who received neoadjuvant therapy before RP were excluded	Biochemical recurrence: not defined $n = NR$	NR	219 with complete methylation data	Surgical specimens, FFPE	APC, TGFβ2, RASSF1A,	Bisulfite conversion, quantitative methylation-specific PCR (MethyLight)
Pierconti/ 2011 [41]	Italy	2006–2008	Cohort design	NR	Consecutive PCa surgical specimens collected from 51 pts at Catholic University School of Medicine Age median (range) 71 (51–79) years	RP No chemo- or immunotherapy before RP	Disease progression, defined as PSA >0.2 ng/ml and or needle biopsy confirmed local or distant failure and/or detection of metastatic lesions $n = NR$	NR	35 Pts with follow-up data available	Surgical specimens, FFPE	SOCS3	Bisulfite conversion, methylation-specific PCR

findings. However, there were several areas that most studies failed to address. For example, study attrition was rarely reported. Additionally, lack of adequate sample size and lack of validation effort were common among studies identified. Figure 2 summaries the counts of studies in each designation for each quality assessment item. Limitations in the current literature are discussed further below for each quality assessment area:

Study population

Common reasons for inadequate reporting of inclusion/exclusion criteria were failure to report the time frame of recruitment ($n = 6$), and lack of explanation as to whether neoadjuvant therapy was considered an inclusion/exclusion criteria ($n = 6$). Inadequate description of the baseline study sample was typically due to lack of information on race/ethnicity by studies conducted within the USA or Canada ($n = 16$).

Study ascertainment and attrition

We found that only six studies adequately reported the study sampling rate. All of which included consecutive patients during a period of time. Furthermore, only three studies reported the proportion of subjects lost to follow-up. Among these three studies, one study reported significant attrition as half of the subjects did not have follow-up information. None of the studies offered discussion on the potential impact of study attrition, although two studies had minimal loss-to-follow-up. Therefore, the potential impact of study attrition on study internal validity was difficult to assess.

Prognostic factor measurement

Most studies provided some information about the specimen handling and assay protocols, but only four provided all the information outlined by reporting guidelines. Four studies that employed methylation assays that produced

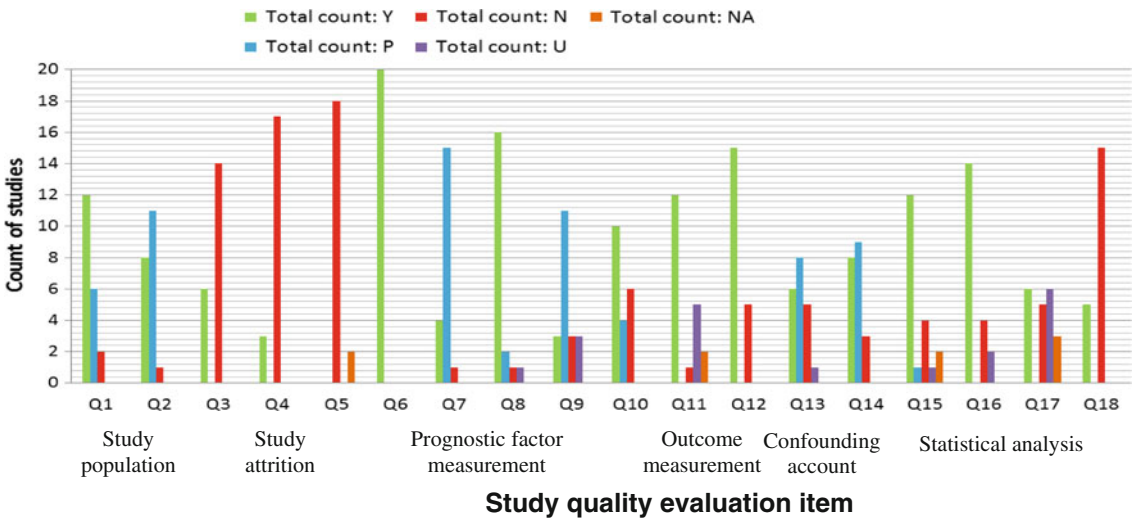

Fig. 2 Summary of findings from study quality evaluation. *Y* Yes, *N* no, *P* partial, *U* unknown due to lack of details presented, *NA* not applicable. *Study population* Q1: Inclusion and exclusion are adequately described, including methods to identify study population and period of recruitment. Q2: Baseline study sample is adequately described for key characteristics: age, race, PSA, clinical and/or pathological stage, biopsy and/or pathological Gleason grade, surgical margin. *Study attrition* Q3: Study reported participation or sampling rate. Q4: Study reported % loss-to-follow-up. Q5: The authors commented on the potential impact of study attrition. *Prognostic factor measurement* Q6: Clear description of measured prognostic factors is provided (e.g., DNA area of methylation measured). Q7: Sufficient information about laboratory procedures, including the information on source of DNA, storage condition, sample volume, specific reagents/kits used for bisulfite conversion and methylation-specific PCR, and sample handling. Q8: Measurement method is sufficient to limit misclassification (i.e., positive/negative PCR and methylation controls, same setting/method for all subjects). Q9: Is methylation level well defined and adequate? Either continuous methylation levels are reported or non-data-dependent cutoffs are used? *Outcome measurement* Q10: Is the outcome clearly defined, including length of follow-up? Q11: If the study has an outcome of biochemical recurrence, has the international definition of biochemical recurrence been used? (PSA >0.2 ng/ml after prostatectomy, or a rise by 2 ng/ml or more above the nadir PSA or three consecutive PSA rises above the nadir following radiotherapy.) Q12: The method and setting for outcome measurement are the same for all study participants (i.e., a standard outcome assessment protocol). *Confounding measurement and account* Q13: Overall, does the model include all classical markers (PSA, stage, Gleason grade, and surgical margin if applicable) so that established prognostic factors are appropriately accounted for? *Statistical analysis* Q14: There is sufficient presentation of data to assess the adequacy of the analysis. Q15: Multivariable analysis findings not selectively reported (i.e., risk estimates and confidence intervals for all methylation markers in the multivariable analyses were reported, regardless of statistical significance). Q16: Statistical modeling is appropriate for the study design. Q17: Adequate the number of events per variable ($\geq$10 was considered acceptable). Q18: The use of internal or external validation

binary output (methylated vs. not methylated), while the reminder used quantitative ms-PCR. However, despite having continuous methylation values, 13 studies modeled methylation level as a dichotomized variable, which likely resulted in the loss of information. Furthermore, two of these studies used an outcome-driven cutoff value to dichotomize methylation, which may have resulted in over-optimism bias.

