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A Liquid Biopsy Assay for Noninvasive Identification of Lymph Node Metastases in T1 Colorectal Cancer

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BACKGROUND & AIMS: We recently reported use of tissue-based transcriptomic biomarkers (microRNA [miRNA] or messenger RNA [mRNA]) for identification of lymph node metastasis (LNM) in patients with invasive submucosal colorectal cancers (T1 CRC). In this study, we translated our tissue-based biomarkers into a blood-based liquid biopsy assay for noninvasive detection of LNM in patients with high-risk T1 CRC. **METHODS:** We analyzed 330 specimens from patients with high-risk T1 CRC, which included 188 serum samples from 2 clinical cohorts—a training cohort (n = 46) and a validation cohort (n = 142)—and matched formalin-fixed paraffin-embedded samples (n = 142). We performed quantitative reverse-transcription polymerase chain reaction, followed by logistic regression analysis, to develop an integrated transcriptomic panel and establish a risk-stratification model combined with clinical risk factors. **RESULTS:** We used comprehensive expression profiling of a training cohort of LNM-positive and LMN-negative serum specimens to identify an optimized transcriptomic panel of 4 miRNAs (miR-181b, miR-193b, miR-195, and miR-411) and 5 mRNAs (AMT, forkhead box A1 [FOXA1], polymeric immunoglobulin receptor [PIGR], matrix metalloproteinase 1 [MMP1], and matrix metalloproteinase 9 [MMP9]), which robustly identified patients with LNM (area under the curve [AUC], 0.86; 95% confidence interval [CI], 0.72–0.94). We validated panel performance in an independent validation cohort (AUC, 0.82; 95% CI, 0.74–0.88). Our risk-stratification model was more accurate than the panel and an independent predictor for identification of LNM (AUC, 0.90; univariate: odds ratio [OR], 37.17; 95% CI, 4.48–308.35; $P < .001$; multivariate: OR, 17.28; 95% CI, 1.82–164.07; $P = .013$). The model limited potential overtreatment to only 18% of all patients, which is dramatically superior to pathologic features that are currently used (92%). **CONCLUSIONS:** A novel risk-stratification model for noninvasive identification of T1 CRC has the potential to avoid unnecessary operations for patients classified as high-risk by conventional risk-classification criteria.

Keywords: Transcriptomic Panel; Risk-Stratification Model; Detection Biomarker; Noninvasive Assay.

In recent years, the diagnosis of invasive submucosal colorectal cancers (T1 CRCs) has increased by up to 15% to 30% due to the implementation of mass CRC screening and frequent patient examinations.[1,2] However, recent advances in endoscopic devices have enabled curative treatment via endoscopic submucosal dissection (ESD) or endoscopic mucosal resection (EMR) for patients with T1 CRC who would have otherwise been treated by radical operations.[3] This has prompted the National Comprehensive Cancer Network to recommend ESD as a preferred treatment modality for patients with suspected T1 CRC.

Successful treatment of patients with T1 CRCs starts with an accurate diagnosis during endoscopy. However, 2 prospective studies recently highlighted that 30% to 40% of these patients are misdiagnosed and that the presurgical discrimination of T1 CRC remains clinically challenging.[4,5] Although some patients can be successfully treated with ESD or EMR, approximately 70% to 80% of patients with T1 CRC require radical operations to achieve a complete cure owing to the potential risk for lymph node metastasis (LNM) after pathologic analysis, which is estimated to occur in as many as 5% to 15% of patients with high-risk T1 CRC.[6–8]

With the implementation of endoscopic treatment for suspected T1 CRCs, identifying the risk of LNM has become necessary to select patients who truly have high-risk disease and require radical surgery while sparing others from overtreatment. The currently used pathologic criteria to identify LNM in patients with T1 CRC include depth of

Abbreviations used in this paper: **AUC, area under the curve; CI, confidence interval; CRC, colorectal cancer; EMR, endoscopic mucosal resection; ESD, endoscopic submucosal dissection; FFPE, formalin-fixed paraffin-embedded; LNM, lymph node metastasis; LNN, lymph node metastasis-negative; LNP, lymph node metastasis-positive; miRNA, micro RNA; mRNA, messenger RNA; MSI, microsatellite instability; OR, odds ratio; PCR, polymerase chain reaction; T1 CRC, invasive submucosal colorectal cancer.**

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WHAT YOU NEED TO KNOW

BACKGROUND AND CONTEXT

Patients with high-risk invasive submucosal colorectal cancers are often recommended radical surgery due to the risk of lymph node metastases (LNM), but the current pathologic criteria for risk-stratification of LNM are inadequate and often lead to overtreatment.

NEW FINDINGS

Serum and matched tumor specimens from independent patient cohorts were used to develop a noninvasive, liquid biopsy transcriptomic assay that can robustly identify patients at risk for LNM before surgery. This biomarker panel was combined with key clinical features to establish a risk-stratification model that exhibited superior accuracy for identification of LNM.

LIMITATIONS

This was a retrospective study, and independent prospective studies are needed to further confirm the diagnostic potential of this diagnostic assay before its translation in the clinic.

IMPACT

This risk-stratification model has a potential to serve as a noninvasive, liquid biopsy assay to identify patients with high-risk invasive submucosal colorectal cancers with LNM before surgery and reduce the overall burden of unnecessary operations and expense associated with these procedures.

submucosal invasion (>1000 μm), presence of lymphatic or vascular invasion, high-grade tumor budding, and poorly differentiated histology.[9–13] If these factors are absent, endoscopic treatment is considered sufficient to cure patients with T1 CRC who have low-risk for LNM.[14,15]

Unfortunately however, if even one of these pathologic risk features is present in clinical settings, the patient is deemed as "high risk for LNM" and is recommended to undergo additional surgery.[3,11,16,17] Such a dichotomized clinical management approach for patients with T1 CRCs has serious drawbacks, because it often leads to overtreatment, even though the positive predictive value for the presence of LNM is quite low.[18] By using the current clinicopathologic criteria, approximately 70% to 80% of patients with T1 CRC are classified as high risk, whereas postsurgical pathologic results demonstrate that only 5% to 15% of these patients actually have LNM.[10,14,16,19–25]

This highlights an important clinical challenge: we need more prudent risk assessment for limiting unnecessary radical surgery in 85% to 95% of patients with T1 CRC. In addition, these data suggest the inadequacy of currently used pathologic risk factors and emphasize the need to develop robust molecular biomarkers that can identify the presence of LNM preoperatively, which would better inform clinical decision making in patients with T1 CRC, minimize the number of operations performed, and reduce the overall burden of costs associated with such invasive procedures.

