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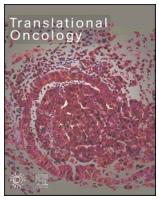

Identification and external validation of tumor DNA methylation panel for the recurrence risk stratification of stage II colon cancer

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ABSTRACT

Background: Tailoring surveillance and treatment strategies for stage II colon cancer (CC) after curative surgery remains challenging, and personalized approaches are lacking. We aimed to identify a gene methylation panel capable of stratifying high-risk stage II CC patients for recurrence beyond traditional clinical variables.
Methods: Genome-wide tumor tissue DNA methylation data were analyzed from 562 stage II CC patients who underwent surgery in Germany (DACHS study). The cohort was divided into a training set ($N = 395$) and an internal validation set ($N = 131$), with external validation performed on 97 stage II CC patients from Spain. DNA methylation markers were primarily selected using the Elastic Net Cox model. The resulting prognostic index (PI), a combination of clinical factors and selected methylation markers, was compared to baseline models using clinical variables or microsatellite instability (MSI), with discrimination and prediction accuracy assessed through time-dependent receiver operating characteristic curves (AUC) and Brier scores.
Results: The final PI incorporated age, sex, tumor stage, location, and 27 DNA methylation markers. The PI consistently outperformed the baseline model including age, sex, and tumor stage in time-dependent AUC across validation cohorts (e.g., 1-year AUC and 95 % confidence interval: internal validation set, PI: 0.66, baseline model: 0.52; external validation set, PI: 0.72, baseline model: 0.64). In internal validation, the PI also showed a consistently improved time-dependent AUC compared with a combination of MSI and tumor stage only. Nevertheless, the PI did not improve the prediction accuracy of CC recurrence compared to the baseline model.
Conclusions: This study identified 27 tumor tissue DNA methylation biomarkers that improved the discriminative power in classifying recurrence risk among stage II colon cancer patients. While this methylation panel alone lacks sufficient prediction accuracy for clinical application, its discriminative improvement suggests potential value as part of a multimodal risk-stratification tool.

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List of abbreviations

CC	colon cancer
PI	prognostic index
MSI	microsatellite instability
AUC	receiver operating characteristic curves
TNM	The tumor-node-metastasis staging
T stage	tumor stage
ctDNA	circulating tumor DNA
LASSO	Least Absolute Shrinkage and Selection Operator
REMARK	Reporting recommendations for tumor marker prognostic studies
PI	prognostic index

Background

Colon cancer (CC) is one of the leading causes of morbidity and mortality worldwide [1]. The tumor-node-metastasis (TNM) staging system remains the primary determinant for prognosis prediction and treatment decisions [2]. Surgical resection is the main treatment for TNM stages I-III CC, with postoperative adjuvant chemotherapy recommended for stage III cases [3–6]. However, the decision to administer chemotherapy after surgery among stage II CC patients remains a clinical challenge [7].

Management of stage II colon patients after curative surgery currently relies on assessing microsatellite instability (MSI) and clinicopathological characteristics such as tumor (T) stage, lymph node count, differentiation grade, intestinal perforation or obstruction, and lymphovascular invasion [7]. Additionally, circulating tumor DNA (ctDNA) has recently emerged as a promising prognostic marker, showing potential in detecting minimal residual disease and predicting recurrence after surgery [8,9].

However, despite these advancements, considerable variability persists in recurrence risk among stage II patients with similar clinicopathological and molecular profiles [10,11]. Consequently, the identification of additional prognostic markers capable of stratifying stage II CC patients based on recurrence risk is imperative for guiding post-surgical treatment and surveillance strategies.

DNA methylation is a process where a small chemical group, called a methyl group, is added to a specific spot on the DNA building block cytosine [12] It is one of the most common epigenetic modifications that regulate gene expression and plays a crucial role in carcinogenesis, cancer progression, and clinical prognosis [12] Emerging evidence highlights the pivotal role of epigenetic alterations, specifically DNA methylation changes in targeted gene regions, in the carcinogenesis and progression of CC [13]. Previous studies using a candidate gene approach have proposed several gene methylation markers in tumor tissue associated with recurrence in stage II CC patients [10,14–16]. However, with the advent of epigenome-wide profiling techniques, researchers can now investigate a wider range of potential methylation markers across the whole genome [17]. This creates methodological challenges, as the enormous number of candidate markers in comparison with relatively small sample size, along with potential multicollinearity among these markers, makes it difficult to pinpoint the most relevant methylation markers to for predicting outcomes [18]. The complexity is further compounded when the endpoint of interest is recurrence, since there are competing events (i.e., death from other causes unrelated to CC) [19]. Ignoring these competing events during analysis can lead to an overestimation of recurrence rates [20,21].

