ColoGuidePro: A prognostic 7-gene expression signature for stage III colorectal cancer patients

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**Translational Relevance**

Colorectal cancer is a common disease with a high mortality rate. Although molecular markers have great potential for prediction of disease outcome, and thereby for guiding choice of adjuvant chemotherapy, post-operative treatment decisions for stage II and III patients are still based on clinicopathological parameters alone. Here, we present ColoGuidePro, a prognostic test for stage II and III colorectal cancer, which is independent of tumor stage in multivariate models, and holds significant prognostic value for stage III patients separately. The classifier is based on the expression of only seven genes, all carrying independent prognostic information. This, and simple test result interpretations, ensures suitability for clinical use.

Stage III patients routinely receive adjuvant treatment. Accordingly, analysis of treatment independent prognosis for these patients requires a careful study design. ColoGuidePro was developed from a historical, non-treated patient series. Treatment independent prognostic value was shown in patient series treated according to current standards. This has great clinical implications for patients whose general health conditions cause reduced eligibility to chemotherapy.
Abstract

Purpose: Improved prognostic stratification of stage II and III colorectal cancer patients is warranted for post-operative clinical decision making. This study was conducted to develop a clinically feasible and robust prognostic classifier for these patients independent of adjuvant treatment.

Experimental Design: Global gene expression profiles from altogether 387 stage II and III colorectal cancer tissue samples from three independent patient series were included in the study. ColoGuidePro, a 7-gene prognostic classifier, was developed from a selected Norwegian learning series (n = 95; no adjuvant treatment) using lasso-penalized multivariate survival modeling with cross-validation.

Results: The expression signature significantly stratified patients in a consecutive Norwegian test series, where patients were treated according to current standards (hazard ratio = 2.9 [1.1, 7.5]; P = 0.03; n = 77), and an external validation series (hazard ratio = 3.7 [2.0, 6.8]; P < 0.001; n = 215) according to survival. ColoGuidePro was also an independent predictor of prognosis in multivariate models including tumor stage in both series (hazard ratio ≥ 3.1; P ≤ 0.03). In the validation series, which consisted of patients from other populations (USA and Australia), 5-year relapse-free survival was significantly predicted for stage III patients only (P < 0.001; n = 107). Here, prognostic stratification was independent of adjuvant treatment (P = 0.001).

Conclusions: We present ColoGuidePro, a prognostic classifier developed for stage II and III colorectal cancer patients. The test is suitable for transfer to clinical use, and has best prognostic prediction potential for stage III patients.
Introduction

Colorectal cancer (CRC) is the third most common type of cancer with a worldwide annual incidence of 1.2 million, and mortality rate of approximately 50% (1). The only available curative treatment for CRC is complete surgical resection of neoplastic tissue (2). Determination of the extent of the disease by clinicopathological tumor staging is the primary prognostic factor for CRC patients (2). Despite the favorable outcome for patients with localized stage II tumors compared to stage III tumors, more than 20% of stage II patients suffer from recurrence (3). Still, investigations of benefit from adjuvant chemotherapy for stage II patients show conflicting results (4-6), and surgery remains the only recommended treatment modality (7). For patients with stage III disease, large clinical trials have consistently showed improved survival with administration of adjuvant chemotherapy, and this constitutes the standard of care for this group of patients (8). However, the significantly poorer survival among patients with stage IIB (T-stage 4, lymph node negative) compared to stage IIIA (T-stage 1-2, lymph node positive) disease, underlines the need for refinements to this prognostic stratification (9).