Outcome measurement

We found that 10 studies did not provide clear outcome definition or study follow-up time. Among the 16 studies that examined the biochemical recurrence outcome, one study applied non-standard definitions and five did not provide the definition of biochemical recurrence. We also found that most studies did not report the protocol for assessing study outcomes, i.e., no information was provided about the frequency for follow-up PSA measurement and/or clinical follow-up visits, and whether or not the same protocol was used for all study subjects. However, most were single center studies, and only five included multiple centers. In summary, we found outcome definition inadequately provided by about half of the studies reviewed.

Confounding measurement and account

We found that only six studies adequately account for and eight studies partially accounted for the established prognostic factors in the multivariable analysis. Five studies did not perform multivariable analysis. For the other study, it was not clear what was included in the multivariable analysis. Thus, current evidence on the incremental prognostic utility of methylation markers beyond established clinical factors is limited.

Statistical analysis

Most studies were based on a small sample size, and five studies [36–40] did not have adequate event to variable ratio (i.e., <10) in the final model. The event to variable ratio was unclear for six studies [33, 34, 41–44]. Most except five studies did not include any validation effort. Overall, small sample size and lack of any validation effort appeared to be the most significant concerns regarding statistical analysis and are likely to affect the validity and generalizability of the study findings.

Summary of current finding on prognostic utility of methylation markers

The associations between prostate cancer disease outcomes and methylation markers for the following genes have been examined in three or more studies and are summarized in Table 2. Glutathione S-transferase pi 1 (*GSTP1)* and adenomatous polyposis coli (*APC*) were the most commonly evaluated genes to date. Other genes included in this review are nuclear receptor protein retinoic acid receptor beta (*RAR-beta),* Ras association domain-containing protein 1 (*RASSF1A*), paired-like homeodomain transcription factor 2 (*PITX2*), prostaglandin-endoperoxide synthase 2 (*PTGS2)*, *cyclin D2 (CCND2*), and endothelin receptor type B (*EDNRB*). Except one study [45], all other studies examined methylation level in the gene promoter region. Level of evidence for each of these genes is presented below in the order of the number of studies available.

GSTP1

GSTP1, or glutathione S-transferase gene, encodes a detoxifying enzyme that catalyzes conjugation reactions with reduced glutathione [46]. GSTP1 plays a role in the metabolism and elimination of potentially harmful xenobiotics, thus protects cells from DNA damage and cancer development. *GSTP1* promoter hypermethylation is the most common epigenetic abnormality observed in prostate cancer [16]. We identified eight studies that examined prostate cancer tissue *GSTP1* methylation levels and prostate cancer progression [36–38, 40, 42–44, 47]. Of the four studies that conducted multivariable analyses, two studies reported an inverse association between *GSTP1* hypermethylation and disease progression (Table 2), while the other two reported lack of association. We also identified three studies that examined the methylation status of *GSTP1* in circulating cell-free DNA and reported a positive association between *GSTP1* hypermethylation and disease progression. However, one of these studies [39] considered *GSTP1* methylation in combination with four other genes (*APC*, *PTGS2*, *MDR1,* and *RASSF1A*), and reported methylation in at least one gene was significantly associated with poor patient outcome. In general, studies that evaluated *GSTP1* methylation failed to conduct the necessary multivariable analyses required to evaluate the incremental prognostic value of *GSTP1* beyond traditional clinical prognostic factors. Most studies also suffered from limitations due to small sample size and were likely underpowered to detect significant effects. The three studies of circulating methylation markers had generally consistent and statistically significant findings, yet all three of these studies were small and are subjected to potential publication bias among other biases. With the limitations in mind, there does not seem to be consistency in the predictive value of circulating *GSTP1* methylation markers and tissue *GSTP1* methylation.

Table 2 Summary of statistical methods and findings on methylation markers evaluated in at least 3 reviewed studies

Study/marker	Statistical analysis	Form of methylation marker	Locations of the CpG loci queried	Covariates adjusted in the analysis	End point	Survival by marker category	Marker effect estimates	*p* value
GSTP1								
Yenasubramanian/2004 [44]	K-M analysis	NIM, analyzed as dichotomous variable, median used as cutoff	Promoter region	Unadjusted analysis	PSA recurrence: Single PSA >0.2 ng/ml	NR	NIM > median was not significantly associated with PSA recurrence. Point estimate NR	Not significant, *p* value NR
Rosenbaum/2005 [42]	Multivariable Cox model	Ratio of promoter methylation of targeted gene over reference gene, analyzed as a dichotomous variable. 75th percentile used as cutoff	Promoter region	Age >60 APC, CCND2	Progression, defined as PSA >0.2 ng/ml, metastasis and/or death.	NR	Upper quartile methylation adjHR = 0.29 (0.11–0.77)	0.01
Woodson/2006 [40]	Bivariate logistic model	Ratio of methylation of targeted gene over reference gene, analyzed as a dichotomous variable. Cutoff choice not clear	Promoter region	Unadjusted analysis	PSA recurrence: PSA >0.2 ng/ml for ≥2 consecutive visits	NR	Hypermethylation unadjOR = 5.31 (0.63–45.07)	0.13
Henrique/2007 [38]	K-M survival analysis	Ratio of promoter methylation of targeted gene over reference gene, analyzed as a dichotomous variable. 75th percentile used as cutoff	Promoter region	Unadjusted analysis	Primary: prostate cancer mortality. Secondary: PSA recurrence: 2 consecutive PSA >0.4 ng/ml	5-yr disease-free survival: 40 and 35 % for men with GSTP1 methylation <75th percentile and ≥75th percentile	NA	0.047 (for KM survival curve)