Methodological studies advocate the Elastic Net model, a machine learning method combining the strengths of LASSO (Least Absolute Shrinkage and Selection Operator) and ridge regression techniques, as particularly effective in handling multicollinearity and selecting meaningful variables from high dimensional time-to-event data with competing risks [22,23]. Building on this approach, the present epigenome-wide study aimed to use the Elastic Net model to identify a novel gene methylation panel that, in combination with traditional clinical variables, can improve the identification of stage II CC patients at a high risk of recurrence.

Methods

Study design and participants

We conducted and reported this study according to the Reporting recommendations for tumor marker prognostic studies (REMARK) [24] and Guidelines and quality criteria for artificial intelligence-based prediction model in health care [25]. The derivation cohort was obtained from the DACHS study, a large population-based case-control and patient cohort study on colorectal cancer [26–29]. Ethical approval for the DACHS study was obtained from the ethics committees of the Medical Faculty of the University of Heidelberg and of the Medical Chambers of Baden-Württemberg and Rhineland-Palatinate. Participants were recruited from 22 hospitals in the Rhine-Neckar region in southwest Germany between 2003 and 2021. Baseline patient information was systematically collected via standardized questionnaires administered by trained interviewers, and tumor characteristics were obtained from medical records and pathology reports. All patients underwent surgical resection for primary colorectal cancer. Detailed data on therapy, comorbidities, and recurrence were obtained from physicians during scheduled follow-up visits at 3, 5, and 10 years after diagnosis. Vital status, date of death, and cause of death were collected from the local population registries and health authorities.

In this study, we included all the available stage II CC patients from the DACHS cohort with available genome-wide tumor tissue methylation information who were diagnosed between 2003 and 2013 and followed up until 2020. Patients who received neoadjuvant or adjuvant therapy were excluded from the analysis. A small proportion of patients (around 2 %) [30] with missing baseline clinical information including TNM stage, tumor location, and treatment were excluded from analyses. For external validation, we analysed data of patients who underwent curative surgery at Bellvitge University Hospital in Barcelona, Spain, between 1996 and 2000 (the Colonomics project) [29].

DNA methylation preprocessing

Molecular tumor tissue analyses involved DNA extracted from formalin-fixed, paraffin-embedded tumor samples. Genome-wide methylation profiling was conducted on tissue DNA using the Illumina Human Methylation 450 Bead-Chip (Illumina, San Diego, CA, USA), which examined over 485,000 CpG sites [31]. The preprocessing of raw DNA methylation data files, produced by the iScan array scanner, involved several steps: filtering low-quality probes, imputing missing values, normalizing type-I and type-II probes, and correcting for batch effect. These tasks were accomplished using the default methods and pipeline provided by the 'CHAMP' R package [32] Methylation levels for each CpG site were quantified as β values, ranging from 0 (completely unmethylated) to 1 (fully methylated). Gene-level methylation was derived by averaging β values from all available CpGs located within the promoter regions (TSS1500, TSS200, 5′-UTR) and the first exon of the respective gene. Gene methylation markers were excluded if <20 % of the CpGs were available from our array.

Statistical analyses

We divided eligible patients from the DACHS cohort into training and internal validation sets in a 3:1 ratio using a stratified approach. This method ensured similar rates of cancer recurrent events across both sets, providing a balanced basis for training and testing. We presented descriptive statistics for patient characteristics for the training, internal validation, and external validation sets. To estimate the median follow-up time among patients in each set, the reverse Kaplan-Meier method

was applied. The outcome of interest was time to recurrence, defined as the duration from curative surgery to either cancer recurrence (including CC reappearance, metastases, or death from CC) or censoring. Deaths due to causes unrelated to CC were considered as competing events [19] Using a competing risks approach, we computed the cumulative incidence of recurrence events [19,33], which offers insights into the probability of recurrence over time. The cause-specific Cox hazard model then provided estimates of recurrence-free time for the CC patients [22,33].