Accordingly, identification of individual patients in need of adjuvant treatment, primarily by optimized prediction of prognosis for stage II and III patients remains a major clinical concern. There are currently no markers in routine clinical use for this purpose (10). Hence, identification of molecular markers for improved prognostic stratification represents a valuable step towards beneficial personalized management of patients with stage II or III disease.
Prognostic gene expression signatures have shown predictive value in cancer management, primarily for patients with breast cancer (11-13). Also for CRC, several studies have reported prognostic gene expression signatures, focusing primarily on stage II and III tumors (14-18). However, these studies have generally been limited by small sample sizes and/or lack of testing in independent sample series (19). More recently, larger studies have shown potential prognostic predictive value for gene expression signatures of varying sizes (20-23). However, no studies have considered the abundance of prognostic information contained within the proposed signatures, possibly resulting in unnecessary large signatures and reduced robustness due to increased risk of over-fitting. Furthermore, testing of independent prediction potential has generally been limited to one patient series.

In this study, we aimed to develop a non-redundant prognostic gene signature for stage II and III CRC based on the expression of only few genes using variable selection by L1 (lasso)-penalization and cross-validation in the Cox proportional hazards model (24). This statistical model has not previously been used for the purpose of subclassification of CRC. The study was designed to clinically validate the new classifier in independent patient series across populations and technology platforms, and to provide prognostic stratification independent of adjuvant treatment in stage III patients.
Materials and methods

Material

Altogether 172 fresh frozen stage II and III CRC tissue samples from two independent Norwegian patient series were analyzed in the study. The learning series included 95 samples taken from patients treated surgically at different hospitals in the Oslo region prior to adjuvant chemotherapy becoming standard treatment for stage III patients, to allow for treatment independent prognostic prediction (Table 1). These patients were selected to include approximately equal numbers of stage II and III tumors, as well as equal numbers of survival events between the stages, again to achieve independent information within each stage. Selection was also based on long term follow-up among survivors (>10 years). The independent test series was consecutively collected (95% inclusion rate) and consisted of 77 patients treated by curative resection at one Norwegian hospital, Aker University Hospital, Oslo (Table 1). These patients received adjuvant treatment according to the current standard, i.e. routine administration of chemotherapy for patients presenting with stage III tumors. RNA was extracted from all samples using the Qiagen AllPrep DNA/RNA Mini Kit (Qiagen, BmbH, Hilden, Germany). Microsatellite instability (MSI)-status of the clinical specimens has previously been determined (25, 26).

The research conformed to the Helsinki Declaration and the research biobanks have been registered according to national legislation (numbers 2781 and 236-2005-16141). The study (amendment number 2010/1805) is part of a project approved by the Regional Committee for Medical and Health Research Ethics (numbers 1.2005.1629 and S-09282c 2009/4958), which requires that informed consent is obtained from patients being enrolled in the study.
Additionally, publicly available gene expression data from two independent series of altogether 215 stage II and III CRC patients were accessed from NCBI’s Gene Expression Omnibus (GEO), accession numbers GSE14333 and GSE17538. There was extensive overlap between samples in the two series (n = 97 stage II and III samples from the H. Lee Moffitt Cancer Center, as found by correlation analyses of the probe cell intensity (CEL) files). Only unique samples (n = 215) were included in the current study, and are herein referred to as the validation series. Clinical information for the patients was obtained from the respective GEO entries (Table 1). There were no significant differences in clinicopathological characteristics between the test and validation series, which are the two patient series used to assess the prognostic test developed in the current study, except for patient age (mean difference 6.9 years, P < 0.001; independent samples t-test) and the distribution of tumor location (P = 0.05; Chi-square test).

Details for experimental performance of exon microarray analyses and preprocessing of gene expression data can be found in the Supplementary Material. Parts of the microarray data have previously been published (27) and can be accessed from GEO (GSE30378 and GSE24550). For the current study, twelve additional samples were included in the learning series (GSM753769-GSM753780), and are amended to the GSE30378 record.

**Development of the prognostic gene expression signature**

A gene expression signature for prediction of prognosis was developed from the learning series of 95 CRC samples. The gene expression dataset was filtered (Supplementary Methods) to
include only genes with variances in expression levels higher than 0.2, and \( P \)-values (Wald test of predictive potential) from univariate Cox proportional hazards analysis lower than 0.5 (\( n = 3,098 \) genes). This gene set was subjected to penalized multivariate Cox proportional hazards survival modeling using an algorithm for variable selection based on L1 penalized (lasso) estimation (Supplementary Methods) (24). Using this model, the genes in the prognostic expression signature were selected \textit{via} cross-validation in the learning series. A penalty parameter, \( \lambda_1 \), reflecting the predictive potential and calculated by cross-validation, was inflicted upon the gene expression signals during survival modeling.

