CHAPTER 5

Validation of the 12-gene Colon Cancer Recurrence Score as a predictor of recurrence risk in stage II and III rectal cancer patients

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ABSTRACT

Background

The 12-gene Recurrence Score assay is a validated predictor of recurrence risk in stage II and III colon cancer patients. We conducted a prospectively designed study to validate this assay for prediction of recurrence risk in stage II and III rectal cancer patients from the Dutch Total Mesorectal Excision (TME) trial.

Methods

RNA was extracted from fixed paraffin-embedded primary rectal tumor tissue from stage II and III patients randomized to TME surgery alone, without (neo)adjuvant treatment. Recurrence Score was assessed by quantitative RT-PCR using previously validated colon cancer genes and algorithm. Data were analysed by Cox proportional hazards regression adjusting for stage and resection margin status.

Results

Recurrence Score predicted risk of recurrence (p=0.011), risk of distant recurrence (p=0.030), and rectal cancer-specific survival (p=0.007). The effect of Recurrence Score was most prominent in stage II patients and attenuated with more advanced stage (interaction $p \leq 0.007$ for each endpoint). In stage II, 5-year cumulative incidence of recurrence ranged from 11% in the pre-defined low Recurrence Score group (48% of pts) to 43% in the high Recurrence Score group (23% of pts).

Conclusions

The 12-gene Recurrence Score is a predictor of recurrence risk and cancer specific survival in rectal cancer patients treated with surgery alone, suggesting a similar underlying biology in colon and rectal cancers.

INTRODUCTION

Before the introduction of the total mesorectal excision (TME) technique, which resulted in a substantial decrease in local recurrences and improved survival, the 5-year local recurrence rate of rectal cancer with conventional surgery was over 20% [1]. Between 1996-1999, the Dutch TME trial investigated the effect of short-term preoperative radiotherapy in combination with TME surgery compared to TME surgery alone in 1861 rectal cancer patients [2]. Five and ten year results of this trial showed improved local recurrence rates in patients treated with preoperative radiotherapy and TME [3-5]. However, no significant effect was seen on distant recurrence and overall survival (OS) [5].

While TME surgery and preoperative therapy have reduced local recurrence, the role of adjuvant chemotherapy in rectal cancer in reducing distant recurrence rates and improving OS remains controversial. In a systematic review and meta-analysis of 21 randomized clinical trials, the use of 5-fluorouracil (5-FU) based adjuvant chemotherapy for rectal cancer patients who received no preoperative therapy was found to improve both OS and disease-free survival (DFS) [6]. However, for rectal cancer patients receiving preoperative chemo- or radiotherapy, most trials did not show a survival benefit for adjuvant chemotherapy [7-10]. Current clinical and pathologic features in rectal cancer are not able to adequately characterize recurrence risk. As such, aggressive approaches combining preoperative chemoradiation, TME surgery, and in some countries, postoperative adjuvant chemotherapy continue to be used in stage III and many stage II rectal cancers, with attendant clinical toxicity, patient burden, and financial cost. There is thus a strong need for new clinical tools which more accurately identify patients with low and high-risk of recurrence; especially for stage II patients, a more individualized approach to balancing risk of recurrence, modest treatment benefit, and therapy-related toxicities should improve treatment decision-making.

The 12-gene Recurrence Score assay (Genomic Health, Redwood City, CA, USA) was developed by using tumor gene expression data from 1851 patients with resected colon cancer from four independent clinical trials [11]. This 12-gene assay, measuring expression of 12 genes (seven recurrence and five reference genes) in fixed, paraffin-embedded (FPE) primary colon tumor tissue, was validated as a predictor of recurrence risk in stage II and III colon cancer patients from QUASAR, Cancer and Leukemia Group B (CALGB) 9581, and National Surgical Adjuvant Breast and Bowel Project (NSABP) C-07 trials [12-14], providing risk discrimination beyond conventional clinical and pathologic factors.

The purpose of this prospectively-designed study was to validate the 12- gene Recurrence Score assay in stage II and III rectal cancer for recurrence risk prediction in patients from the TME alone arm of the Dutch TME trial who received no pre- and postoperative therapy.

METHODS

Patients and Tissue Specimens

Stage II and III rectal cancer patients enrolled in the Dutch TME trial, randomized to surgery alone, underwent radical resection (i.e. R0-R1), were treated per TME trial protocol and had FPE tumor tissue were eligible for the study [3]. Informed consent was obtained from all patients enrolled in the TME trial. The study was approved by the Medical Ethical Committee of the Leiden University Medical Center. Per TME protocol, patients with tumor spillage during operation or tumor-positive resection margin were allowed to receive radiotherapy. Follow-up assessments involved clinical evaluation every three months during the first year after surgery and yearly for at least two more years, including liver imaging and endoscopy. Additionally, chest X-ray/CT, CEA determination and endo-ultrasound were performed on indication.

Pathology and Gene expression

Pathologic T-stage, number of nodes examined and involved by carcinoma, resection margin status, distance from anal verge, and local grade assessments were obtained from the TME clinical database. Positive resection margin (RM) was defined as positive circumferential, distal, proximal, or nodal margin, or presence of the tumor ≤ 1mm from any of these margins. In addition, tumor type and grade were centrally assessed [15] according to WHO guidelines [16] by an academic surgical pathologist specialized in gastrointestinal pathology.