In the training set, we developed an initial prognostic model based solely on clinical variables including age, sex, T stage, and tumor location, to predict recurrence risk using the cause-specific Cox hazard model. Next, we aimed to identify additional gene methylation biomarkers that could enhance the predictive power of the baseline model. To handle the large number of potential methylation markers, we used a two-step feature selection process. First, we applied pre-screening method to reduce the number of candidate genes. Four different prescreening approaches and their combinations were explored (Additional File 1: Methods S1 and Table S1). In the selected pipeline, we began by comparing methylation levels in 44 pairs of patients who were matched by age, sex, and T stage, with and without recurrence during follow-up. We then applied Wilcoxon signed-rank test to identify genes with distinct methylation patterns between the two groups.

To further refine the list of potential biomarkers, we used the Elastic Net Cox model, a technique that allows us to select relevant features from a large dataset by combining clinical factors from the baseline model with gene methylation markers identified in the prescreening step. The model's hyperparameters (λ: regularization strength; α: balance between L1/L2 penalties) were fine-tuned using five-fold cross-validation on the training set. To ensure biological relevance and stability, we performed feature selection using a combination of literature-based approach and a robust penalized shrinkage method. First, three well-established gene methylation biomarkers (*MLH1, WNT5A,* and *EVL*), previously identified in a meta-analysis conducted by our group [34], were included in all selection steps due to their known prognostic significance in colorectal cancer. Second, we applied Elastic Net Cox modeling, a penalized regression approach that optimally balances feature selection and regularization, to identify additional markers that were consistently selected across the five cross-validation folds. The model was then refitted using be optimal settings. The finally selected features were combined to produce a single score for each patient, known as the prognostic index (PI), which quantifies each patient's recurrence risk. The PI score is calculated as a weighted sum of selected features, where the weights were determined by the Elastic Net model's coefficients.

We evaluated the performance of PI in both the internal validation cohort and an external validation cohort. We measured associations between the PI and recurrence risk using univariable and multivariable cause-specific Cox models, adjusted for clinical variables. We determined optimal cut-off points for the PI using a method based on maximally selected rank statistics [35], which allowed us to categorize patients into distinct risk groups. To compare the performance of the PI model and the baseline model, we calculated the time-dependent area under the receiver operating characteristic curves (AUC), which measures the model's ability to discriminate patients who experienced recurrence and those who did not. Additionally, within the subset of the internal validation cohort with MSI data ($N = 115$), we assessed the discriminative power of the PI stratified by MSI status. Besides, we compared the time-dependent AUCs of the PI with T stage alone, T stage combined with MSI status, and PI combined with MSI status to evaluate the potential added value of integrating MSI status. We assessed prediction accuracy using time-dependent Brier scores, which reflect both discrimination and calibration [36] In addition, to evaluate the incremental prognostic value of the PI beyond clinical variables, we calculated the integrated discrimination improvement (IDI) and net reclassification index (NRI), with confidence interval (CI) estimated using 200 bootstrap iterations Statistical analyses were performed using R version 4.2.0, and packages including caret, glmnet, glmnetUtils, randomForestSRC, riskRegression, survival, survminer, and ggplot2.

Results

The flowchart of patient selection is shown in Additional File 1: Fig S1. From the DACHS cohort, 526 eligible patients with stage II CC were identified and then split into a training set ($N = 395$) and an internal validation set ($N = 131$). The external set from the Colonomics project comprised 96 eligible patients. The baseline and follow-up characteristics of the three datasets is presented in Table 1. Compared with patients in the DACHS cohort, patients in the external set were diagnosed in earlier calendar years, had a lower percentage of female patients (26.8 % vs 44.9 % in the DACHS cohort), had a higher percentage of patients with cancer on the distal colon (60.8 % vs 41.8 % in the DACHS cohort), and lower median follow-up time (5.7 vs 10 years in the DACHS cohort).

The cumulative recurrence rates across the three datasets were comparable (Additional File 1: Fig. S2). No competing events were registered in the external cohort, probably because of the prerequisite of a minimum three-year follow-up at the time of selection for eligibility [29].