Table 2 continued

Study/marker	Statistical analysis	Form of methylation marker	Locations of the CpG loci queried	Covariates adjusted in the analysis	End point	Survival by marker category	Marker effect estimates	*p* value
Alumkal/2008 [36]	Multivariable logistic regression	Dichotomous: yes: any methylated band; no: no methylated band	Promoter region	Preoperative PSA, postoperative Gleason score, extracapsular penetration, lymph node involvement, seminal vesical involvement, surgical margin CDKN2A, GSTP1, ASC, CDH13, RUNX3, MGMT	PSA recurrence: single PSA >0.2 ng/ml within 5 years of surgery	NR	GSTP1 methylation: adjOR = 0.30 (0.07–1.24)	0.10
Ellinger/2008 [37]	Multivariable Cox model	NIM, analyzed as dichotomous variable. Cutoff determined based on ROC analysis	Promoter region	NR	PSA recurrence: single PSA >0.2 ng/ml	NR	Hypermethylation of GSTP1 not associated with PSA recurrence by itself. Point estimate NR	NR
Richiardi/2009 [47]	Multivariable Cox model	Methylation versus no methylation from non-quantitative PCR	Promoter region	Source of tumor tissue, Gleason score, follow-up duration (age was the time axis in the model)	Prostate cancer mortality	NR	GSTP1 methylation adjHR = 1.00 (0.64–1.58) in the 1980s cohort. adjHR = 1.44 (0.82–2.54) in the 1990s cohort	NR
Vanaja/2009 [43]	*t* test	Methylation score[a]	Promoter region	NR	PSA recurrence: PSA >0.2 ng/ml followed by a value higher than the 1st PSA, excluding any PSA within 30 days of surgery; local recurrence, or metastasis within 5 years of surgery	NA, case–control design	GSTP1_CpG_9[b] methylation SE = 0.75, SP = 0.73 GSTP1_CpG_21[b] SE = 0.73, SP = 0.73 GSTP1_CpG_19[b] SE = 0.77, SP = 0.82	(*t* test) 0.02 0.02 0.02

Table 2 continued

Study/marker	Statistical analysis	Form of methylation marker	Locations of the CpG loci queried	Covariates adjusted in the analysis	End point	Survival by marker category	Marker effect estimates	*p* value
APC								
Yenasubramanian/2004 [44]	K-M analysis	NIM analyzed as dichotomous variable, median used as cutoff	Promoter region	Unadjusted analysis	PSA recurrence: single PSA >0.2 ng/ml	NR	NIM > median was not significantly associated with PSA recurrence survival. Point estimate NR	NR
Rosenbaum/2005 [42]	Multivariable Cox model	Ratio of promoter methylation of targeted gene over reference gene, analyzed as dichotomous variable. 75th quartile used as cutoff	Promoter region	Age >60 GSTP1	Progression defined as PSA >0.2 ng/ml, metastasis and/or death	8-yr progression-free survival: 56 and 30 % for men with APC upper quadrant methylation and other	Upper quartile APC methylation adjHR = 3.0 (1.42–6.32) Combining APC and Cyclin D2: upper quartile methylation 1 positive: adjHR = 1.84 (0.92–3.72) 2 positive: 4.33 (1.52–12.33)	0.004 0.09 0.01
Henrique/2007 [38]	Multivariable Cox model	Ratio of promoter methylation of targeted gene over reference gene, analyzed as dichotomous variable. 75th quartile used as cutoff	Promoter region	Clinical stage	Primary: Prostate cancer mortality. Secondary: PSA recurrence: 2 consecutive PSA >0.4 ng/ml	4-yr disease-free survival: 65 and 25 % for men with APC methylation <75th percentile and ≥75th percentile 4-yr disease-specific survival: 85 and 55 % for men with APC methylation <75th percentile and ≥75th percentile	Disease-specific survival for upper quartile APC methylation adjHR = 3.51 (1.23–9.96) Disease-free survival: adjHR = 2.58 (1.29–5.16)	0.018 0.008
Alumkal/2008 [36]	Bivariate logistic model	Dichotomous: yes: any methylated band, no: no methylated band	Promoter region	Unadjusted analysis	PSA recurrence: single PSA >0.2 ng/ml within 5 yr of surgery	NR	APC methylation band: unadjOR = 1.26 (0.58–2.74)	0.10
Ellinger/2008 [37]	Multivariable Cox model	NIM, analyzed as dichotomous variable, cutoff determined based on ROC analysis	Promoter region	NR	PSA recurrence: single PSA >0.2 ng/ml	NR for APC methylation status 6 years survival: APC + Reprimo Unmethylated: ~65 % Methylated: ~40 %	Hypermethylation of APC not associated with PSA recurrence in multivariable model. Point estimate NR	NR Log-rank test: 0.0078

