Large-scale external validation and meta-analysis of gene methylation biomarkers in tumor tissue for colorectal cancer prognosis

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Summary

Background DNA methylation biomarkers in colorectal cancer (CRC) tissue hold potential as prognostic indicators. However, individual studies have yielded heterogeneous results, and external validation is largely absent. We conducted a comprehensive external validation and meta-analysis of previously suggested gene methylation biomarkers for CRC prognosis.

Methods We performed a systematic search to identify relevant studies investigating gene methylation biomarkers for CRC prognosis until March 2024. Our external validation cohort with long-term follow-up included 2303 patients with CRC from 22 hospitals in southwest Germany. We used Cox regression analyses to assess associations between previously suggested gene methylation biomarkers and prognosis, adjusting for clinical variables. We calculated pooled hazard ratios (HRs) and their 95% confidence intervals (CIs) using random-effects models.

Findings Of 151 single gene and 29 multiple gene methylation biomarkers identified from 121 studies, 37 single gene and seven multiple gene biomarkers were significantly associated with CRC prognosis after adjustment for clinical variables. Moreover, the directions of these associations with prognosis remained consistent between the original studies and our validation analyses. Seven single biomarkers and two multi-biomarker signatures were significantly associated with CRC prognosis in the meta-analysis, with a relatively strong level of evidence for *CDKN2A*, *WNT5A*, *MLH1*, and *EVL*.

Interpretation In a comprehensive evaluation of the so far identified gene methylation biomarkers for CRC prognosis, we identified candidates with potential clinical relevance for further investigation.

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Keywords: Colorectal cancer; Prognosis; Gene methylation biomarkers; External validation; Meta-analysis

Introduction

Colorectal cancer (CRC) is one of the leading causes of cancer-related morbidity and mortality worldwide, accounting for approximately 0.9 million deaths worldwide in 2020.[1,2] Despite advancements in screening and treatment options, the prognosis of CRC remains highly heterogeneous among patients, underscoring the critical need for reliable prognostic

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Research in context

Evidence before this study

A large number of prognostic gene methylation biomarkers for colorectal cancer (CRC) has been proposed, as summarized in a systematic review published in 2018. However, the majority lack rigorous external validation, impeding their translation into clinical practice. Two previous meta-analyses conducted in 2013 and 2014 found significant associations between *CDKN2A* promoter methylation and adverse prognosis in patients with CRC. For most other proposed methylation biomarkers, relevant meta-analyses are lacking.

Added value of this study

Based on a large clinical cohort including 2303 patients with CRC, we performed an extensive external validation of 180 previously suggested prognostic gene methylation biomarkers identified through an updated systematic review. We were able to confirm the prognostic relevance of 37 single gene and seven multiple gene methylation biomarkers for CRC. In a subsequent meta-analysis, incorporating the newly obtained results from our external validation, we found seven single biomarkers and two biomarker panels for CRC to be prognostically significant. Based on an evidence scoring system, biomarkers demonstrating relatively strong supporting evidence included *CDKN2A, WNT5A, MLH1,* and *EVL*.

Implications of all the available evidence

Our analysis provided a comprehensive assessment and evidence summary of previously suggested gene methylation biomarkers for CRC prognosis. We found several gene methylation biomarkers to be predictive of CRC prognosis, thereby highlighting their potential clinical applicability. Nevertheless, further studies are still needed to establish their added prognostic value beyond the traditional staging system, before their integration into routine clinical practice.

biomarkers to guide more precise clinical decision-making.[3]

Epigenetic modifications, particularly DNA methylation, have been suggested as promising candidates for prognostic biomarkers in CRC.[4] DNA methylation involves the addition of a methyl group to cytosine residues onto CpG dinucleotides, resulting in altered gene expression.[5] Aberrant DNA methylation patterns have been observed in various cancers, including CRC, and have been implicated in tumor development, progression, and response to treatment.[5]

A large number of gene methylation biomarkers have been associated with CRC prognosis in previous studies.[6,7] However, currently, none of these potential prognostic methylation biomarkers have been translated to clinical practice.[4,6] Ensuring the generalizability of these biomarkers requires rigorous validation in large, independent cohorts, a step that has been largely omitted.[4,6] While a few biomarkers have been investigated by multiple studies, they have yielded inconsistent results.[7] A comprehensive systematic review and meta-analysis, synthesizing all available evidence from individual studies, is essential for evidence-based medicine.[8] The most recent systematic review on this topic was published in 2018,[7] but it did not differentiate between different biospecimens (e.g., tumor tissue, blood, or stool), nor did it perform a meta-analysis.[7]

Thus, with this study, we aimed to first contribute new evidence by conducting a comprehensive validation of all previously suggested prognostic gene methylation biomarkers in a large, independent cohort of patients with CRC. Next, we undertook meta-analyses to summarize all available evidence, integrating results from both our external validation and existing literature.

Methods

Systematic search of published DNA methylation biomarkers

This study was performed and reported according to the STROBE[9] and PRISMA[10] guidelines. A systematic search was first conducted to retrieve prognostic gene methylation biomarkers in CRC tissue published as of 26 March 2024. Detailed information on the predefined search strategy, selection criteria, and data extraction process can be found in the eMethods and eTable 1. In brief, we extracted candidate genes with aberrant methylation on the gene level from existing studies (either of a regulatory gene region [e.g., promoter region] or the entire gene) that were statistically significantly associated with survival or recurrence in patients with CRC in at least one study for the validation analysis. When a marker was eligible for the subsequent meta-analysis, we repeated the search to make sure that all studies available for this marker were included in the meta-analysis, whether or not the result was statistically significant.