RNA was extracted from six 5-µm sections, quantified by RiboGreen (Invitrogen, Carlsbad, CA) and analysed by reverse transcriptase quantitative polymerase chain reaction using a standardized, analytically validated process [17]. The 12-gene Recurrence Score results were calculated using prespecified genes and algorithm, as previously validated in QUASAR, CALGB 9581, and NSABP C-07 [12-14]. Prespecified cut points were used to define low, intermediate, and high Recurrence Score groups (i.e., RS<30, 30 to 40, and ≥ 41 respectively) [12].

All centrally-performed pathology and laboratory procedures were prespecified and conducted without knowledge of patient clinical characteristics or outcomes.

Statistical Methods

The prespecified primary study endpoint was recurrence-free interval (RFI), defined as time from surgery to first rectal cancer recurrence (local or distant) or death with a documented recurrence at time of death. Local recurrence was defined as tumor within the lesser pelvis or perineal wound and distant recurrence as tumor in any other area including at the colostomy site or in the inguinal region [3]. Deaths without evidence of recurrence and losses to follow-up were censored. Second primary cancers were

ignored. RFI was chosen as primary endpoint, as opposed to time to local recurrence in the parent TME trial, because gene expression was expected to be associated with any recurrence of the primary tumor and most recurrences in rectal cancer are distant.

Secondary endpoints included distant RFI (DRFI), where local recurrences were neither censored nor considered as events, rectal cancer-specific survival (RCSS), where death is either preceded by rectal cancer recurrence or occurs with documented recurrence, DFS, and OS.

The primary analysis model used Cox proportional hazards (PH) regression to evaluate the association between continuous Recurrence Score results and outcome, adjusted for stage (II, IIIA/B, IIIC corresponding to 0, 1-3 and 4+ positive nodes, respectively) and RM status (RM-negative, RM-positive treated with surgery alone, and RM-positive treated with surgery followed by radiotherapy). A two-sided p-value < 0.05, based on a likelihood ratio test, was considered significant. The hazard ratio for Recurrence Score was reported for an increase of 25 units, consistent with previous studies. Proportional hazards were assessed by examining the relationship between scaled Schoenfeld residuals and time. Non-linearity was assessed by a likelihood ratio test for squared and cubic terms for Recurrence Score results. Stage-specific additive splines that were constrained to be linear in the tails [18] were used to model non-linear effects of the continuous Recurrence Score. Contribution of Recurrence Score beyond prespecified pathologic covariates was evaluated using multivariable Cox PH models. The relationship between Recurrence Score groups and RFI, DRFI and RCCS was characterized by cumulative incidence estimates and Aalen's estimates of variance accounting for death without evidence of recurrence and death due to cancers other than rectal cancer as competing risks [19]. Additionally, Kaplan-Meier methods were used. Relative utility curves and a test tradeoff were computed [20;21]. Analyses used IBM SPSS Statistics 20, R version 2.14.0 (cmprsk and mstate packages) and SAS version 9.2.

RESULTS

Patient characteristics

Tumor tissue was available for 308 (59%) of 518 eligible stage II and III patients in the TME trial who were randomized to surgery alone. Following prespecified procedures for pathology and laboratory processing, 11 (3.6%) patients were excluded, primarily for insufficient tumor tissue (Figure 1). The final evaluable data set contained 297 patients with 128 (43%) recurrences, including 50 (17%) local and 112 (38%) distant recurrences (34 patients had both local and distant recurrence). Recurrences were observed in 34 (26%) of 130 stage II patients, 57 (52%) of 110 stage IIIA/B patients and 37 (65%) of 57