We averaged CpG methylation levels across gene promoter regions, resulting in a total of 18,449 genes. After excluding genes with fewer than 20 % of CpG sites covered, 17,855 gene methylation markers remained. In the training set, we first conducted a prescreening of genes with differential methylation between 44 patient pairs, each matched by age, sex, and TNM stage, with and without cancer recurrence. Additionally, we incorporated three prognostic gene methylation markers based on their strong evidence from previous studies [34]. The initial step reduced the number of gene markers to 672. We then applied an Elastic Net Cox model, including four clinical variables (age, sex, tumor location, and stage) alongside these candidate gene markers, to further refine the selection. Using five-fold cross validation, we determined the

Table 1
Baseline and follow-up characteristics of patients in different cohorts.

Characteristics	DACHS cohort			External cohort ($N = 96$)
	Training set ($N = 395$)	Testing set ($N = 131$)	Overall ($N = 526$)	
Diagnosis year				
Median	2007	2007	2007	NA
Range	2003–2013	2003–2013	2003–2013	1996–2000
Age at diagnosis				
Median (IQR)	71 (64–78)	73 (66–80)	72 (65–78)	72 (66–78)
Sex				
Female	183 (46.3 %)	53 (40.5 %)	236 (44.9 %)	26 (26.8 %)
Male	212 (53.7 %)	78 (59.5 %)	290 (55.1 %)	70 (72.2 %)
TNM stage				
IIA	369 (93.4 %)	122 (93.1 %)	491 (93.3 %)	88 (90.7 %)
IIB/C	26 (6.6 %)	9 (6.9 %)	35 (6.7 %)	8 (8.2 %)
Tumor location				
Proximal colon	230 (58.2 %)	78 (59.5 %)	308 (58.6 %)	37 (38.1 %)
Distal colon	165 (41.8 %)	53 (40.5 %)	218 (41.4 %)	59 (60.8 %)
Outcome events				
Recurrence	70 (17.7 %)	23 (17.6 %)	93 (17.7 %)	22 (22.7 %)
Death from other causes	130 (32.9 %)	42 (32.1 %)	172 (32.7 %)	0 (0.0 %)
Median follow-up (years, IQR)	9.0 (5.2–10.2)	7.8 (5.3–10.2)	10.0 (7.5–10.9)	5.7 (4.8–6.6)

IQR = interquartile range; NA = not available, TNM stage = tumor, node, and metastasis.
[1]Deaths from causes unrelated with colon cancer.

optimal model hyperparameters (alpha = 0.5, lambda = 0.0297), and corresponding cross-validation curves are provided in Additional File 1, Fig S3. Across each cross-validation run, the model consistently selected between 92 and 115 features (median: 108), with 33 features recurrently identified. After finalizing the Elastic Net model with these consistent features and the optimized hyperparameters, 31 features, including four clinical variables and 27 gene methylation biomarkers remained (Table 2). The PI was defined for each patient as a linear predictor based on the final Elastic Net Cox model, with the equation for calculating the PI detailed in Table 2.

In the internal validation set ($N = 131$), a higher PI showed a strong association with recurrence risk. In an unadjusted cause-specific Cox regression model, the hazards ratio was 3.46 (95 % confidence interval [CI]: 1.28 - 9.32), indicating a substantial recurrence risk. This association remained significant after adjusting for age, sex, T stage, and tumor location (3.59,1.31–9.80). A similar association pattern was observed in the external validation cohort ($N = 96$), albeit with limited statistical power to detect significance. In the unadjusted model, a higher PI showed a trend towards increased recurrence risk (1.99, 0.78–5.10), which persisted with a slightly reduced magnitude after adjusting for clinical variables (1.77, 0.63–4.96).

Table 2
Features and coefficients in the final Elastic Net Cox model.

Variable name	Coefficients
Clinical variables	
Age	−0.000876121
Sex	0.158442271
Tumor location	−0.382173283
TNM stage	1.738530808
Gene methylation biomarkers	
WNT5A	−0.5867695
MLH1	−0.527614944
EVL	2.345533703
UCP2	−3.019326117
GP6	2.574318402
SLC12A6	12.45494231
NR2C2AP	−4.699108992
FIS1	2.310761986
ARHGDIG	0.60347267
SP110	10.38254569
DIXDC1	−2.609195752
TMEM170A	−1.09851096
DNAH6	−0.239260267
DEM1	−0.589918785
KIAA1383	−1.292921093
COX16	5.94819615
PGA3	2.939789589
DSC1	−1.091196018
GPR52	−2.735914266
C6orf114	−4.041886777
SCLT1	5.960437261
RAB19	4.166489885
DYNC1LI2	8.120698397
SNORA30	−7.508238508
C1orf216	−5.340546183
LRP2BP	−2.956325546
UGT3A1	−3.466244582