Validation cohort

We used data from the DACHS study as the external validation cohort. The DACHS study is a large population-based case–control and patient cohort study on CRC with recruitment of patients and controls in the Rhine-Neckar region in the southwest of Germany from 2003 to 2021. The study was approved by the ethics committees of the Heidelberg Medical Faculty of Heidelberg University of and of the Medical Chambers of Baden-Württemberg and Rhineland-Palatinate. Further details regarding the DACHS study have been previously reported.[11–13] In brief, patients meeting the following criteria were recruited from a total of 22

hospitals in the study region: first diagnosis of primary CRC, age above 30 years, and physically and mentally capable of participating in a 1-h interview. In the validation study, we included patients recruited up to the year 2013 who had complete information available for genome-wide DNA methylation measured by Illumina 450K, age, sex, tumor location, and tumor-node-metastasis (TNM) stage. These patients were followed up until the year 2020. Seven patients with missing values in key clinical variables including age, sex, TNM stage, and tumor location were excluded from the analysis. Details regarding data collection and DNA methylation preprocessing are provided in the eMethods.

DNA methylation preprocessing

The raw DNA methylation data files generated by the iScan array scanner were read and preprocessed, including normalization and batch effect correction, using the R package 'CHAMP'.[14] To ensure independent validation, no filtering on CpG sites was applied.[15] Instead, we selected all available CpG sites located in the functional region (mostly the promoter region) or the entire gene reported in the identified studies. If the included study did not specify the genomic location where the methylation was measured, we selected the CpGs located in the promoter region, which covers TSS1500, TSS200, 5′UTR, and 1stExon.

The methylation levels of these CpGs were averaged to create a single gene methylation marker. For the single gene methylation marker, we averaged the methylation value of all the available CpG sites located in the genomic region (e.g., promoter region) investigated in the original study to obtain the gene-wise methylation value. For multi-gene methylation markers, if the original studies constructed prognostic models based on single gene methylation levels, we calculated the prognostic scores for each patient using the equation provided by the development studies. Otherwise, we calculated the mean of the methylation levels of available CpGs corresponding to all the genes comprising the multi-marker.

The percentage of available CpGs in our array for each single and multiple gene methylation marker was summarized. To ensure the robustness of our results, external validation analyses were not performed if the available CpG sites was below 20% for the relevant gene region.

Statistical analyses

External validation

Patient baseline characteristics are presented as absolute and relative frequencies for categorical variables, and the median with range for continuous variables. The median follow-up time was computed using the reverse Kaplan–Meier method.

For the validation of each marker, our validation cohort was adapted to match the tumor location (e.g., colon, rectum, or colon and rectum) and stage of patients investigated in the original discovery study. In the case of multiple studies reporting the same marker, we chose the tumor location and stage categories that covered the broadest range (e.g., colon and rectum, stage I-IV). The validation cohort covered all tumor locations and stage categories investigated by any of the single studies.

The association between each identified gene methylation biomarker and the following four prognostic outcomes was examined: 1) overall survival (OS), defined as the time between the date of CRC diagnosis and the date of death or censoring, 2) disease-free survival (DFS), also known as relapse-free survival, defined as the time from date of CRC diagnosis to the time of CRC recurrence (including metastases), death, or censoring,[16] 3) cancer-specific survival (CSS), defined as the time from the date of CRC diagnosis to death from CRC or censoring, with death from other causes considered a competing event, and 4) time-to-recurrence (TTR), defined as the time from date of CRC diagnosis to the time of CRC recurrence or censoring, with death considered a competing event.

The cumulative event rates of OS and DFS were estimated using Kaplan–Meier curves, and the multivariable Cox proportional hazard models adjusted for age, sex, TNM stage, and tumor location were used to assess hazard ratios (HRs) with 95% confidence intervals (CIs). For CSS and TTR, the cumulative incidence function within a competing risks framework was used to calculate the cumulative incidence of events,[17] and cause-specific Cox proportional hazard models were used to measure the association. Following the method used by most of the included studies, the methylation levels of gene biomarkers were dichotomized before entering the Cox regression analysis, with lower methylation serving as the reference group. The optimal cut-off points for each marker in each prognostic analysis were determined using a method based on maximally selected rank statistics.[18] Additionally, for validated methylation biomarkers, two sensitivity analyses were performed by using the median as cut-off values or standardized continuous variables, and then repeated the multivariable Cox regression analyses.

We defined gene biomarkers as validated by our study if they showed a statistically significant association with any of the four prognostic outcomes in the multivariable Cox regression models, and if the directions of their association with prognosis were consistent between the original study and the validation study. Biomarkers were deemed as not validated if they showed statistical significance but had inconsistent directions of association across reported studies. To explore whether these biomarkers retained their prognostic value across patients with varying characteristics,

subgroup analyses were conducted for these biomarkers by sex, tumor location, TNM stage, and any treatment including radiotherapy, chemotherapy, and neoadjuvant therapy. Additionally, to evaluate the added value of validated methylation markers in conjunction with clinical variables including age, sex, TNM stage, and tumor location, we conducted a comparative analysis. This involved evaluating the discriminative power (quantified by time-dependent area under the receiver operating characteristic curves [AUCs]) and predictive accuracy (measured by time-dependent Brier scores) of two Cox models: one comprising solely clinical variables and another integrating both clinical variables and the validated methylation biomarkers.

Meta-analysis

For biomarkers that were either validated or reported in more than one independent study, we calculated the pooled HRs and their 95% CIs using random effects models. The random-effects model was chosen due to the anticipated high potential of heterogeneity across studies.[19] Aside from evidence available in the existing literature, results from our external validation analyses were incorporated in the meta-analyses.