Table 2 continued

Study/marker	Statistical analysis	Form of methylation marker	Locations of the CpG loci queried	Covariates adjusted in the analysis	End point	Survival by marker category	Marker effect estimates	*p* value
Richiardi/2009 [47]	Multivariable Cox model	Methylation versus no methylation from non-quantitative PCR	Promoter region	Source of tumor tissue, Gleason score, follow-up duration (age was the time axis in the model)	PCa mortality	NR	APC methylation adjHR = 1.42 (0.98–2.07) in the 1980s cohort. adjHR = 1.57 (0.95–2.62) in the 1990s cohort. adjHR = 1.49 (1.11–2.00) when both cohorts combined	NR
Liu/2011 [34]	Multivariable Cox model	Percentage of methylated reference (PMR), analyzed as a dichotomous variable, 75th percentile was sued as cutoff	Promoter region	Gleason score, pathological stage, surgical margin, age	PSA recurrence, definition not provided	8-yr PSA-free survival: 55 and 58 % in men with high and low APC methylation (log-rank $p = 0.707$)	APC methylation adjHR = 2.22 (0.78–6.32) APC methylation independently predicted PSA recurrence of pT2 stage patients adjHR = 2.174 (1.04–4.53)	0.137 0.038
RAR-beta								
Rosenbaum/2005 [42]	Bivariate Cox model	Ratio of promoter methylation of targeted gene over reference gene, analyzed as a dichotomous variable. 75th quartile used as cutoff	Promoter region	Unadjusted analysis	Progression defined as PSA >0.2 ng/ml, metastasis and/or death	NR	Upper quartile hypermethylation unadjHR = 1.22 (0.59–2.52)	0.59
Woodson/2006 [40]	Bivariate logistic model	Ratio of methylation of targeted gene over reference gene, analyzed as a dichotomous variable. Cutoff choice not clear	Promoter region	Unadjusted analysis	PSA recurrence: PSA >0.2 ng/ml for ≥2 consecutive visits	NR	RAR-beta2 Methylation unadjOR = 3.34 (0.66–17.29)	0.14

Table 2 continued

Study/marker	Statistical analysis	Form of methylation marker	Locations of the CpG loci queried	Covariates adjusted in the analysis	End point	Survival by marker category	Marker effect estimates	*p* value
Henrique/2007 [38]	K-M survival analysis	Ratio of methylation of targeted gene over reference gene, analyzed as a dichotomous variable. 75th quartile used as cutoff	Promoter region	Unadjusted analysis	Primary: Disease-specific survival (definition not given). Secondary: PSA recurrence: 2 consecutive PSA >0.4 ng/ml	NR	NA	NS, NR
Ellinger/2008 [35]	Multivariable Cox model	NIM, analyzed as dichotomous variable, cutoff determined based on ROC analysis	Promoter region	NR	PSA recurrence: single PSA >0.2 ng/ml	NR	Hypermethylation of RAR-beta not associated with PSA recurrence. Point estimate NR	NR
Vanaja/2009 [43]	*t* test	Methylation score[a]	Promoter region	NR	PSA recurrence >0.2 ng/ml within 5 years, local recurrence, metastasis	Not applicable, case–control design	RAR-beta2 not associated with recurrence. Point estimate NR	(*t* test) NR
RASSF1A								
Yenasubramanian/2004 [44]	K-M analysis	NIM, analyzed as dichotomous variable, median used as cutoff	Promoter region	Unadjusted analysis	PSA recurrence: single PSA >0.2 ng/ml	NR	NIM > median was not significantly associated with recurrence survival. Point estimate NR	NR
Rosenbaum/2005 [42]	Bivariate Cox model	Ratio of promoter methylation of targeted gene over reference gene. 75th percentile used as cutoff	Promoter region	Unadjusted analysis	Progression defined as PSA >0.2 ng/ml, metastasis and/or death	NR	Upper quartile hypermethylation unadjHR = 0.70 (0.31–1.59)	0.39

Table 2 continued

Study/marker	Statistical analysis	Form of methylation marker	Locations of the CpG loci queried	Covariates adjusted in the analysis	End point	Survival by marker category	Marker effect estimates	*p* value
Henrique/2007 [38]	K-M survival analysis	Ratio of promoter methylation of targeted gene over reference gene, analyzed as a dichotomous variable. 75th percentile used as cutoff	Promoter region	Unadjusted analysis	Primary: Disease-specific survival (definition not given). Secondary: PSA recurrence: 2 consecutive PSA 2 >0.4 ng/ml	5-yr disease-free survival: 50 and 20 % for men with RASSF1A methylation <75th percentile and ≥75th percentile	NA	0.019
Liu/2011 [34]	Multivariable Cox model	Percentage of methylated reference (PMR), analyzed as a dichotomous variable, 75th percentile used as cutoff	Promoter region	Gleason score, pathological stage, surgical margin, age	PSA recurrence, definition not provided	8-yr PSA-free survival: 65 and 55 % in men with RASSF1A high and low methylation (log-rank $p = 0.284$)	RASSF1A methylation adjHR = 0.74 (0.42–1.29)	0.284
PITX2								
Vanaja/2009 [43]	*t* test	Methylation score[a]	Promoter region	NR	PSA recurrence >0.2 ng/ml within 5 years, local recurrence, metastasis	Not applicable, case–control design	PITX2_CpG_10 methylation SE = 0.67, SP = 0.64	(*t* test) 0.01866
Weiss/2009 [58]	Multivariable Cox model	Methylation score, analyzed as dichotomous variable using median methylation score in the cohort	Promoter region	Gleason score, pathological stage, preoperative PSA, surgical margins	PSA recurrence (>0.2 ng/ml on 2 consecutive tests) or clinical recurrence or decision to treat based on increased PAS	8-yr PSA-free survival: 94 and 79 % for men with low and high PITX2 methylation	PITX2 methylation adjHR = 2.1 (1.2–3.9)	0.016
Banez/2010 [45]	Multivariable Cox model	Low versus high methylation, cutoff empirically derived from pilot study data	First exon of 2 splice variants	Gleason score, pathological stage, surgical margin, age, preoperative PSA	PSA recurrence: 2 consecutive PSA test >0.2 ng/ml	10-yr PSA-free survival: 80, 50 % for men with low and high PITX2 methylation	High versus low PITX2 methylation adjHR = 2.39 (1.45–3.94)	<0.001