Accumulating evidence indicates that the expression pattern of microRNAs (miRNAs) reflects the physiological and pathologic status of patients with cancer. In fact, several studies have identified the differential expression of specific miRNAs to be directly involved in CRC pathogenesis and have emphasized their potential as circulating biomarkers for CRC.[26–30] Although considerable advances have been made in exploiting miRNAs as noninvasive diagnostic biomarkers,[31–33] using circulating miRNAs to clinically identify high-risk T1 CRCs has thus far not been attempted.

We previously described a panel of tissue-based miRNAs and gene expression biomarkers that allowed robust detection of LNM in patients with T1 CRC.[34,35] However, an ideal clinical application of these biomarkers would be to use them to diagnose patients with high-risk T1 CRC *before* surgery, before such tissue specimens are readily available. Therefore, translating these biomarkers into a "liquid biopsy" assay is attractive, because this would allow a noninvasive, facile, and inexpensive diagnostic assay for LNM in patients with high-risk T1 CRC.

To address this gap in knowledge, we evaluated the feasibility of translating our previously reported transcriptomic biomarkers (miRNAs and messenger [m]RNAs) into a blood-based, noninvasive assay by systematically analyzing blood specimens from multiple cohorts of patients with T1 CRC. As a result, we successfully established a novel, blood-based, transcriptomic signature that robustly identified the presence of LNM in patients with T1 CRC, with an area under the curve (AUC) value of 0.90. This assay allowed reclassification of 75% of high-risk T1 CRCs into the low-risk group, which would obviate the need for unnecessary operations in this significant majority of patients who would have otherwise been subjected to radical operations based on conventional pathologic risk-assessment criteria.

Materials and Methods

Patient Cohorts

We analyzed 330 patient samples, which included 188 serum specimens from patients with high-risk T1 CRCs comprising 2 independent clinical cohorts: a training cohort of 46 patients with 5 LNM-positive (LNP) and 41 LNM-negative (LNN) patients from the National Cancer Center Hospital, Japan, and a validation cohort of 142 patients with 12 LNP and 130 LNN patients from the National Cancer Center Hospital East, Japan (Figure 1*A*). Matched formalin-fixed paraffin-embedded (FFPE) specimens (n = 142), which were obtained after endoscopic or surgical tumor resection, were also obtained from patients within the validation cohort.

All patients were diagnosed as high-risk pathologically. The pathologic criteria included depth of submucosal invasion (>1000 μm), presence of lymphatic or vascular invasion, high-grade tumor budding, and poorly differentiated histology. Radical operations were performed in all patients in the training cohort between January 2017 and December 2017 and in the validation cohort between January 2011 and December 2017. Exclusion criteria were synchronous advanced CRCs,