For each marker eligible for the meta-analysis, we additionally included studies in which their associations with CRC prognosis were not statistically significant.[20–33] For multiple publications from one common study, we only included the latest publication. HRs and their 95% CIs were directly extracted from reports when available, with adjusted HRs extracted preferentially over unadjusted HRs. We unified the reference group as hypomethylation. When the reference group reported in included studies was hypermethylation, reciprocal values of the extracted HRs and 95% CIs were calculated. Log (HR) and their standard errors (SE) were required for the meta-analysis, and the SE was calculated using the following equation: SE = (log (UL)–log (LL))/(2*1.96), in which UL stands for the upper level of the 95% CI and the LL stands for the lower level of the 95% CI. When an HR with a 95% CI were unavailable in an included study, we managed to reconstruct these values by the reported K-M curves. In brief, we extracted data coordinates from the K-M curves using the ScanIt software, which were then uploaded to the IPDfromKM shiny app (https://www.trialdesign.org/one-page-shell.html#IPDfromKM).[34] One author (TY) performed the data extraction for meta-analysis, which was cross-checked by a second author (DW) to ensure accuracy.

The I^2 statistic was used to assess the percentage of variability due to between-study heterogeneity.[35] If a meta-analysis included more than three studies, publication bias was assessed by Egger's test.[36] To explore potential source of heterogeneity, we performed subgroup meta-analyses by study-level characteristics, including study region, sample size, and the percentage of female participants. We also performed subgroup meta-analyses by tumour stage when two or more studies were available for specific subgroup category. Besides, we planned to do subgroup meta-analyses based on the method used for DNA methylation assessment, differentiating between PCR-based low-throughput techniques and epigenome-wide sequencing. Lastly, we conducted sensitivity analyses by restricting to studies reporting HRs adjusted for any potential confounders.

Evidence scoring system

We developed an evidence scoring system to quantify the strength of evidence for biomarkers that were significantly associated with prognosis in the meta-analysis. This scoring system considered the total number of patients, the total number of independent cohorts, and the degree of consistency in the meta-analysis, with details on the calculation provided in the eMethods. For key biomarkers demonstrating a high rank of evidence (top three), we additionally illustrated their Kaplan–Meier curves in our validation cohort and depicted their forest plots in the meta-analysis.

For all analyses, statistical significance was set as a p value less than 0.05. All statistical analyses were performed using R version 4.2.0, and all the R code can be found at: https://github.com/TanweiY/gene_methylation_validation_meta.

Role of the funding source

Study funders had no role in study design, data collection, data analysis, data interpretation, or writing of the report.

Results

Characteristics of eligible studies

Our systematic search identified 121 studies that reported prognostic gene methylation biomarkers for CRC (eFig. 1). The study characteristics and references for each study are provided in the eTables 2–4. The included studies were conducted between 1967 and 2020 and published between 1999 and 2024. Half (50%) of the included studies were from Western Pacific regions, followed by European regions (15%). The majority of studies adopted a candidate gene approach, measuring the methylation level of pre-selected genes mostly by methylation-specific or quantitative methylation-specific PCR (90 studies, 74%). Epigenome-wide arrays (Illumina or MassARRAY) were used by 18 studies (15%). The number of patients with CRC analyzed in each study ranged from 31 to 902 (median 127). The mean or median age of patients varied from 42 years to 74 years (median 64 years). The proportion of females varied from 13% to 63% (median 44%). Most studies (101 studies, 83%) investigated CRC, 17 studies (14%) investigated colon cancer only, and three studies specifically examined rectal cancer. Eighty-five studies (70%) included patients with stage I-IV

cancer, 25 studies (21%) focused on stages I-III, and three studies examined stage IV patients only. Only nine studies (7%) conducted external validation of their findings.

A total of 160 single and 30 multiple gene methylation biomarkers were extracted from the 121 included studies. Notably, 18 single gene and one multiple gene methylation marker were reported in two or more studies. Among these biomarkers, the gene *CDKN2A* was the most extensively investigated, reported in 19 studies, followed by the gene *MLH1*, reported in nine studies.

Characteristics of validation cohort

In our independent patient cohort, the genome-wide methylation array was conducted on a total of 2316 patients. After excluding six patients without follow-up information and seven patients with missing values in tumor location or TNM stage, 2303 patients were included in the analysis. The patients' outcome characteristics for OS, DFS, CSS, and TTR are summarized in the eTable 5 and eFig. 2, respectively. The median follow-up time for the 2303 participants was 10.4 years (interquartile range [IQR]: 10.1–12.4). Patient clinical characteristics are summarized in Table 1.

Variable	Validation cohort *N* = 2303
Diagnosis year	
Range	2003–2013
Median	2007
Age at diagnosis	
Range	30–96
Median (IQR)	69 (62–77)
Gender	
Female	961 (41.7%)
Male	1342 (58.3%)
Stage at diagnosis	
I	417 (18.1%)
II	793 (34.4%)
III	757 (32.9%)
IV	336 (14.6%)
CRC location	
Proximal colon[a]	851 (37.0%)
Distal colon[b]	635 (27.6%)
Rectum	817 (35.5%)
Adjuvant/neoadjuvant treatment[c]	
Yes	1082 (47.0%)
No	1215 (52.8%)
Missing	6 (0.3%)

CRC, colorectal cancer; IQR, interquartile range. [a]Proximal colon including cecum, ascending, hepatic flexure, and transverse colon. [b]Distal colon including splenic flexure, descending, and sigmoid colon. [c]Any treatment including neoadjuvant therapy, chemotherapy, and radiotherapy.

Table 1: **Characteristics of validation cohort.**

The median percentage of available CpG sites for each investigated gene in our validation cohort was 76% (IQR 53–89). Among them, nine single genes had less than 20% available CpG sites and were consequently excluded from the validation analyses. Full details regarding the percentage of available methylation information, validation set sizes, and the optimal cut-off values for all biomarkers is shown in the eTables 6–7.