Table 2 continued

Study/marker	Statistical analysis	Form of methylation marker	Locations of the CpG loci queried	Covariates adjusted in the analysis	End point	Survival by marker category	Marker effect estimates	*p* value
PTGS2								
Yenasubramanian/2004 [44]	Multivariable Cox model	NIM, analyzed as a dichotomous variable, median used as cutoff	Promoter region	Pathological stage, Gleason score	PSA recurrence: single PSA >0.2 ng/ml	10-yr PSA-free survival: 20 and 60 % for men with tissue PTGS2 methylation > and < median	NIM > median adjHR = 4.26 (1.36–13.36)	0.01
Woodson/2006 [40]	Multivariable logistic model	Ratio of methylation of targeted gene over reference gene, analyzed as a dichotomous variable. Cutoff choice not clear	Promoter region	Gleason score	PSA recurrence: PSA >0.2 ng/ml for ≥2 consecutive visits	8-yr PSA-free survival: 55 and 85 % for those with both PSGS2 and CD44 methylated versus neither or 1 methylated	Both PTGS2 and CD44 methylated adjOR = 8.87 (1.85–42.56)	0.006
Ellinger/2008 [37]	Multivariable Cox model	NIM, analyzed as dichotomous variable, cutoff determined based on ROC analysis	Promoter region	NR	PSA recurrence: single PSA >0.2 ng/ml	NR	Hypermethylation of PTGS2 not associated with PSA recurrence in multivariable model. Point estimate NR	NR
CCND2								
Rosenbaum/2005[42]	Multivariable Cox model	Ratio of promoter methylation of targeted gene over reference gene, analyzed as a dichotomous variable. 75th percentile used as cutoff	Promoter region	Age >60, GSTP1	Progression defined as PSA >0.2 ng/ml, metastasis and/or death	8-yr Progression-free survival: 62, 40 and 28 % for men with APC and CCND2 hypermethylation for 0 positive, 1 positive and 2 positive, respectively	Combining APC and Cyclin D2: 1 positive upper quartile methylation: aHR = 1.84 (0.92–3.72) 2 positive upper quartile methylation: 4.33 (1.52–12.33)	0.09 0.01
Henrique/2007 [38]	K-M survival analysis	Ratio of promoter methylation of targeted gene over reference gene, analyzed as a dichotomous variable. 75th quartile used as cutoff	Promoter region	Unadjusted analysis	Primary: Disease-specific survival (definition not given). Secondary: PSA recurrence: 2 consecutive PSA >0.4 ng/ml	NR	NA	Not significant, *p* value NR

Table 2 continued

Study/marker	Statistical analysis	Form of methylation marker	Locations of the CpG loci queried	Covariates adjusted in the analysis	End point	Survival by marker category	Marker effect estimates	*p* value
Weiss/2009 [58]	Bivariate Cox model	Methylation score, analyzed as dichotomous variable using median methylation score in the cohort	Promoter region	Unadjusted analysis	PSA recurrence (>0.2 ng/ml on two consecutive tests) or clinical recurrence or decision to treat based on increased PAS	NR	CCND2 methylation adjHR = 1.4 (0.8–2.2)	0.23
EDNRB								
Yegnasubramanian/2004 [44]	K-M analysis	NIM, analyzed as a dichotomous variable, median used as cutoff	Promoter region	Unadjusted analysis	PSA recurrence: single PSA >0.2 ng/ml	NR	NIM > median was not significantly associated with PSA recurrence survival. Point estimate NR	NR
Alumkal/2008 [36]	Bivariate logistic regression	Dichotomous: yes: any methylated band, no: no methylated band	Promoter region	Unadjusted analysis	PSA recurrence: single PSA >0.2 ng/ml within 5 years of surgery	NR	EDNRB methylation band: unadjOR = 1.49 (0.59–3.74)	0.40
Ellinger/2008 [37]	Multivariable Cox model	NIM, analyzed as a dichotomous variable, cutoff determined based on ROC analysis	Promoter region	NR	PSA recurrence: single PSA >0.2 ng/ml	NR	Hypermethylation of EDNRB not associated with PSA recurrence. Point estimate NR	NR
Serum markers								
GSTP1								
Bastian/2005 [31]	Multivariable Cox model	Hypermethylation index (continuous), analyzed as a dichotomous variable. Cutoff determination not clear	Promoter region	Surgical margin, pathologic lymph node status	PSA recurrence: single PSA >0.2 ng/ml	3-yr PSA-free survival: 41 and 74 % for men with positive or negative serum DNA GSTP1 hypermethylation, log-rank $p = 0.01$	Serum GSTP1 hypermethylation (positive versus negative) adjHR = 4.4 (2.2–8.8)	<0.001

Table 2 continued

Study/marker	Statistical analysis	Form of methylation marker	Locations of the CpG loci queried	Covariates adjusted in the analysis	End point	Survival by marker category	Marker effect estimates	*p* value
Roupret/2008 [66]	Chi-square test and multivariable linear model (data not shown)	Ratio of promoter methylation of targeted gene over reference gene. Continuous variable	Promoter region	NR	PSA recurrence: single PSA >0.2 ng/ml	Not applicable, case–control design	GSTP1 methylation was 100 % (concentration 47.5) and 91 % (47.6) in the group with recurrence and without recurrence	<0.0001
Okegawa/2010 [39]	Multivariable Cox model	NIM, analyzed as a dichotomous variable, cutoff based on ROC analysis	Promoter region	PSA level, Gleason score, extent of disease, PSA doubling time, circulating tumor cells	Overall survival among hormone-refractory patients	NR for GSTP1. Median survival: 12 versus 48 mos for those with and without methylated marker of GSTP1, APC, PTGS2, MDR1 and RASSF1A	≥1 methylated marker versus no methylated marker: adjHR = 1.83 (1.07–2.96)	<0.001