These stable features were consistently selected in all five cross-validation folds. The equation to calculate the Prognostic Index (PI) for each patient is as follow: PI = Age * $\text{Coefficient}_{\text{Age}}$ + Sex score + Tumor location score + TNM stage score + Methylation value of *WNT5A** $\text{Coefficient}_{WNT5A}$ + Methylation value of *MLH1** $\text{Coefficient}_{MLH1}$ + ... Methylation value of *UGT3A1** $\text{Coefficient}_{UGT3A1}$.
in which:.
Tumor Location score: proximal tumor = $\text{Coefficient}_{\text{Tumor location}}$ (−0.382173283); distal colon = 0.
TNM stage score: IIB/*C* = $\text{Coefficient}_{\text{TNM stage}}$ (1.738530808); IIA = 0.

Both the internal and external validation analyses showed that the time-dependent AUC of the PI consistently outperformed that of the baseline clinical model across the follow-up period (Fig. 1a). Although these improvements were consistent, they lacked statistical significance due to limited sample size and statistical power, as 95 %*CIs* were overlapping (Additional file 1: Table S2). For example, within the internal validation cohort, the AUC values and their corresponding 95 % *CIs* for the PI at 1, 3, and 5 years were as follows: 0.66 (0.50–0.81), 0.66 (0.55–0.77), and 0.62 (0.52–0.73), respectively. In contrast, the baseline model showed lower AUC values across the same time points: 1-year: 0.52 (0.24–0.79), 3-year: 0.61 (0.46–0.76), 5-year: 0.55 (0.41–0.68). Similarly, in the external validation set, the 1-year AUC value for the PI (0.72 [0.67–0.77]) surpassed that of the baseline model (0.64 [0.4–0.89]). In the internal validation set, where MSI status information was available, the one-year AUC of the PI was 0.68 (0.62–0.73) in microsatellite stable patients ($N = 85$) and 0.87 (0.78–0.95) in MSI patients ($N = 31$). Besides, our PI consistently showed higher AUC values compared to models based on T stage alone or T stage combined with MSI status (Fig. 2). Additionally, the performance of the PI alone was comparable to that of a combined model incorporating both the PI and MSI status, indicating that our PI may offer valuable prognostic information even without the inclusion of MSI status. Nevertheless, it is important to note that despite these improvements, the discriminative power for all models were at best moderate. Besides, the time-dependent Brier scores indicate that the PI did not enhance the prediction accuracy of recurrence when compared with the baseline model (Fig. 1b), highlighting the poor calibration of the gene methylation model. Similarly, across follow-up time points, there was no statistically significant improvement in IDI or NRI when adding PI to the baseline clinical model (Additional file 1: Table S3).

Discussion

In this study, we identified 27 gene methylation biomarkers that improved discriminative capability compared with traditional clinical variables and MSI status among stage II CC patients. Despite rigorous internal and external validation, the discriminative power remained moderate. Notably, the 27-gene methylation panel did not enhance absolute risk predicative accuracy, limiting its immediate clinical applicability as a standalone tool for recurrence risk stratification.

The existing body of literature has predominantly proposed two types of DNA methylation biomarkers for cancer prognosis. First, the methylation status of individual CpG sites [30], offering detailed information about specific methylation patterns. However, they are susceptible to technical variations that can affect their reliability and reproducibility [37], as demonstrated in a recent systematic review of CpG biomarkers for colorectal cancer prognosis, where none of the 300 previously proposed CpG biomarkers were consistently reported across studies [30]. Second, gene methylation biomarkers, focusing on average methylation levels across functional regions of genes [38], that offer more stability and reproducibility [37]. In this study, we focused on gene methylation at CpG sites located on the promoter regions, which play a crucial role in regulating gene expression and thus provide a more interpretable and straightforward approach for analysis in our study.