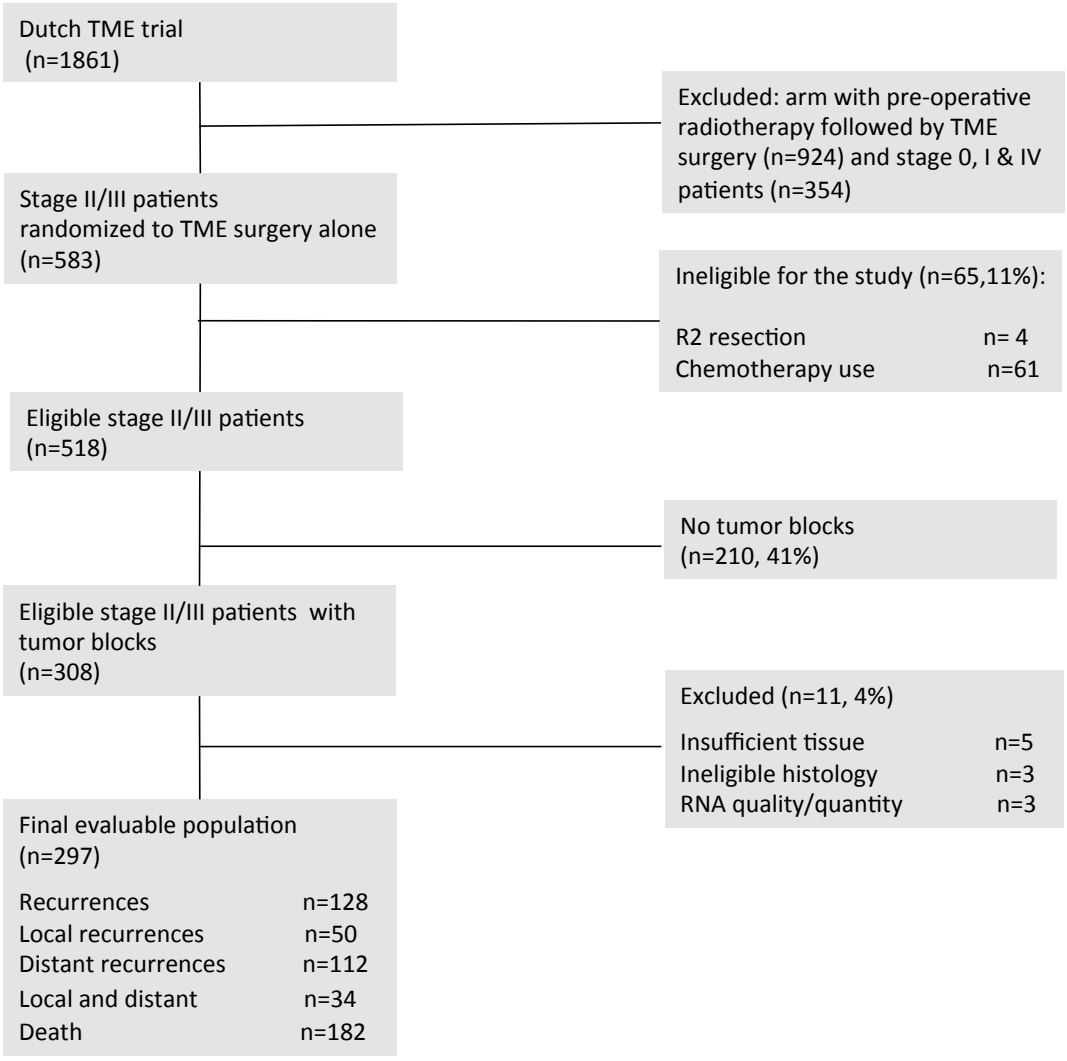


Figure 1: Study flow diagram.
TME, Total Mesorectal Excision

stage IIIC patients. A total of 182 patients died, including 120 (66%) patients who died after recurrence of rectal cancer.

Patient characteristics were representative of a contemporary rectal cancer population, with median age of 66 (range 23-92), the majority being male (63%), and receiving a low anterior resection (LAR) (64%) (Table I). Most patients had T3-T4 tumors (90%) and 30% of the tumors were high grade. The median number of nodes examined was 9 (range 1-52) and 36% of the patients had ≥12 nodes examined (Table I). Importantly, a quarter of patients had positive resection margins, with the proportion of RM-positive patients increasing from 16% in stage II to 53% in stage IIIC (Table I).

The demographic and pathologic characteristics of patients evaluated in this study were similar to those of eligible stage II and III patients in the parent trial without FPE tissue (Supplemental Table I). RFI was comparable as well (logrank *p*-value 0.507).

Table I: Baseline Clinical and Pathologic Characteristics for the total cohort and stratified for stage.

Characteristic	Values	All N(%) 297 pts	Stage II (N%) 130 pts	Stage III A/B (N%) 110 pts	Stage III C (N%) 57 pts
Year of surgery	<1998	157 (52.9)	69 (53.1)	56 (50.9)	32 (56.1)
	≥1998	140 (47.10	61 (46.9)	54 (49.1)	25 (43.9)
Age	<60	102 (34.3)	50 (38.5)	35 (31.8)	17 (29.8)
	60 to <70	89 (30.0)	33 (25.4)	41 (37.3)	15 (26.30
	70+	106 (35.7)	47 (36.2)	34 (30.9)	25 (43.9)
Gender	Female	111 (37.4)	56 (43.1)	34 (30.9)	21 (36.8)
	Male	186 (62.6)	74 (56.9)	76 (69.1)	36 (63.2)
Resection type	LAR	191 (64.3)	80 (61.5)	77 (70.0)	34 (59.6)
	APR	106 (35.7)	50 (38.5)	33 (30.0)	23 (40.4)
Resection margin status	R0	223 (75.1)	109 (83.8)	87 (79.1)	27 (47.4)
	R1 no RT	37 (12.5)	15 (11.5)	9 (8.2)	13 (22.8)
	R1+RT	37 (12.5)	6 (4.6)	14 (12.7)	17 (29.8)
Distance from anal verge*	<5 cm	103 (34.7)	49 (37.7)	36 (32.7)	18 (31.6)
	5-9.9 cm	110 (37.0)	42 (32.3)	49 (44.5)	19 (33.3)
	10+ cm	84 (28.3)	39 (30.0)	25 (22.7)	20 (35.1)
T-Stage	T1	1 (0.3)		1 (0.9)	0 (0.0)
	T2	29 (9.8)		22 (20.0)	7 (12.3)
	T3	248 (83.5)	123 (94.6)	82 (74.5)	43 (75.4)
	T4	19 (6.4)	7 (5.4)	5 (4.5)	7 (12.3)
Number of lymph nodes examined	<12	190 (64.0)	95 (73.1)	74 (67.3)	21 (36.8)
	12+	107 (36.0)	35 (26.9)	36 (32.7)	36 (63.2)
Grade **	High	88 (29.6)	22 (16.9)	38 (34.5)	28 (49.1)
	Low	209 (70.4)	108 (83.1)	72 (65.5)	29 (50.9)
Tumour type	Mucinous	16 (5.4)	4 (3.1)	8 (7.3)	4 (7.0)
	Adenocarcinoma	281 (94.6)	126 (96.9)	102 (92.7)	53 (93.0)
Obstruction or perforation	Present	21 (7.1)	7 (5.4)	6 (5.5)	8 (14.0)
	Absent	276 (92.9)	123 (94.6)	104 (94.5)	49 (86.0)

Abbreviations: RT=Radiotherapy, R0= Radical resection, R1: residual disease after resection