External validation of prognostic gene methylation biomarkers

Detailed results of the external validation for all biomarkers are provided in the eTables 8–10. We were able to confirm the prognostic value of 37 single and seven multiple gene methylation biomarkers for CRC (Fig. 1). These biomarkers showed significant associations with OS, CSS, DFS, or TTR in multivariable Cox regression analyses, which were consistent regarding the direction of association. Among the 44 biomarkers or panels, 18 showed significant associations with all four prognostic outcomes, maintaining their prognostic value even in sensitivity analyses conducted using the median as cut-off points or standardized continuous methylation values (eTable 11). Throughout the 10-year follow-up period, the inclusion of the validated 44 methylation biomarkers enhanced the discriminative power and predictive accuracy of the Cox model beyond clinical variables alone, albeit with modest improvement in magnitude (Fig. 2).

Nine single marker and one multiple marker signature were statistically significantly associated with CRC outcomes, but their reported directions of association with prognosis were inconsistent across included studies (Fig. 3). In particular, in our validation cohort, *MLH1* promoter hypomethylation were found to be statistically significantly associated with increased risk of OS (HR 0.87, 95% CI 0.78–0.98), DFS (0.86, 0.77–0.96), CSS (0.58, 0.40–0.83), and TTR (0.55, 0.39–0.79). This confirms the findings from four previous studies,[37–40] while five other studies reported that hypermethylation of *MLH1* promoter was associated with poor CRC prognosis.[41–45]

In stratified analyses by clinical characteristics (eTable 12), associations of the successfully validated gene biomarkers were consistent across most subgroups by sex, tumor location, and treatment. However, we noted that for gene methylation biomarkers originally analyzed in specific tumor stages, especially in stage IV CRC, their prognostic relevance either diminished or disappeared in other stage groups.

Meta-analysis of all available evidence

We were able to meta-analyze a total of 23 single and six multiple gene methylation biomarkers across 64 studies (eTable 13), of which seven single biomarkers and two multiple gene biomarkers were significantly associated with CRC prognosis (Fig. 4). The strongest associations