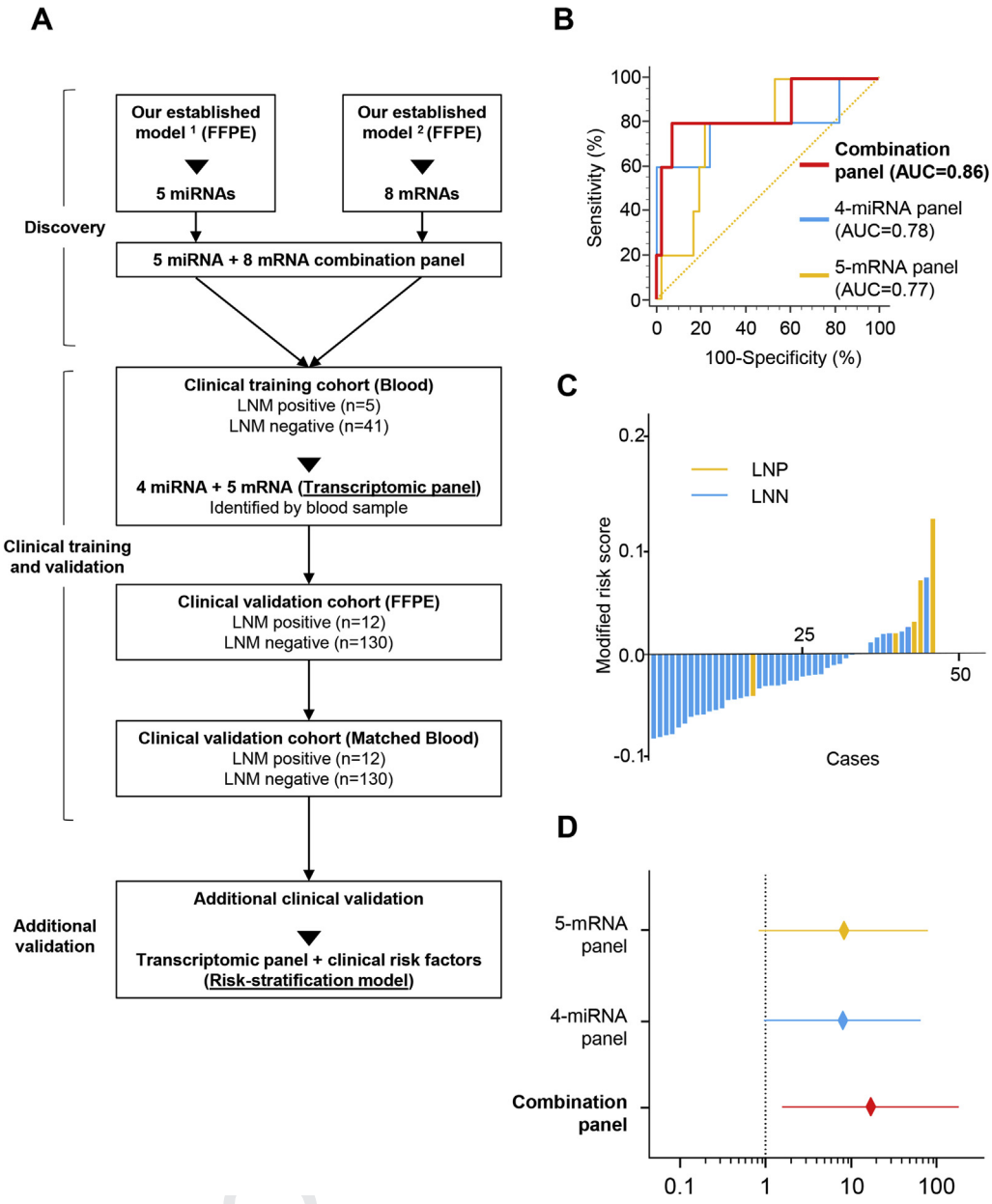

Figure 1. Training phase of a transcriptomic panel for the identification of LNM in patients with T1 CRC. (*A*) Overview of the study. (*B*) A receiver operating characteristic curve for a 4-miRNA and 5-mRNA panel in serum from training cohort patients (LNP = 5, LNN = 41; AUC: 0.78 for 4-miRNA panel; 0.77 for 5-mRNA panel; 0.86 for combination panel). (*C*) Risk score distribution plot in training cohort patients. Modified risk scores were obtained from individual risk scores by using Youden's index values from the risk model. (*D*) Forest plots with ORs for each panel risk score status in univariate logistic regression analysis in training cohort patients (OR: 8.62 for 4-miRNA panel; 8.44 for 5-mRNA panel; 14.22 for combination panel).

presence of distant metastases, hereditary or inflammation-associated CRC, nonadenocarcinoma, or nonavailability of serum specimens.

All patients underwent standard endoscopic and surgical procedures (resection of affected segment of colon or rectum and regional lymphadenectomy), and all specimens were evaluated by pathologists at each participating institution, according to the Seventh Edition of the American Joint Committee on Cancer TNM grading system. The study was conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from all patients, and the study was approved by the institutional review boards of all participating institutions.

RNA Extraction From Serum and Formalin-Fixed Paraffin-Embedded Specimens

Total RNA extraction from all serum specimens was performed using the Qiagen miRNeasy Kit (Qiagen, Hilden, Germany). Briefly, 200 μL of serum was thawed on ice and

centrifuged at 3000*g* for 5 minutes to remove cell debris. Next, 200 μL of the supernatant was lysed in 5 times the volume of QIAzol solution (Qiagen). To normalize any inadvertent sample-to-sample variations during the RNA isolation procedure, 3.5 μL of synthetic *Caenorhabditis elegans* miRNA (cel-miR-39) was spiked into each denatured sample. Total RNA was subsequently enriched and purified following the manufacturer's protocol. For FFPE specimens, 10-μm-thick sections were manually microdissected to enrich for cancer cells (>75% of tumor cells), and the RNA was extracted using the AllPrep DNA/RNA FFPE Kit (Qiagen). Extracted RNA from serum and FFPE specimens was processed for the generation of complementary DNA (cDNA) before polymerase chain reaction (PCR) assays.

Real-Time Quantitative Reverse-Transcription Polymerase Chain Reaction Assays

For miRNA, synthesis of cDNA from total RNA was performed using the TaqMan microRNA Reverse Transcription Kit (Thermo Fisher Scientific, Waltham, MA). For mRNA, a high-capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific) was used to convert RNA into cDNA. Real-time reverse transcription quantitative PCR analysis was performed using the SensiFAST probe Lo-ROX Kit (Bioline, London, United Kingdom) on the QuantStudio 7 Flex Real Time PCR System (Applied Biosystems, Foster City, CA), and expression levels were evaluated using the corresponding software system. The relative abundance of target transcripts was evaluated and normalized to the expression of miR-16 for miRNA and β-actin for mRNA as internal controls, using the $2^{-\Delta DCt}$ method. DCt represents the difference of cycle threshold (Ct) values between the miRNA of interest and the internal normalizing gene. Normalized expression values were $\log_{10}$ transformed[36] before downstream statistical analysis. All primers for miRNAs analyzed in this study were purchased from Thermo Fisher Scientific. The catalog number for all miRNA primers was 4427975, and the assay IDs of individual miRNAs were as follows: Hsa-miR-16: 391, Hsa-miR-32-5p: 2109, Hsa-miR-181b: 1098, Hsa-miR-193b-3p: 2367, Hsa-miR-195-5p: 494, and Hsa-miR-411-5p: 1610. The primer sequences for the target genes used in the present study are listed in Supplementary Table 1.

Statistical Analysis

Clinicopathologic characteristics of the patient cohorts are shown as patient number and ratio except for age (median and range) (Table 1). The cutoff thresholds for continuous variables were divided by the median value in the total participants. Several clinicopathologic characteristics were compared between LNP and LNN patients by using the χ^2 or Mann-Whitney *U* test for categorical data. Binary logistic regression was used to train a classifier based on the expression of 4 miRNAs and 5 mRNAs. Of note, once the model was trained in the training cohort, the same statistical model variables (weights and cutoff thresholds) were applied in the validation cohort. The LNM risk score for all patients was calculated based on the individual biomarker coefficients derived from the training cohort as follows: Logit (P) = (−0.318 × MIR181b) + (−0.762 × MIR193b) + (−1.019 × MIR195) + (−0.627 × MIR411) + (−0.135 × AMT) + (−0.010 × forkhead box A1 [FOXA1]) + (0.241 × matrix metalloproteinase 1 [MMP1]) + (−0.776 × matrix metalloproteinase 9 [MMP9]) + (0.231 × polymeric immunoglobulin receptor [PIGR]) − 8.363. The cutoff threshold for the LNM risk score was chosen as 0.08, which was determined by Youden's index.