** To inferior margin of tumor*

*** Centrally assessed by a pathologist at Genomic Health*

Association of Recurrence Score Result with Outcomes

Recurrence Score values ranged from 0 to 72 with a median score of 32 (interquartile range, 24 to 42) and a mean ± SD of 33.3 ± 12.7. In the primary analysis, the continuous Recurrence Score result was significantly associated with recurrence risk, when controlling for stage and RM status, with a hazard ratio (HR) of 1.57 for a 25-unit increase in the score (95% CI 1.11-2.21, p=0.011). The proportional hazards assumption held (p=0.52). An interaction between Recurrence Score result and stage was observed (p=0.002), with evidence of nonlinearity in the relationship between the continuous score and the log hazard of recurrence (p<0.001). Adjusting for stage and RM status and accounting for interaction with stage and non-linearity, the Recurrence Score result was associated

with risk of recurrence in stage II (HR defined as ratio of the hazards at the 75th and 25th percentile of RS, 3.27, 95% CI 1.52-7.01, $p<0.001$) and stage IIIA/B (HR, 1.87, 95% CI 1.18-2.95, $p=0.007$) (Figure 2). The Recurrence Score result was not associated with recurrence risk in stage IIIC (HR, 0.75, 95% CI 0.46-1.21, $p=0.243$). The pre-defined high Recurrence Score group had higher recurrence risk than the low group in stage II (HR, 5.81, 95% CI 2.33-14.50, $p<0.001$) but not in stage IIIA/B (HR, 1.62, 95% CI 0.82-3.19, $p=0.169$) or stage IIIC (HR, 0.64, 95% CI 0.29-1.41, $p=0.272$): the effect of the Recurrence Score was most prominent in stage II and attenuated in more advanced stage (Figure 3). In the stage II patients, cumulative incidence estimates of 5-year recurrence risk for the low- (63 patients, 48%), intermediate- (37 patients, 28%), and high (30 patients, 23%) Recurrence Score groups were 11% (95% CI 6-22%), 27% (95% CI 16-46%) and 43% (95% CI 29-65%), respectively (Table II). Recurrence risk estimates by Kaplan-Meier methods were similar for low group and higher for the high score group (Supplemental Table II and Figure 1).

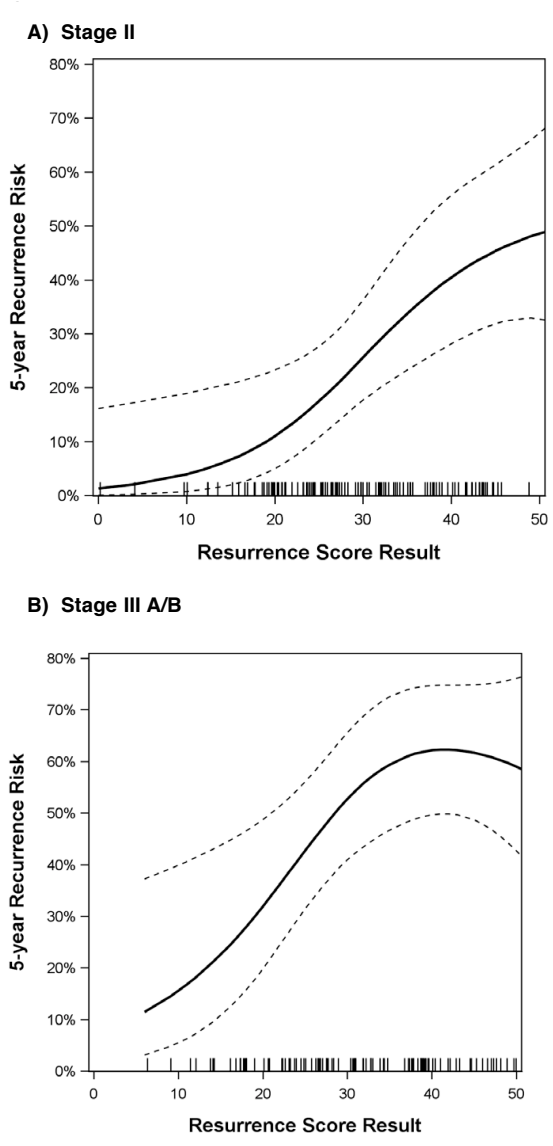
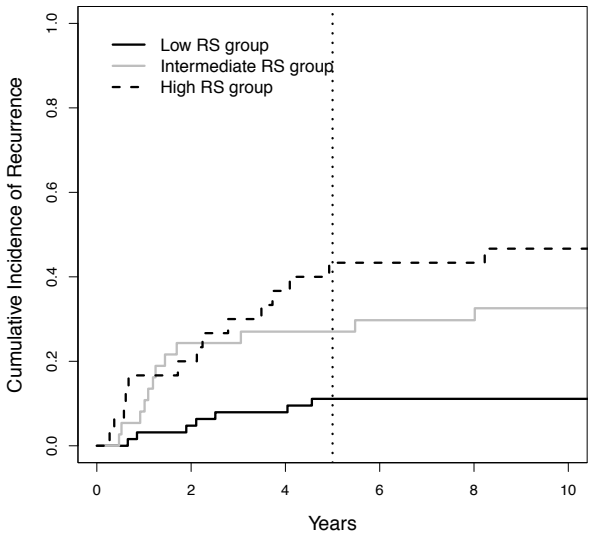


Figure 2: Relationship between risk of recurrence and continuous Recurrence Score in patients with negative resection margins.
Relationship between risk of recurrence and continuous Recurrence Score in 297 rectal cancer patients with negative resection margins. A) stage II, B) stage IIIA/B (1-3 positive lymph nodes). The solid line represents risk of recurrence; the dotted lines represent 95% confidence intervals. A rug plot depicting the distribution of Recurrence Score values is included at the bottom of each figure.