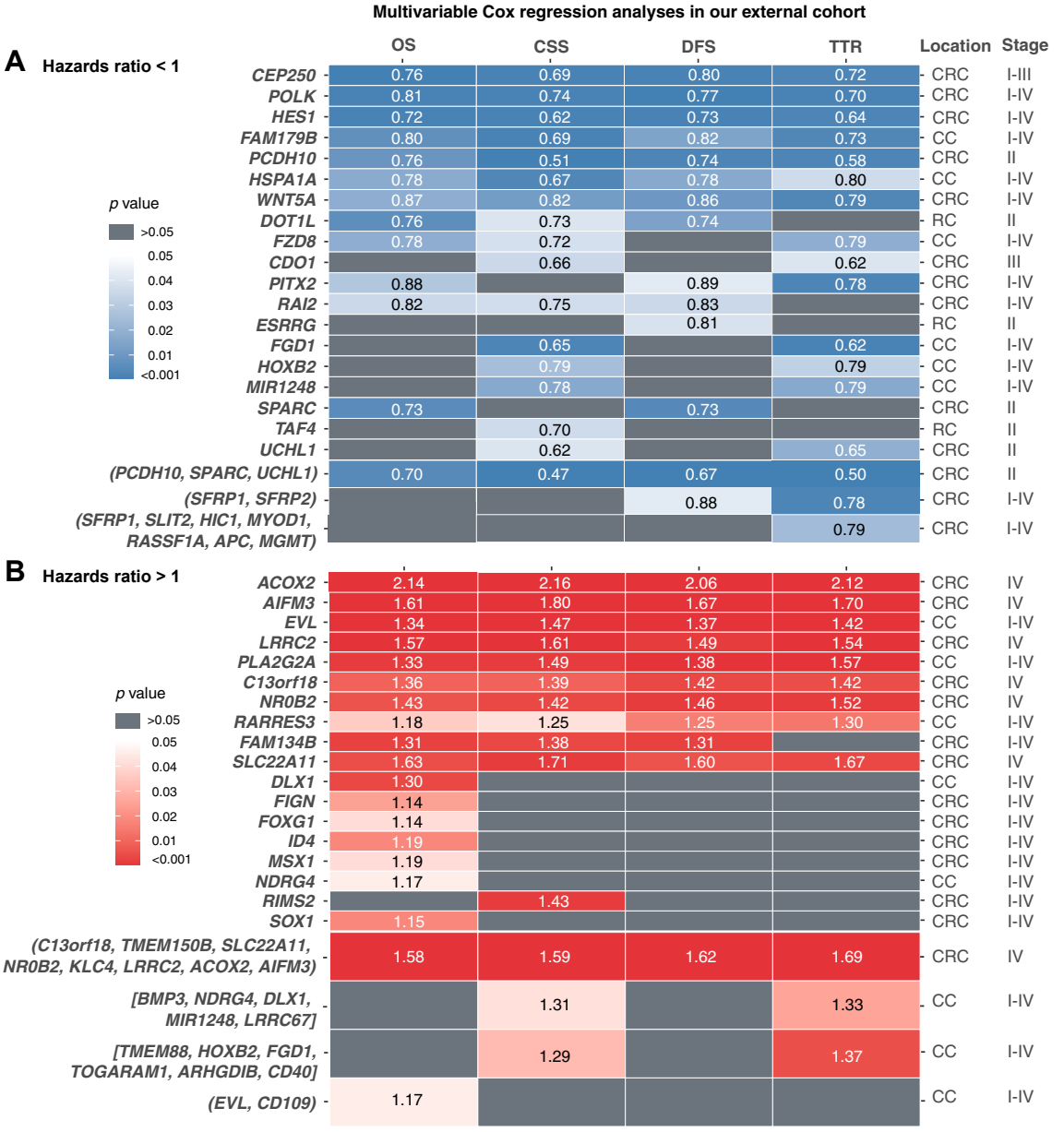

Fig. 1: **Gene methylation biomarkers that could be validated in our DACHS cohort. A) Higher methylation value of gene biomarkers associated with better prognosis; B) Higher methylation level of gene biomarkers or prognostic scores associated with poorer prognosis.** *p* values derived from the multivariable Cox regression analysis measuring the association between gene methylation biomarkers and prognostic outcomes, adjusting for age, sex, TNM stage, and tumor location. Values in each non-gray block represent the point estimate of adjusted hazards ratio. The 'Stage' and 'Location' columns represent the tumor stage and location of patients in both the original derivation cohort in the external validation cohort. Multiple gene biomarkers inserted in the "()" represent co-methylation in a panel, and those inserted in the "[]" represent a prognostic index constructed from the methylation values of each gene. The direction of association for each validated marker was consistent between the original study and the validation study. CC, colon cancer; CRC, colorectal cancer; RC, rectal cancer; OS, overall survival; DFS, disease-free survival; CSS, cancer-specific survival; TTR, time to recurrence.

with better CRC prognosis were observed for *CDO1* (HR 0.56, 95% CI 0.39–0.78), followed by *MLH1* (0.71, 0.52–0.97) and *HES1* (0.71, 0.59–0.85). The eight-gene methylation panel (*C13orf18, TMEM150B, SLC22A11, NR0B2, KLC4, LRRC2, ACOX2, AIFM3*), studied in patients with stage IV CRC,[46] showed the strongest association with poorer prognosis (2.60, 1.29–5.23). The extensively investigated *CDKN2A* gene methylation was

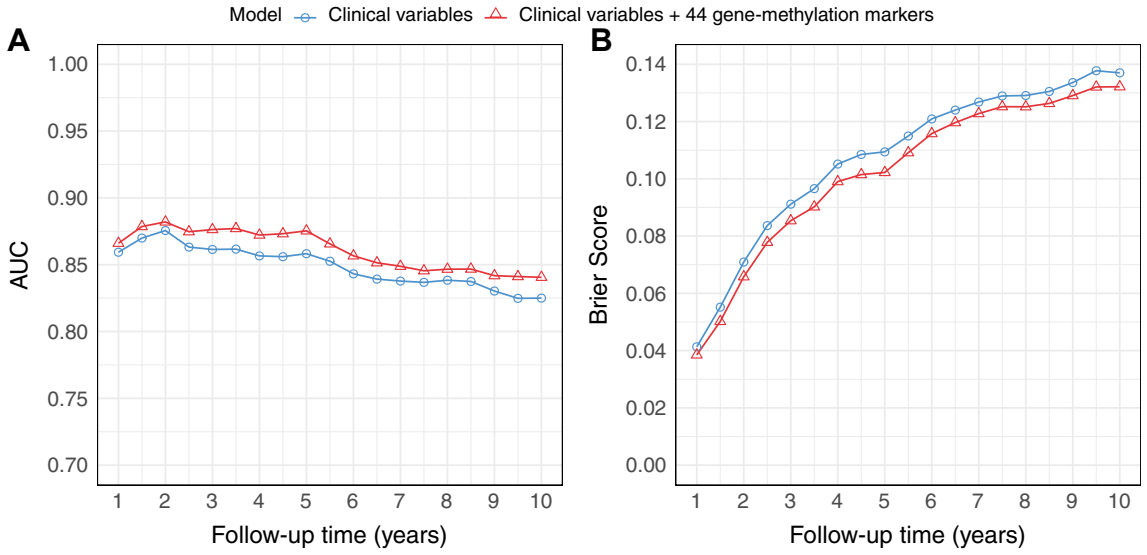

Fig. 2: **Comparison of Cox models: clinical variables only vs. clinical variables and 44 validated gene methylation biomarkers. A) Time-dependent AUCs; B) Time-dependent Brier Scores**. The Cox models were evaluated for the cancer-specific survival in stage I-IV colorectal cancer patients. Clinical variables, including age, sex, TNM stage, and tumor location, were considered in both models. Time-dependent AUCs measure the discriminative power of model at different follow-up time points. An AUC of 0.5 indicates no discriminative power, while an AUC of 1 represents perfect discrimination. The Brier score measures predictive accuracy, with a lower score indicating better predictive accuracy. AUC, area under the receiver operating characteristic curve.

associated with poorer DFS (1.98, 1.19–3.28) and OS (1.38, 1.11–1.71). Four biomarkers (*CEP250, WNT5A, MLH1*, and *CDKN2A*) retained their significant associations with CRC prognosis when the meta-analysis was restricted to studies adjusted for any potential co-founders (eTable 14).

In the subgroup meta-analyses by tumor stage (eTable 15), among patients with non-metastatic CRC, *MLH1* hypomethylation (CSS, 0.57, 0.39–0.84) and *CDKN2A* hypermethylation (DFS, 2.08, 1.34–3.22) were also associated with prognosis, respectively. Notably, *GFRA1* methylation was significantly associated with

Fig. 3: **Gene methylation biomarkers that show significant association with prognosis in our DACHS cohort but the direction of association was inconsistent in existing literature**. *p* values derived from the multivariable Cox regression analysis measuring the association between gene methylation biomarkers and prognostic outcomes, adjusting for age, sex, TNM stage, and tumor location. Values in each non-gray block represent the point estimate of adjusted hazards ratio. OS, overall survival; DFS, disease-free survival; CSS, cancer-specific survival; TTR, time to recurrence.