For all cohorts, receiver operator characteristic curves and AUC values were used to evaluate the performance of the panel for LNM detection in patients with T1 CRC. A *P* value of <.05 was considered statistically significant. Statistical analyses were performed using JMP Genomics 9.0 statistical software

Table 1. Clinicopathologic Characteristics of Clinical Cohorts

Characteristics	Training cohort (n = 46)	Validation cohort (n = 142)	*P*
Age, *y*			
Median (range)	70 (38–90)	67 (24–85)	.16
Sex			
Male	24 (52)	86 (61)	
Female	22 (48)	56 (39)	.32
LNM			
Positive	5 (11)	12 (8)	
Negative	41 (89)	130 (92)	.62
MSI status			
MSI-H		10 (7)	
MSI-L		5 (4)	
MSS		127 (89)	…
Tumor location			
Right side	17 (37)	41 (29)	
Left side	29 (63)	101 (71)	.3
Tumor size, *mm*			
≥20	23 (50)	65 (46)	
<20	23 (50)	77 (54)	.18
Submucosal invasion, *mm*			
≥1000	43 (93)	140 (99)	
<1000	3 (7)	2 (1)	.16
Budding grade			
≥2	9 (20)	26 (18)	
1	27 (58)	104 (74)	
Unavailable	10 (22)	12 (8)	.52
Lymph invasion			
Positive	13 (28)	53 (37)	
Negative	33 (72)	89 (63)	.26
Vascular invasion			
Positive	13 (28)	49 (35)	
Negative	33 (72)	93 (65)	.43
Differentiation			
Well	22 (48)	97 (68)	
Moderate	21 (46)	43 (31)	
Poor	2 (4)	0 (0)	

NOTE. Data are shown as n (%) unless indicated otherwise. MSI, microsatellite instability; MSI-H, high-frequency microsatellite instability; MSI-L, low-frequency microsatellite instability; MSS, microsatellite stable.

(SAS Institute Japan, Tokyo, Japan), Medcalc 16.2.0 statistical software (Medcalc Software bvba, Ostend, Belgium), GraphPad Prism 7.0 (GraphPad Software, San Diego, CA), and R 3.5.0 (R Development Core Team, https://cran.r-project.org/).

Results

Patients Within the Training and Validation Cohorts Exhibited Similar Clinicopathologic Features, Including the Rate of Lymph Node Metastasis

To develop a blood-based, noninvasive assay, we first confirmed that both cohorts of patients with T1 CRC showed similar clinicopathologic characteristics. The training cohort consisted of 46 patients with T1 CRC, which included 5 patients (11%) with LNM; the patients in this cohort were a median age of 70 years. The validation consisted of 142 patients with T1 CRC, which included 12 patients (8%) with LNM; the patients in this cohort were a median age of 67 years. The detailed clinicopathologic characteristics of the patients in these cohorts are provided in Table 1. We observed no statistically significant difference in the prevalence of LNM rates or any other clinicopathologic characteristic between the 2 cohorts, which eliminates any inadvertent bias between the patient cohorts examined in our study.

A Noninvasive Transcriptomic Risk-Assessment Model Identifies Lymph Node Metastasis in Patients With T1 Colorectal Cancer

By undertaking a systematic and comprehensive biomarker discovery and validation effort, we previously reported tissue-based miRNA and mRNA signatures for the identification of LNM in patients with T1 CRC.[34,35] In these studies, we described a panel of 5 miRNAs (miR-32, miR-181b, miR-193b-3p, miR-195-5p, and miR-411-5p) and 8 genes (AMT, FOXA1, MMP1, MMP9, LYZ, C2CD4A, RCC1, and PIGR) that could identify LNM in patients with T1 CRC. However, an ideal clinical application of these biomarker signatures would be in a noninvasive, liquid biopsy, diagnostic platform. Such a platform would obviate the need for analysis of tissue specimens, which are generally not available from most patients in preoperative settings. Therefore, in this study, we focused on translating the tissue-based biomarkers into a blood-based assay that could yield a clinically attractive assay for noninvasive diagnosis of LNM in patients with T1 CRC.

Accordingly, we evaluated the expression of our transcriptomic biomarkers in 2 independent cohorts of patients with T1 CRCs. First, we evaluated the feasibility of detecting the miRNA and mRNA markers using real-time quantitative reverse transcription PCR in serum specimens from the training cohort of patients (5 LNP and 41 LNN). One miRNA (miR-32-5p) and 3 mRNAs (LYZ, C2CD4A, and RCC1) were not detectable in serum specimens, which led us to establish a panel of 4 miRNAs (miR-181b, miR-193b-3p, miR-195-5p, and miR-411-5p) and 5 mRNAs (AMT, FOXA1, MMP1, MMP9, and PIGR).

Next, we systematically interrogated the diagnostic accuracy of our transcriptomic panel for its ability to detect LNM in patients with T1 CRC. Using logistic regression analysis, we trained a risk-assessment model in the training cohort of patients that allowed robust identification of LNM in patients with T1 CRC using the 4 miRNAs (AUC, 0.78; 95% CI, 0.64–0.89) or the 5 mRNAs (AUC, 0.77; 95% CI, 0.62–0.88) (Figure 1*B* and *C* and Supplementary Table 2). Identification of LNM was notably superior when we used all 4 miRNAs and 5 mRNAs to establish a combined transcriptomic panel (AUC, 0.86; 95% CI, 0.72–0.94).

We performed univariate analysis to confirm that each of the biomarker panels was quite robust individually (miRNA panel: odds ratio [OR], 8.62; $P = .06$; mRNA panel: OR, 8.44; $P = .05$) (Figure 1*D*, Supplementary Table 3). However, the combined panel was significantly superior in diagnosing the presence of LNM in patients with T1 CRC (OR, 14.22; $P = .02$). We developed this risk-assessment scoring model based on the coefficients derived from individual markers by using the logistic regression analysis as the following model parameters: Logit (P) = $(-0.318 \times \text{MIR181b}) + (-0.762 \times \text{MIR193b}) + (-1.019 \times \text{MIR195}) + (-0.627 \times \text{MIR411}) + (-0.135 \times \text{AMT}) + (-0.010 \times \text{FOXA1}) + (0.241 \times \text{MMP1}) + (-0.776 \times \text{MMP9}) + (0.231 \times \text{PIGR}) - 8.363$. Taken together, these data show we successfully established a novel transcriptomic panel for noninvasive identification of LNM in patients with T1 CRC.

Transcriptomic Biomarkers and a Risk Nomogram Identify Lymph Node Metastases in Patients With T1 Colorectal Cancer in an Independent Validation Cohort

After the encouraging results of our blood-based transcriptomic panel for the noninvasive detection of LNM, we next confirmed its robustness and accuracy by applying the same statistical coefficients in serum specimens from a large, independent, validation cohort of 12 LNP and 130 LNN patients. To further assess our ability to assay transcriptomic biomarkers shed by the primary cancer into the systemic circulation, we also used matched endoscopically or surgically resected FFPE tissue specimens from patients within this cohort. We first evaluated the diagnostic accuracy of our transcriptomic panel in these FFPE surgical specimens and were enthused to observe that its diagnostic performance was comparable to that observed in serum specimens in the training cohort (AUC, 0.83; 95% CI, 0.75–0.89) (Figure 2*A*). When we evaluated the performance of the signature in matched blood serum specimens, the diagnostic accuracy was in line with the findings from tissue specimens (AUC, 0.82; 95% CI, 0.74–0.88) (Figure 2*B* and *C*). This highlights the clinical significance of our transcriptomic panel in identifying the presence of LNM in patients with T1 CRC.