Mos Months, *NA* not applicable, *NIM* normalized index of methylation, ranging from 0–100 % [38], *NR* not reported, *PSA* prostate-specific antigen, *years* years

[a] Methylation score in this study was defined as the difference of the distance for each class of the methylation value for each CpG weighed by the class average CpG methylation and further divided by the class methylation variance to half the class average CpG unit methylation

[b] Selected CpG locus in the GSTP1 promoter region. CpG site location information for Alumkal et al. [36] was obtained from contacting the corresponding author

APC

APC, a tumor suppressor gene, encodes the protein adenomatous polyposis coli which plays a critical role in several cellular processes, including cell division, adhesion, and cell migration [48]. Mutations in this gene are known to increase risk of colorectal cancer [49]. We identified seven studies that examined *APC* methylation status in prostate cancer tissue and risk of prostate cancer disease progression [34, 36–38, 42, 44, 47] Of the five studies that conducted a multivariable analysis, all except one study reported a significantly (or marginally significant) elevated hazard ratio with *APC* hypermethylation. Therefore, although none of the studies accounted for all known prognostic factors, there appeared to be some suggestion of the prognostic utility of *APC* methylation status for prostate cancer progression.

RAR-beta

The human *RAR-beta* gene encodes the nuclear receptor protein retinoic acid receptor beta. *RAR-beta* is a nuclear transcriptional regulator mediates cellular signaling in embryonic morphogenesis, cell growth, and differentiation [50]. It is thought that this protein functions as a tumor suppressor by limiting growth of many cell types [51]. We identified five studies that examined the association between prostate cancer tissue *RAR-beta* methylation status and risk of prostate cancer disease progression [37, 38, 40, 42, 43]. In the only study [37] where multivariable analysis was conducted, *RAR-beta* was not significantly associated with biochemical recurrence. The other four studies also reported a lack of statistical significance in their unadjusted findings. All of these studies had a sample size less than 100 men.

RASSF1A

The *RASSF1A* gene encodes the Ras association domain-containing protein 1. The encoded protein was found to interact with DNA repair protein XPA as well as inhibit the accumulation of cyclin D1 and thus induce cell cycle arrest [52]. Loss or altered expression of this gene has been implicated in the development of various cancers, suggesting the tumor suppressor role of this gene [53]. We identified four studies that examined the association between prostate cancer tissue *RASSF1A* methylation status and risk of prostate cancer disease progression [34, 38, 42, 44]. Only one study [34] examined the association between *RASSF1A* and biochemical recurrence in multivariable analysis and found that *RASSF1A* was not associated with biochemical recurrence. Of the three studies that reported the crude association only, two did not find any significant association and one reported an inferior 5-year biochemical recurrence-free survival for those with *RASSF1A* hypermethylation. Of these four studies, three studies suffered from limited sample size, and one study did not report the number of events.

PITX2

The human *PITX2* gene encodes the protein called paired-like homeodomain transcription factor 2, also known as pituitary homeobox 2. This protein acts as a transcription factor and regulates procollagen lysyl hydroxylase gene expression [54]. *PITX2* hypermethylation has been observed in several tumor types, including acute myeloid leukemia [55], lung [56], and breast [57]. We identified three studies that examined the association between prostate cancer tissue *PITX2* methylation status and risk of prostate cancer disease progression [43, 45, 58]. All three studies reported a significant positive association between *PITX2* hypermethylation and risk of progression. While one study only examined the crude association, the other two studies had relatively larger sample sizes (i.e., >200 subjects) and number of outcome events. Both of these studies had accounted for all important established prognostic factors for prostatectomy patients. As such, there is some evidence for the prognostic utility of *PITX2* based on two studies that appear to have better quality and greater internal validity when compared to other included studies.

PTGS2

Human *PTGS2* gene encodes protein prostaglandin-endoperoxide synthase 2, also known as cyclooxygenase-2 (*COX-2*). *PTGS2* converts arachidonic acid to prostaglandin-endoperoxide H2 and is involved in all stages of carcinogenesis [59]. PTGS2 elicits cell-autonomous effects on tumor cells resulting in stimulation of growth, increased cell survival, enhanced tumor cell invasiveness, stimulation of neovascularization, and tumor evasion from the host immune system [59]. Elevated levels of *PTGS2* expression also facilitate a pro-inflammatory environment. We identified three studies that examined the association between prostate cancer tissue *PTGS2* methylation status and risk of biochemical recurrence, all of which reported multivariable adjusted associations with biochemical recurrence [37, 40, 44]. Of these, two studies suggested a positive association with PTGS2 methylation and disease progression, while the other one reported lack of association. It should be noted that all three studies had a small sample size, and most did not report the factors accounted for in the multivariable analysis. These limitations call for caution in interpreting the results of these studies.

CCND2

The human *CCND2* gene encodes the protein called G1/S-specific cyclin-D2. The cyclin proteins are regulators of cyclin-dependent kinases and mediate the transition of cells from G1 to S phase and thus promote cell cycle progression and chromosomal instability [60]. We identified three studies that examined the association between prostate cancer tissue *CCND2* methylation status and risk of prostate cancer disease progression [38, 42, 58]. *CCND2* methylation status was not found to be associated with disease progression in two studies in the crude analysis. In the other study where multivariable analysis was conducted [42], *CCND2* was evaluated along with *APC* methylation status. Hypermethylation (≥75th percentile) of both genes was associated with disease progression [hazard ratio = 4.33 (1.52–12.33)] during ≥8 years of follow-up. Again, two of the three studies had limited sample size of less than 100 subjects.