A



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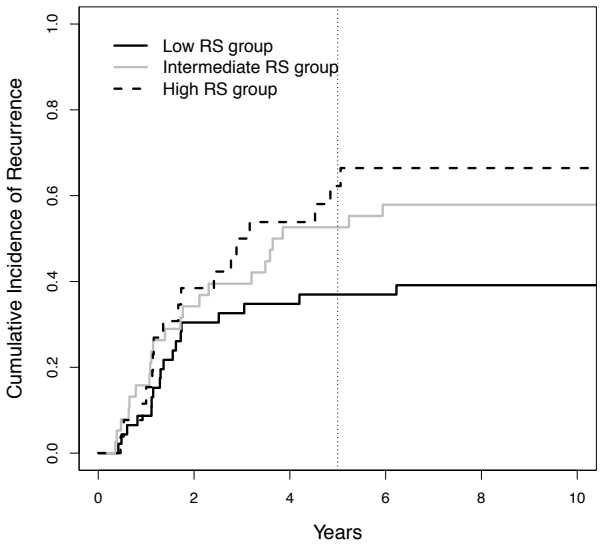

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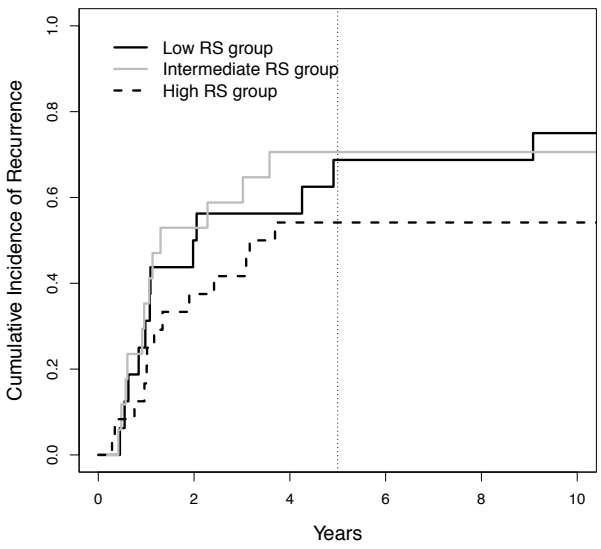

Figure 3: Cumulative incidence for recurrence by stage and Recurrence Score group Cumulative incidence curves for recurrence in 297 rectal cancer patients by Recurrence Score group based on prespecified cut-points and separated by stage. A) stage II, B) stage IIIA/B (1-3 positive lymph nodes), C) stage IIIC (4 or more positive lymph nodes).
Solid black line represents low Recurrence Score group, solid grey line – intermediate Recurrence Score group and dashed black line - high Recurrence Score group.

Table II: Five-year Estimates of Cumulative Incidence in Stage II Rectal Cancer Patients (n=130)

Recurrence Score group	N (%) pts	Cumulative Incidence for Recurrence (95% CI)	Cumulative Incidence for Distant Recurrence (95% CI)	Cumulative Incidence for Rectal Cancer Specific Mortality (95% CI)
Low	63 (48.5%)	11.1% (5.5%, 22.3%)	7.9% (3.4%, 18.4%)	4.8% (1.6%, 14.4%)
Intermediate	37 (28.5%)	27.0% (15.9%, 45.8%)	24.3% (13.8%, 42.9%)	18.9% (9.7%, 36.9%)
High	30 (23.1%)	43.3% (28.8%, 65.2%)	33.3% (20.1%, 55.2%)	30.0% (17.4%, 51.8%)

Similar results were observed for DRFI and RCSS: in the pre-specified main-effects models, the Recurrence Score result was significantly associated with DRFI (HR for 25 unit increase in the score of 1.50, 95% CI 1.04-2.17, p=0.030) and RCSS (HR of 1.64 (95% CI 1.15-2.34, p=0.007). Significant interaction between Recurrence Score result and stage and non-linearity were also observed for these endpoints. In stage II patients, cumulative incidence estimates of 5-year recurrence ranged from 8% (95% CI 3-18%) to 33% (95% CI 20-55%) for DRFI and from 5% (95% CI 2-14%) to 30% (95% CI 17-52%) for RCSS for low vs. high score groups, respectively (Table II).

The Recurrence Score result was not significantly associated with DFS (p=0.118) and OS (p=0.111) in the pre-specified analyses, similar to one of the colon cancer validation studies [13] where most deaths were not cancer-related. Notably, in this study, 52% of deaths in stage II patients were not due to rectal cancer.

Recurrence Score in the Context of Conventional Clinical and Pathologic Factors

When clinical and pathologic factors were examined (Supplemental Table III), higher age (p=0.041) and higher T-stage (T4N0, T3-4N1 vs. T3N0, T1-2N1, p=0.016) were associated with recurrence in analyses adjusted for stage and resection margin. Type of surgical resection and distance from anal verge showed an interaction with stage (p=0.026 and p=0.049, respectively), with LAR and greater distance from the anal verge associated with lower risk of recurrence in stage IIIC (both p<0.005) but not in stage II or stage IIIA/B. While resection margin status was significantly associated with outcome in the univariate analysis (p=0.015), its effect was attenuated after adjustment for stage in the multivariable analyses, paralleling what was observed for resection margin status in all eligible stage II-III surgery alone patients in the TME trial.