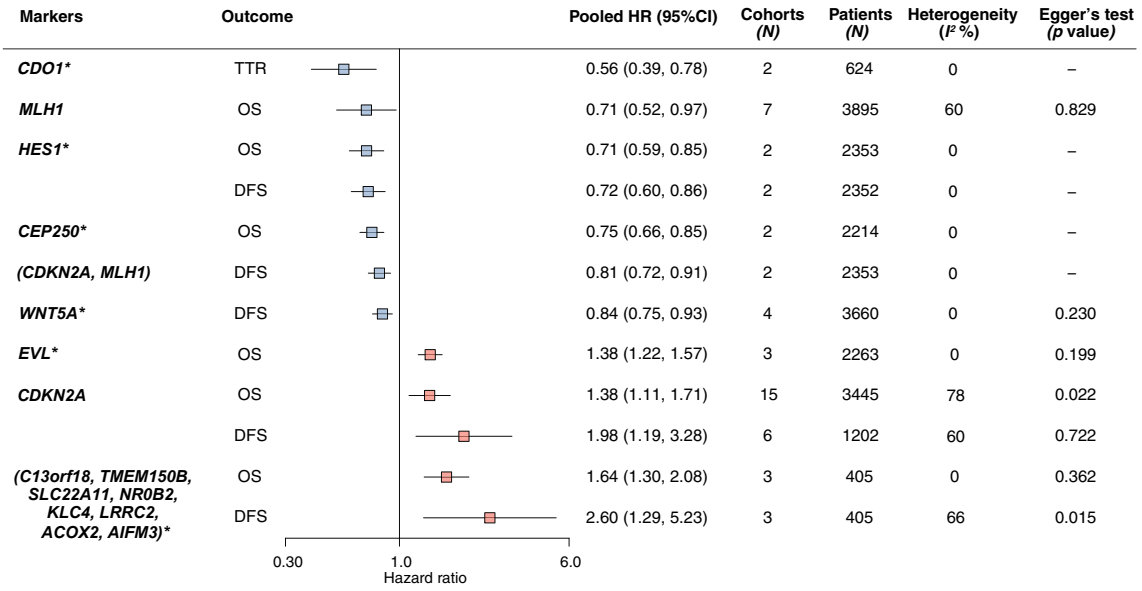

Fig. 4: **Gene methylation biomarkers that showed significant associations with prognosis in meta-analyses (high vs. low methylation).** * Gene methylation biomarkers also successfully validated in our DACHS cohort. CC, colon cancer; CRC, colorectal cancer; RC, rectal cancer; OS, overall survival; DFS, disease-free survival; CSS, cancer-specific survival; TTR, time to recurrence.

shorter OS (1.64, 1.10–2.42) in stage IV patients, a finding not observed in the main analyses covering stage I-IV patients. But this finding was based on two studies only and should be interpretated with caution.

We observed moderate to high heterogeneity in four of the 12 (33%) meta-analyses showing statistical significance (I^2 range 60–78%). Subgroup analyses by study-level characteristics were only possible for the *CDKN2A* methylation and *MLH1* methylation (eTable 16). For both markers, heterogeneity was somewhat lower in studies undertaken in the Western Pacific region (I^2 range 0–54%) compared to European studies or as observed in other subgroups defined by sample size and sex. Nearly all studies included in the meta-analysis used a PCR-based methods to measure DNA methylation. Notably, the subgroup meta-analysis of studies using PCR-based methods was only possible for *MLH1* methylation, which showed a similar association with CRC prognosis as the overall meta-analysis (0.64, 0.42–0.98, eFig. 3).

Indication of publication bias was found in studies reporting associations between *CDKN2A* methylation and OS (p = 0.022 by Egger's test) and the eight-gene methylation panel[46] for DFS (p = 0.015 by Egger's test).

Evidence scoring system

According to our evidence scoring system, methylation in *CDKN2A* emerged as the most robust indicator for CRC prognosis, followed by *WNT5A*, *MLH1*, and *EVL* (Table 2). The forest plots depicting the meta-analysis results of the associations between these gene methylation biomarkers and colorectal cancer prognosis are shown in eFig. 4, and their Kaplan–Meier curves for OS and DFS in our validation cohort are shown in Fig. 5.

Discussion

In our external validation study involving a large patient cohort, we were able to corroborate the prognostic value of 44 out of 180 previously proposed gene methylation biomarkers for CRC. Through subsequent meta-analysis, we identified nine biomarkers that showed significant associations with CRC prognosis. Notably, *CDKN2A, WNT5A, MLH1, and EVL* showed relatively strong levels of evidence of their prognostic value, underscoring their potential clinical relevance.

CDKN2A promoter methylation was the most extensively investigated marker. While we were unable to evaluate this marker in our external cohort due to data constraints, our meta-analysis unequivocally showed its association with poorer CRC prognosis. Moreover, this increased risk pattern remained consistent across sensitivity analyses and subgroup analyses. These findings were in line with two previous meta-analyses published in 2013[47] and 2014,[48] respectively. Indeed, *CDKN2A* is a well-established tumor suppressor gene,[49] and its promoter methylation silences gene expression, thereby fostering uncontrolled cell proliferation and facilitating tumor development and progression.[47]

MLH1 promoter methylation was the second most reported marker and was found to be associated with good CRC prognosis both in our external validation and meta-analysis. *MLH1* is a crucial gene involved in DNA mismatch repair. Epigenetic silencing of *MLH1* is

Gene methylation markers	Tumor location, stage[a]	Out-come	Evidence score				
			Patients[b]	Cohorts[c]	Consis-tency[d]	Evidence score[e]	Rank
CDKN2A	CRC, I-IV	OS	4	3	0.93	6.5	1
		DFS	2	3	0.83	4.2	
WNT5A	CRC, I-IV	DFS	4	2	1	6.0	2
MLH1	CRC, I-IV	OS	4	3	0.71	5.0	3
EVL	CC, I-IV	OS	3	2	1	5.0	3
HES1	CRC, I-IV	OS	3	1	1	4.0	4
		DFS	3	1	1	4.0	
CEP250	CRC, I-III	OS	3	1	1	4.0	4
(CDKN2A, MLH1)	CRC, I-IV	DFS	3	1	1	4.0	4
(C13orf18, TMEM150B, SLC22A11, NR0B2, KLC4, LRRC2, ACOX2, AIFM3)	CRC, IV	OS DFS	1 1	2 2	1 1	3.0 3.0	5
CDO1	CRC, III	TTR	1	1	1	2.0	6