For an easier translation of the biomarker panel into the clinic, we evaluated its performance along with other pathologic risk features (ie, lymphatic or vascular invasion, high-grade tumor budding) and established a nomogram

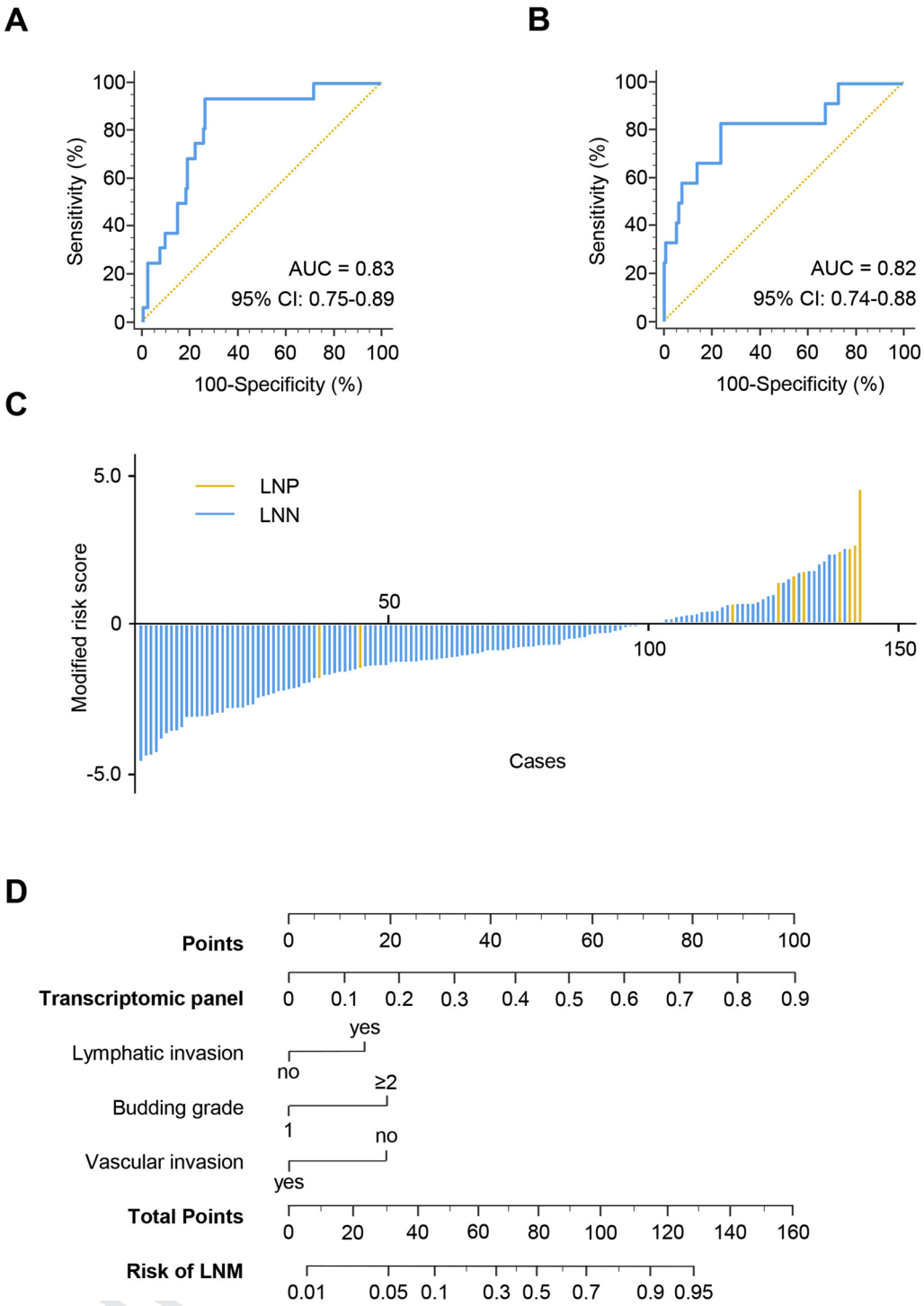

Figure 2. Validation phase of the transcriptomic panel for the identification of LNM in patients with T1 CRC. A receiver operating characteristic curve for the transcriptomic panel in (*A*) tissue specimens from validation cohort patients (LNP = 12, LNN = 130; AUC, 0.83) and (*B*) in matched serum samples in validation cohort patients (LNP = 12, LNN = 130; AUC, 0.82). (*C*) Risk score distribution plot in serum specimens from validation cohort patients. (*D*) A nomogram illustrates the probability of LNM risk. For clinical purposes, the scores of each covariate are added, and the total score is depicted on the total score point axis.

for predicting the diagnostic probability for the presence of LNM from validation cohort. Through ranking the effect estimators, point scores were assigned to each risk factor. The total points accumulated from all the risk factors corresponded to the predicted probability of LNM for individual patients. We incorporated all pathologic and

molecular risk features and determined that although other pathologic risk-assessment features added some weight to the model, our panel had the highest weight in this model and was an independent and the most significant predictor for the presence of LNM in patients with T1 CRC (Figure 2*D*).

A Risk-Stratification Model That Combines Transcriptomic Biomarkers and Current Risk-Assessment Features Significantly Improves Diagnosis of Lymph Node Metastasis in Patients with T1 Colorectal Cancer

Considering the current landscape of widely used clinical risk factors for identifying patients with T1 CRC, we asked whether a risk-stratification model that includes some of the currently used pathologic risk features (ie, lymphatic and vascular invasion, tumor budding grade, and depth of tumor invasion) along with our transcriptomic biomarkers might further improve diagnostic accuracy in detecting LNM in patients with T1 CRC. Because 12 patients lacked clinical information, 130 patients were included in risk-stratification model. When we performed such an analysis in the patients within the serum specimens of validation cohort, this led to a significant improvement in its diagnostic sensitivity and specificity for the identification of LNM (AUC, 0.90; 95% CI, 0.83–0.95) (Figure 3*A* and Table 2).