In pre-specified multivariable analysis adjusted for stage, RM status, T-stage, grade and number of nodes examined, the Recurrence Score result was a significant predictor of recurrence risk in stage II (p<0.001) and stage IIIA/B (p=0.019), but not Stage IIIC (p=0.122) (Table III). Similar results were observed when age, the only other covariate associated with RFI, was added to the model, and when the analysis was adjusted for circumferential (radial) margin status only. The model with Recurrence Score and

Table III: Multivariable Analysis: Contribution of Recurrence Score to Prediction of Recurrence Risk beyond Clinical and Pathologic Covariates

Variable	HR	HR (95% CI)	*p*-value
Stage			
IIIA/B vs. II	1.36	(0.71-2.95)	0.36
IIIC vs. II	2.48	(1.22-5.02)	0.01
Resection margin status			
R1 no RT vs. R0	1.02	(0.59-1.75)	0.95
R1 + RT vs.R0	1.01	(0.62-1.67)	0.96
T-Stage			
T4N0, T3-4N1 vs. T3N0, T1-2N1	2.03	(1.14-3.60)	0.01
Grade *			
High vs. low	0.99	(0.67-1.45)	0.95
Number of nodes examined			
12+ vs. <12	1.10	(0.75-1.62)	0.63
RS contribution**			
RS in stage II	3.40	(1.58-7.30)	<0.001
RS in stage IIIA/B	1.75	(1.11-2.77)	0.02
RS in stage IIIC	0.69	(0.42-1.12)	0.12

** Centrally assessed by a pathologist at Genomic Health*

*** Includes stage specific linear and spline terms (2 d.f.) to account for non-linearity. Hazard Ratio for Recurrence Score is the ratio of the hazards at the 75th and 25th percentiles of Recurrence Score*

conventional measures identified 25% of stage II patients with 5-year recurrence risk below 15% and 39% of patients with risks above 30% while the model based on the conventional measures alone assessed the risk for 95% of stage II patients to be in the 15%-30% range and 5% of patients with risk above 30%. Addition of the Recurrence Score assay to conventional measures resulted in higher relative utility (Figure 4). A test tradeoff calculation [21] illustrates the value of the assay for different treatment paradigms. If default strategy is treating everyone, testing 14 to 18 patients is required for every correct prediction of recurrence to increase the net benefit of risk prediction compared to conventional measures alone (risk thresholds 25-30%). If therapy is not routinely recommended, testing 37 to 45 patients is required (risk thresholds 45-50%).

The Recurrence Score result predicted DRFI (stage II p=0.009, stage IIIA/B p=0.020) and RCSS (stage II p<0.001 and stage IIIA/B p=0.034) after adjustment for these additional covariates.

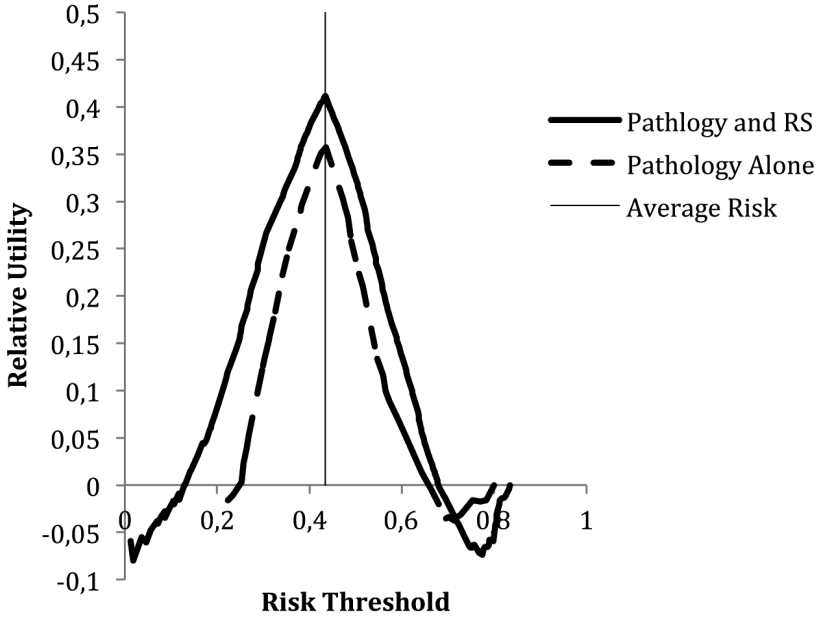

Figure 4: Relative utility curves for recurrence risk prediction using the models with and without Recurrence Score in all patients.

Relative utility curves for recurrence risk prediction in 297 rectal cancer patients. Relative utility is the maximum net benefit of prediction divided by the net benefit of perfect prediction. Risk threshold is the recurrence risk at which a patient is indifferent to the use of a treatment (e.g. post-operative chemotherapy). Solid black line represents Cox regression model with Recurrence score, N and T stage, resection margin status, number of nodes examined and grade. Dashed black line represents Cox regression model with N and T stage, resection margin status, number of nodes examined and grade.

DISCUSSION

In this prospectively-designed study, the 12-gene Recurrence Score was validated as a predictor of recurrence in stage II and III rectal cancer patients treated with TME surgery alone, providing information beyond conventional clinical and pathologic factors [12-14]. There was a significant interaction between Recurrence Score and stage, with the Recurrence Score providing the greatest discrimination of recurrence risk in stage II disease and little discrimination in stage IIIC.

Consistency of these rectal cancer results with 3 large validation studies of the Recurrence Score assay in colon cancer supports the association of this score with metastatic potential of large bowel cancers, and demonstrates the presence of common biological determinants of recurrence across tumors arising from the colon as well as the rectum.