We identified 27 gene methylation biomarkers that showed potential in refining the classification of recurrence risk beyond traditional clinical variables. Among these markers, the methylation of *MLH1, WNT5A,* and *EVL* have been previously associated with colorectal cancer prognosis in previous studies [39–46]. Additionally, many other gene markers have been previously implicated in cancer prognosis and treatment response. For instance, the *ARHGDIG* gene has been implicated in tumor migration and epithelial–mesenchymal transition, with high expression correlating with colorectal cancer recurrence [47]. Similarly, the *RAB19* gene, a member of *RAS* oncogene family, has shown significant upregulation in colorectal cancer tumor tissues compared to normal tissues, potentially influencing cell cycle and

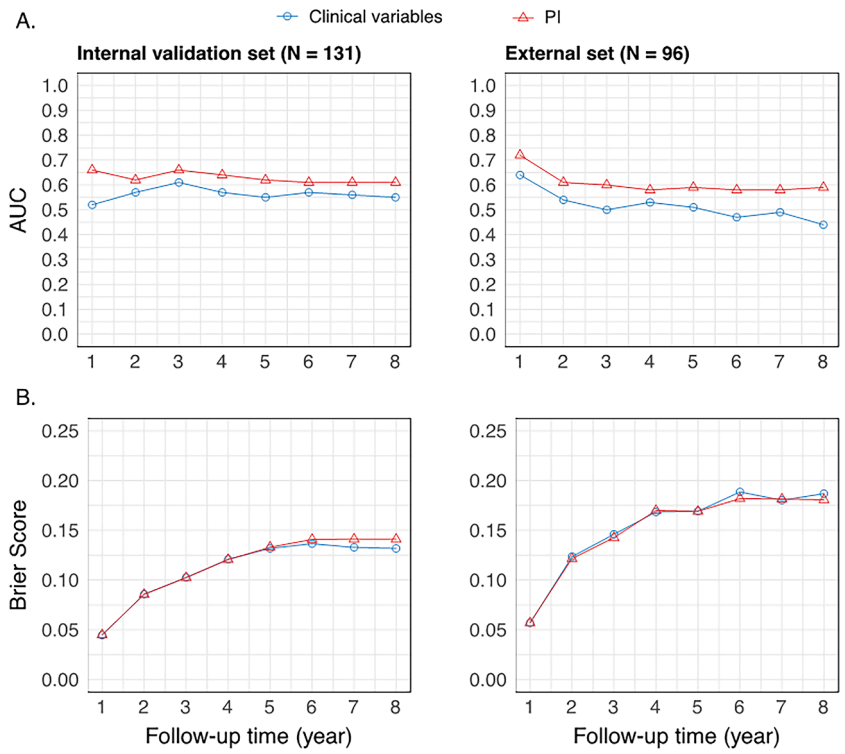

Fig. 1. Internal and external validation of prognostic index in comparison with clinical variables. A) Discriminative power measured by time-dependent AUC; B) Prediction accuracy measured by time-dependent Brier Score.
Higher AUC stands for better discriminative power, while for Brier score, the lower is better. The baseline model was based on age, sex, TNM stage and tumor location, and the prognostic index was calculated based on age, sex, TNM stage, tumor location, and 27 gene methylation biomarkers. The baseline risks for the internal and external sets were recalibrated when calculating the Brier Score.
AUC = area under the receiver operating characteristic curves. PI = prognostic index.

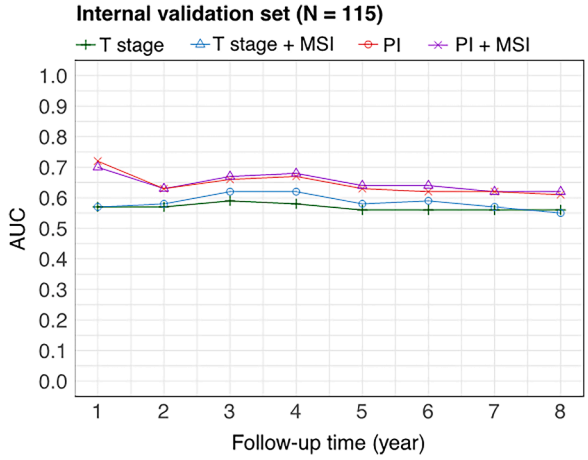

Fig. 2. Comparison of the time-dependent AUC between prognostic index Internal and T stage and MSI status in the internal validation set.
T stage = tumor stage, MSI = microsatellite instability, PI = prognostic index,