We next determined specific diagnostic correlates for our combined biomarker panel in blood samples from the validation cohort: its sensitivity, specificity, positive predictive value, and negative predictive value were 83.3%, 76.2%, 24.4%, and 98.0%, respectively (Table 2). When we performed a similar analysis of the newly established risk-stratification model that also included pathologic risk features, its performance was significantly superior: its sensitivity, specificity, positive predictive value, and negative predictive value were 90.0%, 81.4%, 29.0%, and 99.0%, respectively. This highlights the superiority of the risk-stratification model for identifying LNM in patients with T1 CRC.

We next categorized all patients into high- and low-risk groups using cutoff thresholds derived from Youden's index for the 9 miRNA and mRNA biomarkers. Accordingly, we performed univariate logistic regression analysis which revealed that our transcriptomic panel emerged as an independent predictor for LNM in patients with T1 CRC in both clinical cohorts compared with any single clinical risk factor (training cohort: OR, 14.22; 95% CI, 1.41–143.68; $P = .025$; validation cohort: OR, 15.97; 95% CI, 3.32–76.82; $P < .001$ (Table 3).

Further, univariate and multivariate logistic regression analysis revealed that our novel risk-stratification model was superior compared with the panel and an independent predictor of LNM (univariate: OR, 37.17; 95% CI, 4.48–308.35; $P < .001$; multivariate: OR, 17.28; 95% CI, 1.82–164.07; $P = .013$) in the validation cohort of patients (Figure 3*B* and *C* and Table 3). Collectively, these data highlight the potential clinical significance of our risk-stratification model for diagnosis and risk assessment in the identification of LNM.

Our noninvasive risk-assessment model is significantly superior to currently used pathologic risk factors for identifying patients with high-risk T1 CRC and reducing the burden of unnecessary surgical treatments

The ultimate goal of our study was to determine the clinical usefulness of our transcriptomic panel in non-invasively identifying patients who truly have LNM and sparing the rest from unnecessary operations. In this study, we only enrolled patients who were deemed high-risk based on the currently used pathologic risk factors. However, only 8% of "high-risk" patients (12 of 142) were actually high risk, indicating that 92% of patients (130 of 142) were erroneously categorized as high risk and underwent unnecessary radical operations (Figure 3*D*, *left panel*).

In contrast, when we analyzed the same patients using our transcriptomic classifier and divided into high and low risk by Youden's index, it stratified 29% of patients into the high-risk category (41 of 142). Among these, 10 patients (7%) had LNM, indicating that only 22% of the entire cohort (31 of 142) received overtreatment, which is notably superior to potential overtreatment compared with the currently used pathologic features (92% vs 22%) (Figure 3*D*, *middle panel*). Our newly established risk model was even more accurate than the panel, as it stratified only 25% of patients into the high-risk group (32 of the 130), and the remaining 75% (98 of the 130) of patients were deemed as low risk. Of the 32 patients who were classified as high risk, 9 patients (7%) had LNM, indicating that only 18% (23 of 130) of all patients with T1 CRC were potentially overtreated, which is dramatically superior compared with currently used pathologic features (92% vs 18%) (Figure 3*D*, *right panel*). This highlights the potential for using our liquid biopsy-based risk-assessment model in patients with high-risk T1 CRC.

Discussion

The presence of LNM is an important risk factor for additional surgery after curative endoscopic treatment in patients with T1 CRC. Our present study overcomes the inadequacy of clinicopathologic risk features that are currently used in the clinic to identify LNM in "high-risk" subsets of patients with T1 CRC. Our data demonstrate that a blood-based, transcriptomic assay can be used to accurately estimate risk in preoperative settings, has a tremendous clinical potential for more robust risk-stratification for the identification of LNM, and can lead to a dramatic reduction in the number of unnecessary operations that are currently being performed in these patients. Identifying true high-risk patients and saving others from such unnecessary treatment will reduce patient complications, physician burdens, and associated health care costs.[37–39]

In this study, our newly established noninvasive risk model exhibited a significantly superior diagnostic accuracy for LNM (AUC, 0.90) vs the currently used clinical risk models (AUC, 0.73 [training] and 0.76 [validation] (Supplementary Figure 1). Although all patients enrolled in our study were deemed to be high-risk for LNM and