Improved risk discrimination with the Recurrence Score result in stage II and IIIA/B rectal cancer should have clinical relevance for patients and physicians considering individualized approaches to pre-operative and post-operative treatment. In the United States the standard recommendation for treatment of stage II and III rectal

cancer patients includes neoadjuvant chemoradiation followed by TME surgery and postoperative adjuvant chemotherapy, based on extrapolation from trials in colon cancer [22;23]. By contrast, in most countries in Europe, adjuvant chemotherapy is not routinely recommended in rectal cancer. The benefit of adjuvant chemotherapy in patients with combined chemoradiation before surgery is controversial [7-10]. Across these treatment paradigms, the ability of Recurrence Score to identify patients with widely different risks of recurrence may enable tailored approaches, directing use of pre-operative and post-operative chemotherapy and radiation to patients at high risk of tumor recurrence and less aggressive treatment for low risk patients. In this regard, the low recurrence risk observed in our study for the large sub-group of stage II rectal cancer patients with low Recurrence Score results may be particularly impactful, as these patients demonstrated excellent outcomes without any pre- or post-operative chemotherapy or radiation. In moderate risk patients, the decision for more aggressive treatment should be discussed by patient and physician taking into account potential recurrence risk, morbidity associated with treatment, comorbidities and patient preferences. It is important to note that the ability of the Recurrence Score to predict neoadjuvant or adjuvant chemotherapy benefit in rectal cancer has not been studied. This study focused on patients who did not receive pre-operative chemotherapy or radiation, and the assay's ability to differentiate risk for patients with neoadjuvant therapies should be addressed in future studies.

The results of this validation study are consistent with recent analyses by the Cancer Genome Atlas Network [24], demonstrating similarity of colon and rectal cancers at the genomic level. A number of recent studies have suggested the existence of different subtypes of colorectal cancer [25-29]. All support the notion that colorectal tumors with a stromal response signature (EMT/TGFbeta signalling) have the worst outcome. Our results reaffirm the clinical relevance of two key biological pathways measured by the Recurrence Score assay - stromal response and cell cycle control, which is consistently reflected across multiple subtyping and genomic profiling efforts in the literature.

This prospectively-designed validation study demonstrates that the 12-gene colon cancer assay, can assess risk of recurrence in rectal cancer patients. The low exclusion rate observed during sample processing was consistent with QUASAR (3.6%), CALGB (3.1%) and C-07 (3.1%), indicating a precise and robust analytical process [12-14]. Limitations should also be acknowledged. First, blocks for only 59% of eligible patients were collected, although the demographics for those with blocks and without blocks were similar. Second, risk discrimination by Recurrence Score was attenuated in stage IIIA/B and IIIC, and Recurrence Score was not an independent recurrence risk predictor in stage IIIC. The reason for this attenuation is unclear, but may relate to challenges with achieving a complete resection of tumor at higher stage, which may affect recurrence rates beyond the biology of the tumor itself. Furthermore, the total study size is modest in absolute numbers and some subgroup analyses may be underpowered, but this is

one of the largest cohorts of well-characterized rectal cancer patients to be studied with a gene expression assay.

The use of adjuvant chemotherapy in rectal cancer is still under debate, and efforts are underway to study reduced-intensity approaches, including those that spare radiation and even surgery. Incorporation of the Recurrence Score assay into clinical trials, along the lines of the TAILORx and RxPonder trials in breast cancer[30;31], may enable these efforts through improved patient stratification for risk-adapted treatment strategies. Our results highlight the importance of understanding the underlying biology of rectal tumors for individual patients in assessing risk and potentially guiding treatment decisions in this disease.

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SUPPLEMENTAL MATERIAL

Supplemental Table I: Comparison of patient characteristics for eligible patients with and without blocks from the TME trial

Characteristic	Values	Evaluable patients in this study (N=297)	Eligible TME trial patients without blocks (N=210)	*p*-value*
Age	<60	102 (34.3)	58 (27.6)	0.07
	60 to <70	89 (30.0)	63 (30.0)	
	70+	106 (35.7)	89 (42.4)	
Gender	Female	111 (37.4)	83 (39.5)	0.62
Number of Nodes Examined	<12	190 (64.0)	133 (63.9)	0.99
Number of	0 (Stage II)	130 (43.8)	111 (53.4)	0.17
Nodes Involved	1-3 (Stage IIIA/B)	110 (37.0)	57 (27.4)	
	4+ (Stage IIIC)	57 (19.2)	40 (19.2)	
T-Stage	T1-T2	30 (10.1)	14 (6.7)	0.85
	T3	248 (83.5)	190 (90.9)	
	T4	19 (6.4)	5 (2.4)	
Obstruction or Perforation	Present	21 (7.1)	9 (4.3)	0.19
Grade**	High	73 (24.6)	35 (28.2)	0.43
Resection margin	R1	74 (24.9)	38 (18.3)	0.08

** p-values are from the chi-square and Cochran-Mantel-Haenszel chi-square tests for nominal categorical and ordered categorical variables, respectively*

*** Locally assessed during TME trial; available for 124 patients without blocks.*

Supplemental Table II: Five-year Estimates of Risk based on Kaplan Meier analysis in Stage II Rectal Cancer Patients (n=130)