EDNRB

The human *EDNRB* gene encodes the protein endothelin receptor type B. Endothelin receptor type B is a G protein-coupled receptor which activates a phosphatidylinositol-calcium second messenger system [61]. This receptor regulates several critical biological processes, including the development and function of blood vessels, the production of certain hormones, and the stimulation of cell growth and division [62]. We identified three studies that examined the association between prostate cancer tissue *EDNRB* methylation status and risk of biochemical recurrence [36, 37, 44], all of which reported a lack of statistical significant association. Only one study conducted multivariable analysis for *EDNRB*. However, all three studies used dichotomized methylation status and suffered from limited sample size, which may not have sufficient power to detect a significant association.

Summary of larger study findings of methylation discovery

Five studies that evaluated methylation markers in prostate cancer tissue met the sample size requirement of 200 or more. Cotterll et al. [63] conducted a case–control study of 304 men with radical prostatectomy, with an additional 223 men in an independent validation set. Subjects with a median age of 60 were ascertained from four hospitals from the USA and Germany. This study performed a genome-wide search for prognostic methylation markers. Among the top five candidate markers identified, 3 markers, *G protein-coupled receptor (GPR7), or neuropeptides B/W receptor 1 (*NPBW1), *epoxide hydrolase 3 (ABHD9)*, and *an expressed sequence tag on chromosome 3 (Chr3-EST*) significantly distinguished patients with and without early recurrence. ABHD9 and Chr3-EST were further analyzed among an independent validation set of patients with 59 early biochemical recurrence and 134 without recurrence. In multivariable regression, *ABHD9* and *Chr3-EST* were both significantly associated with recurrence, adjusting for Gleason score, pathology stage, and surgical margin. The strengths of the study include the use of an independent validation set. Lacking the outcome definition used at each institute as well as racial information renders it difficult to assess to whom the study results may apply. However, the consistency in the associations with these two markers in different patient subsets provides some preliminary evidence for the prognostic utilities of these two genes.

Weiss et al. [58] conducted a cohort study of 605 patients aged 40–80 years who were treated with radical prostatectomy between 1993 and 2000 at three medical centers in the USA. Weiss and colleagues examined the associations between methylation status of six genes previously shown to be predictive of prostate cancer outcomes: *ABHD9*, *CCND2*, *Chr3-EST*, *GPR7*, *histone cluster 2, H2bf (HIST2H2BF*), and *PITX2* and biochemical recurrence during a median follow-up period of 66 months. A total of 65 biochemical recurrence events were observed. Except *CCND2*, all markers were significantly associated with biochemical recurrence in bivariate analysis. *PITX2* had the strongest association and was further evaluated in the multivariable analysis [hazard ratio = 2.1 (1.2–3.9)], adjusting for Gleason score, pathological stage, preoperative PSA, and surgical margin status. Furthermore, *PITX2* methylation status split the patients with intermediate Gleason score seven into two groups with significantly separated survival curves.

Banez et al. [45] conducted a multicenter cohort study to examine the predictive utility of *PITX2* for biochemical recurrence in prostate cancer patients treated with prostatectomy between 1995 and 2001. This study was conducted in the USA and Netherlands. A total of 476 men with localized prostate cancer from four medical centers were included in the analytical cohort. About half of these men were white (56 %), and 25 % were black. There were a total of 106 biochemical recurrence events, although the median length of follow-up was not reported. This study represents a validation effort of previous findings on *PITX2* and included an independent sample that was not used in previous analysis. Study results and limitation were discussed in the previous section for *PITX2*.

Kron et al. [35] conducted a cohort study of 232 patients diagnosed at a mean age of 61 years between 1998 and 2001 and who underwent radical prostatectomy at the one institute in Canada. The authors examined the association

between *homeobox D3 (*HOXD3) promoter hyper-methylation (>75th percentile) and biochemical recurrence. Mean follow-up time was 1,600 days in this study, with a total of 85 patients developing biochemical recurrence during follow-up. In multivariable analysis adjusting for Gleason score, pathological stage, surgical margin status, and age, HOXD3 hyper-methylation was not a significant predictor for biochemical recurrence [HR = 0.50 (0.19–1.33)]. However, the racial composition and definition for biochemical recurrence were not provided, rendering it difficult to assess to whom results may apply.

Liu and colleagues conducted a cohort study of 219 patients which was a subset of the population included in Kron et al. [35]. The authors examined the associations between promoter methylation status of an additional three genes: *APC*, *transforming growth factor-beta 2 (TGF-beta2*), *RASSF1A,* and biochemical recurrence [34]. In multivariable analyses adjusting for Gleason score, pathological stage, surgical margin, and age, methylation status of all three genes were not significantly associated with biochemical recurrence. However, APC and TGF-beta2 methylation predicted biochemical recurrence in patients with pT2 and pT3a stage disease, respectively. The combination of three markers, *APC*, *TGF-beta2,* and *HOXD3,* was then examined. Hypermethylation (i.e., ≥75th percentile) of two or more genes was significantly predictive of the biochemical recurrence. However, in addition to the limitations identified in Kron et al. [35], the number of events observed was not provided for this study, although there appeared to be up to 8 years of follow-up. Overall, this study suffered from poor reporting quality, which hindered the assessment of the validity of their results.