Multiple gene biomarkers inserted in the "()" represents co-methylation, CC, colon cancer; CRC, colorectal cancer; RC, rectal cancer; OS, overall survival; DFS, disease-free survival; CSS, cancer-specific survival; TTR, time to recurrence. [a]The tumor location and stage that the gene methylation marker was mostly analyzed for. [b]Points for the total number of patients in the meta-analysis: <1000 (1), 1000–2000 (2), 2000–3000 (3), and >3000 (4). [c]Points for the total number of independent cohorts included in the meta-analysis: 2 (1), 3–5 (2), and >5 (3). [d]Consistency degree: the number of estimates/cohorts with the direction of association consistent with the final pooled estimate divided by the total number of estimates in the meta-analysis. [e]Evidence score = (Points $_{\text{number of patients}}$ + Points $_{\text{number of independent cohorts}}$) * Consistency degree.

Table 2: **Rank of evidence for gene methylation biomarkers that showed significant associations with prognosis in meta-analyses.**

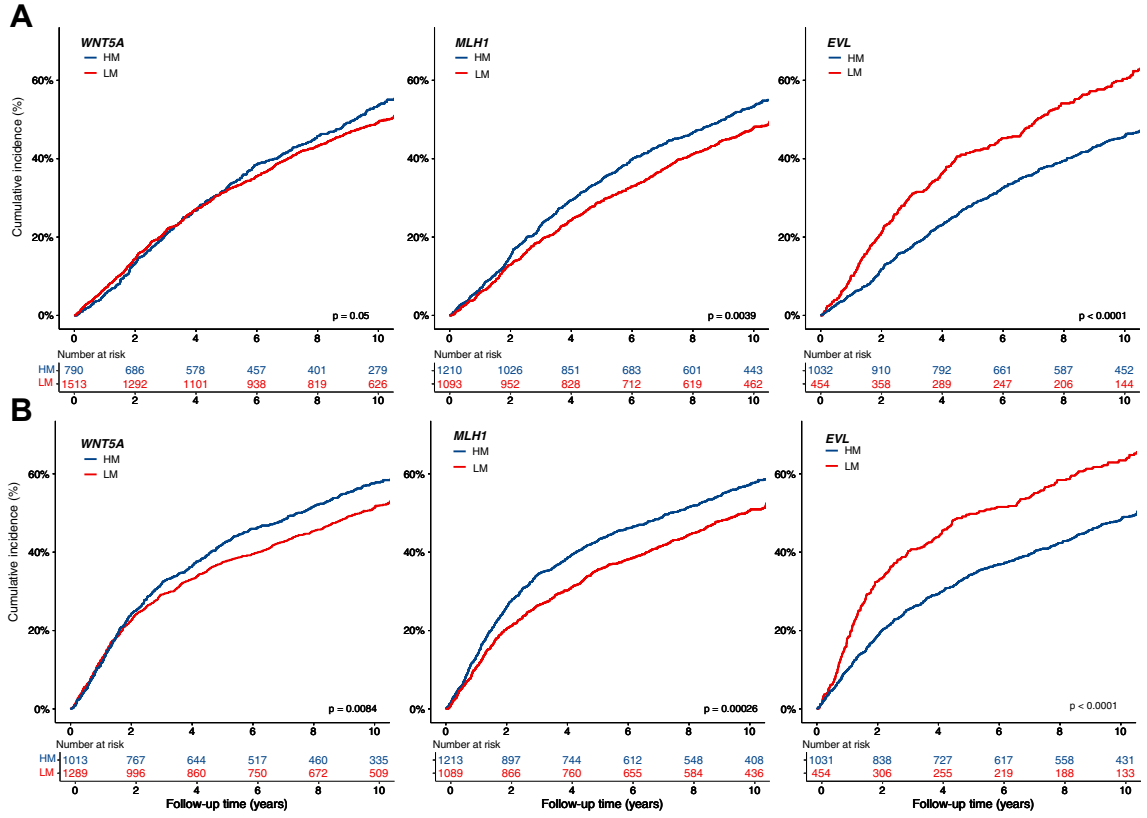

Fig. 5: **Kaplan–Meier survival curves for three gene methylation biomarkers with high evidence level (A) overall survival (B) disease-free survival**. HM, higher methylation level; LM, lower methylation level.

frequently linked to microsatellite instability-high CRC, which tends to exhibit a better prognosis compared to microsatellite-stable tumors.[50] This phenomenon may be attributed to the accelerated accumulation of mutations in tumors with deficient DNA mismatch repair due to epigenetic alterations, resulting in an increased production of aberrant surface proteins (neoantigens).[51] This, in turn, may trigger a heightened immune response against tumor cells, contributing to improved prognosis.[51]

WNT5A promoter hypomethylation was found be associated with poorer CRC prognosis, with relatively strong supporting evidence both in our external validation and in existing literature. *WNT5A* gene belongs to WNT signaling pathway, the activation of which is often associated with tumorigenesis in CRC.[52] *EVL* hypermethylation also had relatively strong level of evidence and possible biological explanation for worse CRC prognosis. Specifically, the *EVL* gene is involved in the extracellular matrix pathway, which was found to be silenced in CRC.[53] Dysfunction of this pathway could enhance tumor metastatic behavior.[53,54] This assumption was supported by our subgroup analyses showing a significant association between *EVL* methylation and poor CRC prognosis in early-stage patients but not in stage IV patients.