Recurrence Score group	N (%) pts	Recurrence Risk (95% CI)	Distant Recurrence Risk (95% CI)	Rectal Cancer Specific Mortality (95% CI)
Low	63 (48.5%)	12.4% (6.1%, 24.3%)	9.1% (3.9%, 20.4%)	5.3% (1.8%, 15.7%)
Intermediate	37 (28.5%)	28.7% (16.6%, 46.8%)	25.8% (14.4%, 43.8%)	20.3% (10.2%, 37.9%)
High	30 (23.1%)	52.7% (34.7%, 73.2%)	45.9% (27.6%, 68.8%)	37.0% (21.2%, 59.1%)

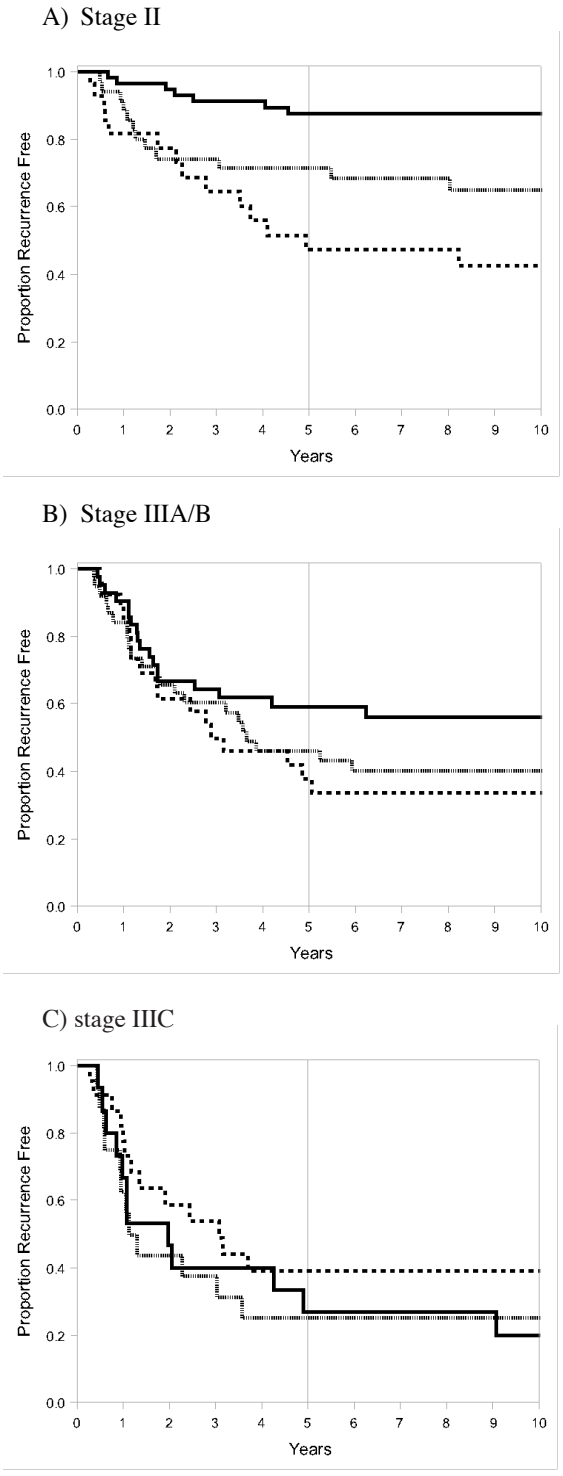
Supplemental Table III: Association of conventional clinical and pathologic factors with risk of recurrence.

Characteristic	Values	HR	HR 95% CI	*p*-value*	*p*-value for interaction with stage***
Age	Continuous, per 1 year increase	1.02	(1.00,1.03)	0.04	0.92
Grade, central	High vs low	1.01	(0.68,1.49)	0.96	0.61
Grade, local	High vs low	1.06	(0.71,1.57)	0.78	0.74
Nodes examined	<12 vs. 12+	1.18	(0.80,1.74)	0.40	0.67
Gender	Male vs. Female	1.09	(0.75,1.58)	0.64	0.58
T Stage	T4N0, T3-4N1 vs. T3N0, T1-2N1	1.89	(1.10,3.25)	0.02	0.28
Surgery	APR vs. LAR	1.44	(1.00,2.06)	0.05	0.03
Distance from anal verge	5-9.9 vs. <5	0.93	(0.62,1.39)	0.72	0.05
	10+ vs. <5	0.62	(0.39,0.99)	0.04	
Residual disease**	R1 vs. R0	1.28	(0.86,1.92)	0.23	0.30
Resection margin status**	R1 no RT vs. R0	1.18	(0.69,2.04)	0.55	0.52
	R1 + RT vs. R0	1.37	(0.85,2.22)	0.21	
CRM margin (<1 mm)**	Positive vs Negative	1.34	(0.89,2.00)	0.17	0.27
CRM margin (<2 mm)**	Positive vs Negative	1.28	(0.87,1.87)	0.21	0.21

**Based on Cox PH models including a given covariate, stage and RM status.*

***Based on Cox PH models including a given covariate and stage.*

****Based on Cox PH models including a given covariate, stage and interaction of covariate and stage.*



Supplemental Figure 1: Kaplan Meier analysis for recurrence-free interval by stage and Recurrence Score group.

Kaplan Meier curves for Recurrence Free Interval (RFI) in 297 rectal cancer patients stratified for Recurrence Score group based on prespecified cut-points and separated by stage. A) stage II, B) stage IIIA/B (1-3 positive lymph nodes), C) stage IIIC (4 or more positive lymph nodes).

Solid black line represents low Recurrence Score group, dashed black/grey line-intermediate Recurrence Score group and dotted black line-high Recurrence Score group.