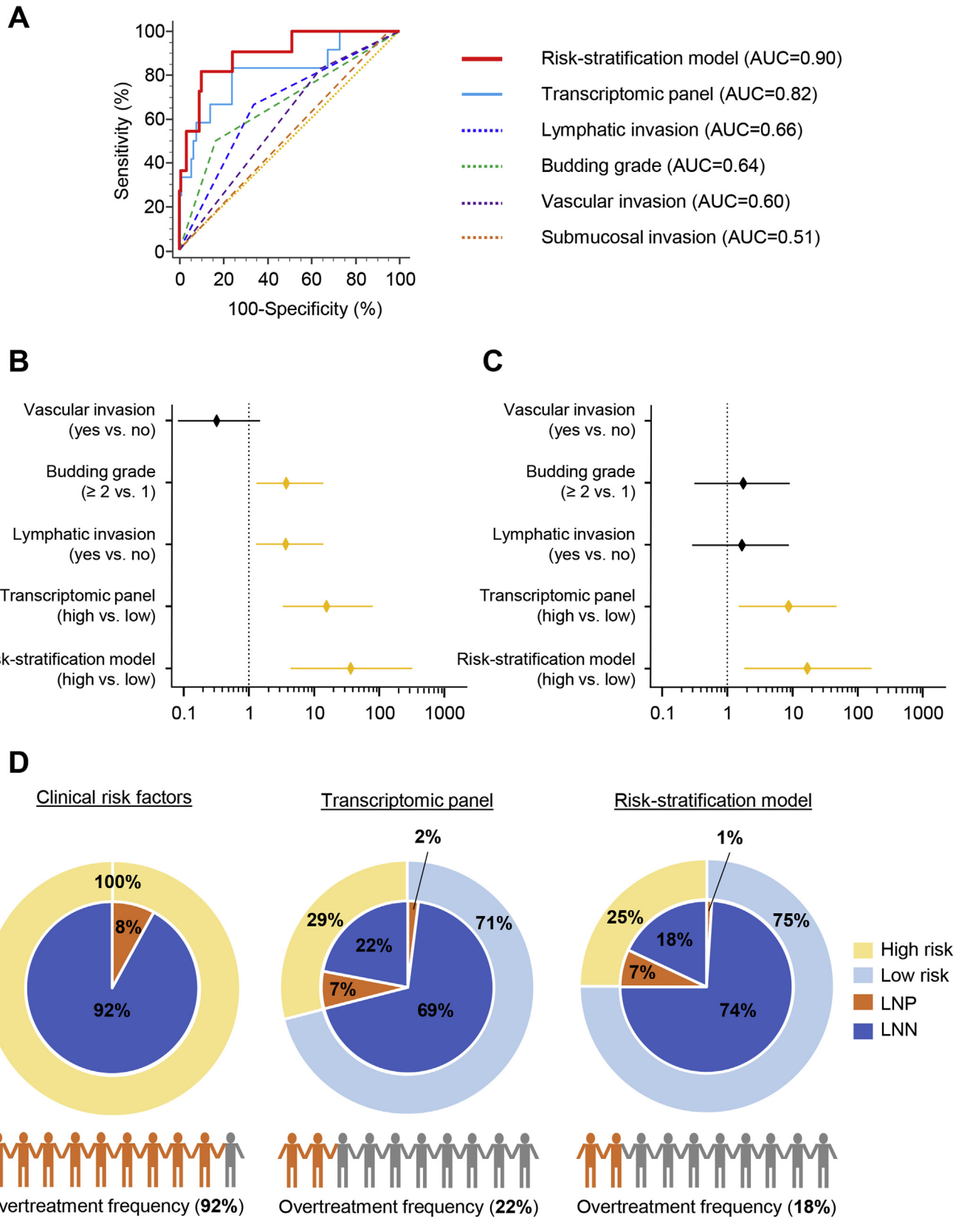

Table 2. Model Performance in Estimating the Risk of Lymph Node Metastasis

Variable	Value (95% CI)			
	Training cohort (Blood)	Validation cohort (FFPE)	Validation cohort (Blood)	Risk-stratification model (Blood)
Cutoff value	0.08	0.05	0.08	0.08
Sensitivity, %	80.0 (28.4–99.5)	91.7 (61.5–99.8)	83.3 (51.6–97.9)	90.0 (55.5–99.7)
Specificity, %	92.7 (80.1–98.5)	73.9 (65.4–81.2)	76.2 (67.9–83.2)	81.4 (73.1–87.9)
AUC, %	85.5 (71.8–94.0)	82.6 (75.4–88.5)	81.5 (74.1–87.5)	90.0 (83.4–94.6)
PPV, %	57.1 (29.2–81.2)	24.4 (18.8–31.2)	24.4 (17.8–32.4)	29.0 (21.0–38.6)
NPV, %	97.4 (86.8–99.5)	99.0 (93.6–99.8)	98.0 (93.3–99.4)	99.0 (93.7–99.8)

NPV, negative predictive value; PPV, positive predictive value.

received radical surgery, postsurgical pathologic analyses identified that only 9% (17 of 188 [46 in the training cohort and 142 in the validation cohort]) of patients were LNP and that 91% of patients underwent unnecessary operations. Our newly established diagnostic signature revealed that only 18% were overtreated, which is dramatically better for identification of LNM.

Several reports have indicated the potential of ESD for diagnosing LNM in patients with T1 CRC[40,41]; however, others suggest its diagnostic accuracy for LNM is still inadequate.[42] Because current clinical guidelines consider the presence of LNM an important risk factor for classifying a patient with T1 CRC as high risk, this highlights the need to develop robust biomarkers for LNM before treatment, which would be clinically transformative in selecting patients who truly require such invasive and radical surgical treatments. Our ability to successfully validate our signature in pretreatment serum samples underscores its clinical significance for improved treatment strategies in patients with T1 CRC, especially the ones who truly have LNM. Our previous studies similarly highlighted the clinical use of pretreatment serum samples for diagnostic purposes in patients with CRC; however, none of the previous studies used these samples directly for diagnosing LNM status, which could have a profound impact in the selection of treatment strategies.[31–33,43] Preoperative application of our transcriptomic biomarkers as a robust, facile, and inexpensive assay will lead to minimized risks from surgical procedures, including perforation or bleeding, and a reduction in the overall health care burden from such expensive surgical procedures.

Our study has some potential limitations because our retrospective study design might result in a potential selection bias. First, owing to the limited sample size (especially the small number of positive cases) in the present study, we evaluated our signature in a moderately sized clinical cohort. Thus, a prospective clinical trial with larger patient cohorts is required to further confirm the diagnostic accuracy of our risk-stratification model.

Second, our study used training and validation cohorts of patients from Japan, who showed similar clinicopathologic characteristics; such characteristics could potentially vary if we were to analyze patient populations from other countries. Therefore, it will be important to validate the selected biomarkers and our risk-stratification model in patient cohorts from other countries to further reinforce the generalizability of our findings.

Finally, we established the risk-stratification model which included miRNAs, mRNAs, and clinical factors. However, previous reports showed that the patients with the consensus molecular subtypes and DNA mutations were related to the risk for LNM.[44,45] Because fewer factors have the potential for an easier clinical application, future studies may need to explore other factors such as consensus molecular subtypes or DNA mutations to evaluate whether these offer additional diagnostic accuracy for LNM detection. Nonetheless, our study provides an important proof for detecting LNM in patients with T1 CRC, and these findings are potentially an important major step toward the availability of robust molecular biomarkers for the risk assessment and management of a lethal malignancy.

Figure 3. Clinical validation of the risk-stratification model in patients with T1 CRC. (*A*) The risk-stratification model, which combines the transcriptomic panel and pathologic risk factors, outperformed detection accuracy of the transcriptomic panel or risk factors alone in serum specimens from validation cohort patients (AUC, 0.90). Forest plot with ORs of clinicopathologic variables, transcriptomic panel, and risk-stratification model in (*B*) univariate and (*C*) multivariate logistic regression analysis in validation cohort patients. (*D*) Currently used pathologic factors led to the overtreatment of 92% patients with T1 CRC (*left panel*). The patients in validation cohort using our transcriptomic classifier divided into high (*yellow*) and low (*light blue*) risk by Youden's index. The pie chart shows LNM status of LNP (*orange*) and LNN (*dark blue*). The transcriptomic panel would have led to the overtreatment of only 22% patients with T1 CRC (*middle panel*), and the risk-stratification model would have led to the overtreatment of only 18% patients with T1 CRC (*right panel*).