immune-related pathways [48]. Studies have indicated that the deficiency of *UCP2* protein, encoded by the *UCP2* gene, may enhance colon tumorigenesis by increasing levels of oxidized glutathione and proteins within tumors [49]. Moreover, over-expression of the *DIXDC1* gene has been linked to increased colon cancer cell proliferation via the PI3K pathway [50]. The *GP6* gene, encoding a platelet membrane glycoprotein, is implicated in colon cancer metastasis through interaction with cancer cell-derived galectin-3 [51]. Notably, *PGA3* gene expression has been associated with immune cell infiltration [52], which often correlates with a more favorable prognosis in colorectal cancer [53]. The *UGT3A1* gene, involved in the UDP-glucuronosyltransferase pathway, is noteworthy due to reports of *UGT* gene mutation affecting cancer progression and drug resistance [54]. Furthermore, aberrant expression of *DSC1 gene, which* mediates cell-cell adhesion, has been observed in colorectal adenocarcinomas [55]. However, some identified genes markers (e.g., *COX16, DYNC1LI2, SLC12A6*) lack extensive documentation regarding their roles in cancer development and progression, warranting further investigation.

Our methodological approach was meticulously designed to prevent data leakage during feature selection and evaluation processes, and various pre-screening strategies were explored. Additionally, to ensure the model's generalizability, we fitted the Cox model to the training cohort and then used the derived equation to calculate the prediction index for validation purposes [56]. However, despite these rigorous methodologies, we consistently observed that the discriminative power for the clinical variables augmented with the addition of the 27 methylation markers remained at a moderate level, with a C index mostly below 0.70, across independent cohorts. The moderate discrimination aligns with the inherent challenges of stage II colon cancer prognostication, where tumor heterogeneity and subtle molecular differences limit predictive performance even for established biomarkers like MSI [57]. Our findings underscore the importance of methodological transparency in biomarker research, particularly to avoid over-optimistic claims and misdirected clinical efforts.

The model's Brier score did not show improvement in absolute prediction error, which was further supported by other metrics including IDI and NRI. However, our model's ability to stratify patients into distinct risk groups remains clinically relevant. Previous studies have proposed DNA methylation markers, suggesting their potential to enhance prognostic accuracy of recurrence risk among early-stage colorectal cancer patients [14–16,58]. However, it is important to

note that these studies primarily relied on Cox models to establish the association between their methylation markers and recurrence [14–16], or they reported AUC values solely within the discovery cohort, leading to data leakage and potentially overestimated performance [58]. Importantly, none of these aforementioned studies used appropriate metrics to measure calibration or prediction accuracy for their methylation markers [14–16,58]. This underscores the necessity for a more rigorous adherence to methodological and reporting guidelines in studies involving DNA methylation markers for cancer prognosis. Our rigorous approach—external validation, avoidance of overfitting, and evaluation of both discrimination and prediction accuracy—provides a more realistic assessment of biomarker utility. Future studies with larger cohorts are needed to further refine model performance.

Several plausible reasons might contribute to the limited role of DNA methylation biomarkers in enhancing prognostication among patients with stage II colon cancer. First, CC is a multifaceted disease characterized by a spectrum of genetic and epigenetic alterations that contribute to its progression. While DNA methylation patterns offer valuable insights, they represent just one facet of epigenetic changes within the broader context, which also includes histone modifications and various non-coding RNA species [13]. Moreover, beyond epigenetic alterations, other molecular factors such as gene mutations, protein expression variations, and influences from the tumor microenvironment, collectively contribute to the recurrence dynamics in CC. These molecular components interact in intricate ways, forming a complex network that influence CC progression [13]. Consequently, DNA methylation markers, when considered in isolation, may not sufficiently encapsulate the nuanced intricacies and inherent heterogeneity of the disease, thereby limiting their prognostic power. Lastly, an inherent challenge in analyzing high-dimensional omics data, like DNA methylation profiles, is the reliance on relatively small sample sizes during the development of these biomarkers. The limited sample sizes can pose inherent constraints in accurately capturing the diverse and comprehensive molecular landscape.

This study has some limitations. First, we were unable to fully integrate certain key clinical prognostic markers, particularly MSI status, into our model due to incomplete data availability. However, our PI incorporated *MLH1* methylation, which plays a crucial role in the sporadic MSI pathway. Additionally, our internal validation demonstrated that the PI consistently outperformed a combination of MSI and T stage in terms of discrimination during patient follow-up. The ability to use our methylation panel independent of MSI testing could enhance its generalizability.