Similarly, the eight-gene panel used to assess metastatic CRC has already been externally validated in the original report,[46] the hypermethylation of which also showed a strong association with poor prognosis in our external cohort. When individually assessing each component of the eight-gene panel, six genes (*C13orf18, SLC22A11, NR0B2, LRRC2, ACOX2, AIFM3)* retained their significant associations across all four prognostic outcomes. However, this strong prognostic value was only confined to patients with metastatic CRC and was not observed in our subgroup analyses of stage I-III patients. This underscores the potential need for developing stage-specific DNA methylation biomarkers door, at the very least, considering tumor stage as a crucial factor when assessing the association between gene methylation and CRC prognosis.

Our meta-analyses also found that CRC prognosis was associated with methylation of *CEP250, HES1,* and *CDO1*, respectively. All associations with these biomarkers were confirmed in our external cohort, with their biological implications extensively discussed in the original studies.[52,55–57] *CEP250* is an inflammation-related gene[56] and its prognostic value was found to be restricted to stage I-III patients in our subgroup analyses. *HES1*, encoding a transcriptional repressor in the Notch signaling pathway, has been shown to enhance the invasive potential of CRC cells via the STAT3-MMP14 pathway in laboratory studies.[55,58] Finally, the association between the tumor suppressor gene *CDO1* methylation with better CRC prognosis was confined to stage III patients receiving postoperative adjuvant chemotherapy in the original study.[57] One possible explanation is that the expression of *CDO1* gene in tumor cells may confer clinical resistance to chemotherapy.[57]

Compared with the previous systematic review on this topic[7] or meta-analyses that focused on only single gene methylation markers,[47,48] results from our study extend and summarize current knowledge about the prognostic value of gene methylation biomarkers in CRC. First, we systematically compiled all previously suggested tumor gene methylation biomarkers for CRC prognosis and then externally assessed them all in a large well characterized patient cohort with long-term follow-up. Furthermore, we conducted meta-analyses to synthesize the available evidence for each marker and panel, incorporating the newly obtained evidence from our external validation. Lastly, we developed an evidence scoring system to delineate the strength of evidence for prognostic gene methylation biomarkers in the current literature.

Nevertheless, this study has some limitations. First, due to technical limitations of our epigenome-wide methylation array, we had to exclude nine genes with less than 20% methylation information available from the external validation analysis, including the most investigated *CDKN2A* gene, to ensure the quality of the results. Second, differences between our validation cohort and the original studies in terms of patient demographics and technical methods for DNA methylation measurement may impact the validity of our validation results. In particular, while the majority of the included studies used PCR-based low throughput techniques for measuring DNA methylation levels, our validation cohort used epigenome-wide sequencing (Illumina 450k). Epigenome-wide sequencing techniques, such as Illumina 450k, are frequently used to identify potentially relevant methylation markers, followed by PCR-based methods for cross-validation of targeted markers.[59–61] Heterogeneity of measurement methods might thus be a limitation in our analyses. On the other hand, if candidate genes were successfully validated in our study and meta-analysis despite differences in patient cohorts and measurement techniques, it further strengthens the evidence regarding their prognostic value and robustness. Third, our validation cohort was based on patients with CRC from Germany only, with potentially limited generalizability. Fourth, only a few studies were included in some meta-analyses and subgroup categories. Findings from these meta-analyses should be considered preliminary and warrant further investigation when more data become available. Fifth, despite the high level of consistency of studies, we found moderate to high between-study heterogeneity and evidence of publication bias for some of the gene methylation markers that showed statistically significant associations in meta-analyses. Lastly, we did not evaluate the risk of bias for each included study.

However, if the risk of bias in the original studies would significantly influence the outcomes, it is unlikely that the reported prognostic biomarkers could be validated and remain their prognostic value in meta-analysis.

More well-designed clinical cohort studies in different clinical settings are warranted to further confirm the prognostic value of the promising gene methylation biomarkers reported in this study. These validation studies should ideally involve large sample sizes, sufficiently long follow-up periods, consider major characteristics of the patient population (e.g., tumor location and stage), and use techniques capable of capturing all possible CpG sites within the genes of interest. In addition to biomarkers showing strong associations with CRC prognosis in either the external validation or the meta-analyses, further research should also evaluate the nine biomarkers that we were unable to assess in our external validation cohort. Lastly, the development of new gene methylation panels and their investigation in guiding treatment regimen selection could yield valuable insights for both future research endeavors and clinical practice. Future studies in this domain could build upon our findings by prioritizing methylation biomarkers with strong level of evidence from previous literature, rather than relying solely on data-driven feature selection methods.[62]

In conclusion, we provide new empirical evidence regarding the prognostic potential of several previously suggested gene methylation biomarkers and panels for CRC prognosis. However, further rigorous validation efforts are essential to establish their definitive clinical value beyond the traditional staging system. Future studies aiming at developing panels of gene methylation markers or evaluating their specific clinical value can build upon our findings by prioritizing biomarkers that have shown promise in this study.

Contributors

TY and MH were involved in the study concept and design. MH supervised this work. MH, HB, and TY had access to and verified all the underlying data. TY conducted study selection, data extraction, did all the analyses, designed the figures and wrote the first draft of the manuscript. DW contributed to data extraction. DE and JNK provided consultation regarding methodology. KET, WR, BHM, AB, MK, HB, MH, and BB were involved in the acquisition of data. All authors were involved in the revision of the manuscript for important intellectual content and approval of final version.

Data sharing statement

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Use of generative AI

The AI-assisted technology (GhatGPT-3.5) was used by the first author to improve the readability and language of the first draft.

Declaration of interests

JNK declares consulting services for Owkin, France; DoMore Diagnostics, Norway, Panakeia, UK, Scailyte, Switzerland, and Histofy, UK; furthermore he holds shares in StratifAI GmbH, Germany, and has received honoraria for lectures by AstraZeneca, Bayer, Eisai, MSD, BMS, Roche, Pfizer and Fresenius.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.ebiom.2024.105223.

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