Table 3. Univariate and Multivariate Logistic Regression Analysis for Lymph Node Metastasis

	Univariate analysis			Multivariate analysis		
Factors	OR	95% CI	P^a	OR	95% CI	P^a
Training cohort (n = 46)						
Age (≥67 vs <67)	0.22	0.02–2.10	.19			
Sex (male vs female)	0.58	0.09–3.82	.57			
Tumor location (right vs left)	0.39	0.04–3.82	.42			
Tumor size (≥20 mm vs <20 mm)	1.58	0.24–10.44	.64			
Submucosal invasion (≥1000 μm vs <1000 μm)	<0.01		.99			
Budding grade (≥2 vs 1)	3.57	0.42–30.10	.24			
Lymph invasion (positive vs negative)	1.82	0.27–12.38	.54			
Vascular invasion (positive vs negative)	4.65	0.68–31.91	.12			
Transcriptomic panel (high risk vs low risk)	14.22	1.41–143.68	**.025**			
Validation cohort (n = 142)						
Age (≥67 vs <67)	0.97	0.30–3.16	.96			
Sex (male vs female)	1.33	0.38–4.66	.65			
MSI status (MSI-H vs MSI-L, MSS)	1.22	0.14–10.56	.86			
Tumor location (right vs left)	0.81	0.21–3.15	.76			
Tumor size (≥20 mm vs <20 mm)	7.82	0.98–62.35	.05			
Submucosal invasion (≥1000 μm vs <1000 μm)	<0.01		.99			
Budding grade (≥2 vs 1)	3.89	1.08–13.95	**.037**	1.70	0.32–9.06	.53
Lymph invasion (positive vs negative)	3.78	1.08–13.23	**.038**	1.60	0.29–8.69	.59
Vascular invasion (positive vs negative)	0.35	0.07–1.68	.19			
Transcriptomic panel (high risk vs low risk)	15.97	3.32–76.82	**<.001**	8.13	1.43–46.29	**.018**
Risk-stratification model (high risk vs low risk)	37.17	4.48–308.35	**<.001**	17.28	1.82–164.07	**.013**

MSI, microsatellite instability; MSI-H, high-frequency microsatellite instability; MSI-L, low-frequency microsatellite instability; MSS, microsatellite stable

[a]Bold *P* values are statistically significant ($P < .05$).

Conclusion

We have identified and developed a novel risk-stratification model that allows identification of LNM in a liquid biopsy assay for more robust and accurate identification of patients with high-risk T1 CRC. Pending validation in future prospective studies, our findings highlight the potential clinical impact of our model for improved selection of patients with high-risk T1 CRC, which will reduce the overall burden of unnecessary operations and expense associated with these procedures and improve the overall management of patients with this malignancy.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at https://dx.doi.org/10.1053/j.gastro.2021.03.062.

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CRediT Authorship Contributions Q15
Yuma Wada, MD, PhD (Conceptualization: Equal; Data curation: Lead; Formal analysis: Lead; Methodology: Lead; Validation: Lead; Writing – original draft: Lead; Writing – review & editing: Equal). Mitsuo Shimada, MD, PhD (Formal analysis: Supporting; Writing – review & editing: Supporting). Tatsuro Murano, MD, PhD (Resources: Equal; Validation: Supporting; Writing – review & editing: Equal). Hiroyuki Takamaru, MD, PhD (Resources: Equal; Validation: Supporting; Writing – review & editing: Supporting). Yuji Morine, MD, (Data curation: Supporting; Validation: Supporting; Writing – review & editing: Supporting). Tetsuya Ikemoto, MD, PhD (Formal analysis: Supporting; Writing – review & editing: Supporting). Yu Saito, MD (Methodology: Supporting; Writing – review & editing: Supporting). Francesc Balaguer, MD, PhD (Conceptualization: Supporting; Resources: Supporting; Writing – review & editing: Equal). Luis Bujanda, MD (Resources: Supporting; Validation: Supporting; Writing – review & editing: Supporting). Maria Pellise, MD (Resources: Supporting; Writing – review & editing: Supporting). Ken Kato, MD (Resources: Equal). Yutaka Saito, MD, PhD (Resources: Equal; Writing – review & editing: Supporting). Hiroaki Ikematsu, MD (Resources: Equal; Writing – review & editing: Equal). Ajay Goel, PhD (Conceptualization: Lead; Funding acquisition: Lead; Writing – review & editing: Lead).

Conflicts of interest Q3
The authors disclose no conflicts.

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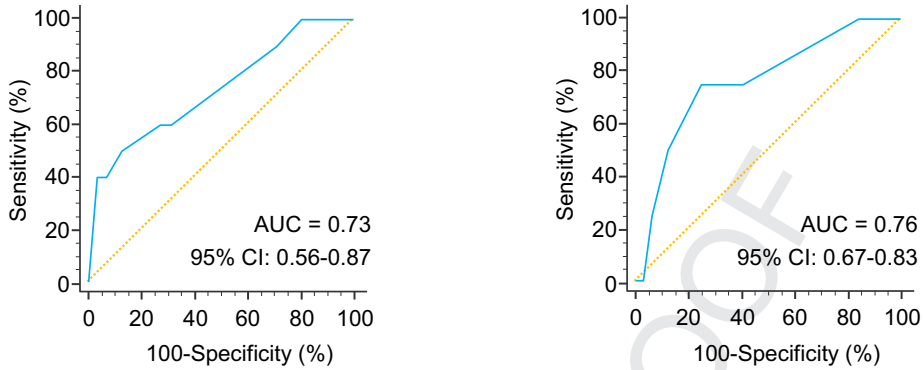

Supplementary Figure 1. Receiver operating characteristic curves (ROCs) for the detection of LNM in the T1 CRC training and validation cohorts. (*A*) ROC curve for combined current clinical risk factors of depth of submucosal invasion (>1000 μm), presence of lymphatic or vascular invasion, high-grade tumor budding, and poorly differentiated histology for LNM without the transcriptomic panel in the training cohort (AUC, 0.73). (*B*) ROC curve for the current clinical risk factors for LNM without the transcriptomic panel in the validation cohort (AUC, 0.76).