Second, while our PI demonstrated good discriminative power when stratified by MSI status, we were unable to further evaluate its performance in other high-risk groups (e.g., T4 patients) or directly compare its efficacy with other molecular biomarkers, such as ctDNA and mutation profiles (e.g., *KRAS/BRAF*). Third, although our study focused on the prognostic role of methylation markers, their potential utility in predicting response to adjuvant chemotherapy remains unexplored. Future research should assess whether integrating methylation-based risk stratification with treatment data could inform personalized chemotherapy decisions in stage II colon cancer. Fourth, our focus on recurrence led us to categorize deaths unrelated to colon cancer as competing events. Despite rigorous methods employed to ensure data quality, the possibility of misclassifying causes of death cannot be entirely eliminated. Fifth, our exclusive focus on methylation levels at gene promoter regions may overlook potential impacts from methylation in other genomic regions, which could also influence cancer recurrence [58].

Sixth, although multiple measures (e.g., prescreening, stability selection, and external validation) were implemented to mitigate overfitting, the limited training cohort size may still introduce residual overfitting. Larger cohorts are needed to confirm the stability of our methylation panel. Seventh, while the inclusion of an external validation cohort is a strength, its small sample size ($N = 97$) limits statistical power, as evidenced by the relatively wide confidence intervals, and generalizability. Lastly, Demographic and clinical differences between training and external validation cohorts (e.g., recruitment period) may also introduce bias. However, these differences also allowed us to evaluate our model's robustness across heterogeneous populations. Regression analyses adjusted for key variables (i.e., age, sex, T stage, and tumor location) and stratified analysis by MSI status confirmed consistent discriminative power, supporting generalizability. Nonetheless, further validation in a larger prospective patient cohort specifically targeting stage II CC patients would be desirable.

The lack of prediction accuracy improvement highlights that methylation markers, as a standalone tool, cannot yet guide clinical decisions. Nevertheless, our 27 DNA methylation markers could serve as valuable components in multi-omics approaches for risk prediction in stage II CC. The integration of these markers with mutation profile (e.g., *KRAS/BRAF*), additional omics layers (e.g., genomics, transcriptomics, and proteomics) and other modalities (e.g., ctDNA, immune profiling, histological imaging) may enhance prediction accuracy and clinical utility. Future studies should integrate multi-omics approaches to explore how methylation markers might synergize with ctDNA and other modalities to refine clinical risk stratification.

In conclusion, our rigorously developed and externally validated tumor methylation panel improves discriminative power for recurrence risk stratification in stage II CC compared to traditional clinical variables. However, the absence of enhanced prediction accuracy highlights that methylation biomarkers alone are insufficient for clinical decision-making. Rather than dismissing its utility, our marker panel may serve as a component of future multimodal models.

Contributors

TY, MH, DE, EG, LS and HP were involved in the study concept and pipeline design. MH supervised this work. MH, HB, and TY had access to all the data. DE, EG, LS, HP, XJ and JK provided consultation regarding methodology. TY analyzed the data, designed the figures and wrote the first draft of the manuscript. KET, WR, BHM, AB, MK, HB, and MH were involved in the acquisition of data. All authors were involved in the revision of the manuscript for important intellectual content and approval of the final version.

CRediT authorship contribution statement

Tanwei Yuan: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Methodology, Investigation, Formal analysis, Conceptualization. **Dominic Edelmann:** Writing – review & editing, Supervision, Methodology, Conceptualization. **Víctor Moreno:** Writing – review & editing, Validation, Resources. **Elisabeth Georgii:** Writing – review & editing, Validation, Methodology. **Lisa Barros de Andrade e Sousa:** Writing – review & editing, Methodology. **Helena Pelin:** Writing – review & editing, Methodology. **Xiaofeng Jiang:** Writing – review & editing, Methodology. **Jakob Nikolas Kather:** Writing – review & editing, Methodology. **Katrin E. Tagscherer:** Writing – review & editing, Data curation. **Wilfried Roth:** Writing – review & editing, Resources. **Melanie Bewerunge-Hudler:** Writing – review & editing, Resources. **Alexander Brobeil:** Writing – review & editing, Resources. **Matthias Kloor:** Writing – review & editing, Resources. **Hendrik Bläker:** Writing – review & editing, Resources. **Hermann Brenner:** Writing – review & editing, Supervision, Resources, Funding acquisition, Conceptualization. **Michael Hoffmeister:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing 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. No other conflicts of interest are declared by any of the authors.

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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.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.tranon.2025.102405.

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