Discussion

Level of current evidence on the prognostic utility of DNA methylation markers

In this systematic review of 20 studies that examined the prognostic utility of DNA methylation markers in prostate cancer, we identified several common limitations in the quality of the study design as well as the quality of reporting. Overall, many of the available studies appeared to be conducted as a secondary analysis and thus were not based on a robust study design. Many studies did not report on the racial composition of the study population. Similarly, the sampling scheme and subject selection methods were often not reported, raising concerns about potential selection bias that may be inherent in these studies. Furthermore, rate of loss-to-follow-up was not reported by many studies, making it difficult to assess the potential bias introduced by attrition. In terms of laboratory assay, most studies used standard bisulfite conversion and ms-PCR as the method to measure DNA methylation status. Most studies employed adequate quality control procedures. However, many studies dichotomized the continuous methylation level which might result in loss of information. In terms of outcome measurement, several studies failed to describe the length of follow-up, as well as the definition for clinical disease progression. However, for studies that examined the biochemical recurrence, most used the standard definition for biochemical recurrence. Most studies did not include all established clinical prognostic factors, and hence did not allow the evaluation of the incremental prognostic utility of methylation markers beyond clinical factors. Lastly, most studies suffered from a small sample size, as only five studies had a sample size greater than 200. Small sample size is a serious limitation when interpreting the non-significant findings from these studies. Finally, most studies lacked any validation effort.

Given these limitations noted, it is not presently possible to draw strong inference for any of these markers. Thus, no formal recommendation can be made as which markers should receive higher priority for evaluation. However, based on the review of current evidence, the more promising marker of choice for further evaluation would be *PITX2*, *APC*, *ABHD9,* and *Chr3-EST*, due to the availability of independent validation as well some consistency in the literature available to date. Notably, findings from several studies are also suggestive of the prognostic utility of a combined test of methylation of several genes.

We identified four studies that examined circulating DNA methylation markers. Disseminated tumor cells and DNA from apoptotic and necrotic tumor cells are released into the bloodstream early in tumor development [64]. Analyses of circulating tumor cells or cell-free DNA allow the detection of tumor-related genetic and epigenetic alterations that are relevant to cancer development and progression [65]. Serum markers can also be obtained repeatedly and monitored longitudinally, theoretically allowing close monitoring of disease progression and treatment response. The selection of appropriate tumor-related genes that are known to have a distinct tumor-related methylation profile is critical in the search for clinically useful tests. Among the four studies identified in this systematic review [30, 31, 39, 66], genes examined included *GSTP1, APC*, *MDR1*, *EDNRB*, *CD44*, *NEP*, *PTGS2*, *RASSF1A*, *RAR-beta*, *ESR1*, *CDH1*, *DAPK*, *MGMT*, *p16*, *p14*, and *TIMP3*. *GSTP1* methylation was found to be associated with prostate cancer outcome in three studies. However, as previously described, significant variability and validity concerns exist in study population and study methods. Therefore, the prognostic role of serum methylation markers, especially *GSTP1*, in prostate cancer needs to be more rigorously examined, particularly by adequately powered studies.

Knowledge gaps and future directions

In addition to the study limitations identified, we also identified knowledge gaps in the literature that may inform the direction of future studies. First, we found that most studies included patients who underwent radical prostatectomy. Evaluation of methylation markers in radiation treated patients, and men without treatment is lacking. As such, there is a lack of research for overall prognostic markers that may inform prognosis without the influence of treatment. For this purpose, cohort of patients who are under active surveillance or watchful waiting will be needed. An even better design is to utilize archived specimens from randomized clinical trials of active surveillance to minimize potential selection bias associated with treatment choice. Both methylation markers in circulating DNA or prostate cancer tissue derived from diagnostic biopsy cores are viable candidates for the search of overall prognostic markers as well as for predictive markers in the context of radiation therapy.

Second, it should be noted that not all biochemical recurrence will be clinically meaningful; and distant metastasis should be the most critical outcome to evaluate. Therefore, future research should pursue longer follow-up to study the most clinically meaningful outcomes with adequate power. Third, there is a lack of studies that primary focuses on African-American men. African-American men are not only at higher risk of developing prostate cancer, but they are also at increased risk of dying from prostate cancer. The mechanism of this racial disparity in prostate cancer has not been fully elucidated. However, many studies have suggested that biological factors contribute to the racial disparity observed. To this end, previous studies have found different methylation profiles in prostate cancer tissue of Caucasian versus African-American men [67, 68]. Therefore, the search for a prognostic algorithm should consider potential racial/ethnic variations with stratified analyses.

Limitations of present systematic review

There are several limitations of this systematic review that should be mentioned. First, in the literature search process, the initial title and abstract screening were not done by duplicates. Instead, two investigators [MC and MP] split the literature search and the title/abstract/full-text screening. However, these investigators were asked to obtain consensus should they encounter any uncertainty. Second, the variations in study design and poor quality in reporting made it challenging to compare study results. Our results were therefore limited to qualitative summary of currently available data, as opposed to literature synthesis. Lastly, due to the small number of studies available for any given marker, we did not formally evaluate the likelihood of and the potential impact of publication bias.

Conclusion

In conclusion, this review demonstrates that the current literature is inconclusive regarding the prognostic and predictive value of DNA methylation markers in prostate cancer. Like in many other systematic reviews of prognostic markers, critical concerns in internal validity and reporting quality are identified. As such, it is important to reinforce the need for adequate study design and adherence to reporting recommendations in order to facilitate the development of useful clinical tumor markers. Several areas found to be limited in the current literature deserve particular attention in future studies. These include sample size, inclusion of African-Americans, inclusion of patients under active surveillance or watchful waiting (e.g., using prostate cancer tissue from diagnostic biopsy as the tissue source), efforts to minimize loss-to-follow-up, use of continuous methylation levels, and accounting for all established clinical prognostic factors to evaluate incremental prognostic utility of the novel marker. Furthermore, given the advancement in technology, evaluation of prognostic methylation markers should move toward multiplex assays and consider multiple markers simultaneously to assess the utility of a multi-marker test.

Conflict of interest The authors declare that they have no conflict of interest.

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