Genes in the resulting prognostic expression signature were considered to be associated with poor patient survival at expression levels above the 80\textsuperscript{th} percentile across the dataset for genes with univariate hazard ratios (HR) > 1, and below the 20\textsuperscript{th} percentile for genes with univariate HR < 1. To obtain a simple classification rule, patients were stratified into prognostic groups according to the number of genes in the prognostic signature being expressed at levels associated with poor prognosis. For comparison, sample-wise prognostic indices (PI) were also calculated based on expression values and lasso-penalized multivariate regression coefficients for genes in the signature, and used for prognostic stratification (Supplementary Methods).

Further statistical analyses were done using the SPSS 16.0 software (SPSS Inc, Chicago, IL). These include independent samples t-tests, Chi-square tests, standard univariate and multivariate Cox proportional hazards analyses (estimation of HR and corresponding 95\% confidence intervals (CI)), Wald test for predictive potential, generation of Kaplan-Meier survival plots, and Pearson correlation analysis. Two-sided \( P \)-values \( \leq 0.05 \) were considered significant. For survival
analyses, the end point was relapse-free survival. Relapse or death from CRC was regarded
events, and patients with no events were censored. Generation of the correlation heatmap was
Results

Development of the prognostic gene expression signature

Lasso-penalized multivariate Cox proportional hazards modeling was performed on a filtered genome-wide expression dataset obtained from a learning series of 95 stage II and III CRCs (Supplementary Methods). There were several survival models accommodating optimal penalty conditions from cross-validation (Supplementary Figure S1 and Supplementary Methods). Across 1,000 iterations, seven different gene expression signatures accommodated optimal survival prediction in the learning series more than 50 times each (size range 1 to 12 genes; Figure 1). These different signatures expanded on the same sets of genes, and the smaller signatures were subsets of the larger. For all signatures (except the 1-gene signature), there were significant associations between patient survival and increasing numbers of genes expressed at levels associated with poor survival (HRs ranging from 1.6 to 1.9, \( P < 0.04 \)).

Dichotomization of patients to good and poor prognosis groups was tested for all possible stepwise increases in amounts of genes with associations to poor survival within each signature (Figure 1). For the 28 possible poor prognosis groups, 22 (79%) had significant associations with poor patient survival (univariate Cox proportional hazards analyses, HR ranging from 3.0 to 11.5; \( P < 0.04 \)). To further assess which of the seven signatures had the best predictive potential in independent samples, the signatures were tested also in the test series. Here, five of the dichotomizing stratification rules resulted in significant prognostic stratification (univariate Cox proportional hazards analyses; HRs ranging from 2.9 to 5.8; \( P < 0.04 \)). The best performing stratification rule across both the learning and test series (by rank of \( P \)-values), assigned patients
to a poor prognosis group when expressing (any) three or more genes in the 7-gene signature at levels associated with poor prognosis. This constitutes the ColoGuidePro prognostic test. The genes included in the signature were \textit{OLFM4, CXCL9, DMBT1, UGT2B17, SEMA3A, NT5E}, and \textit{WNT11} (further described in SupplementaryTable S1 and SupplementaryFigure S2). In accordance with the initial selection criteria for genes entered into the lasso algorithm, as well as for the algorithm itself, the seven genes had strong univariate associations with patient survival in the learning series, as well as large variation and low correlation in gene expression (Figure 2).

\textbf{Assessment of the prognostic gene expression signature}

In addition to the learning and test series (\(n = 95\) and 77, respectively), the performance of the 7-gene expression signature was assessed on an external validation series of stage II and III CRC patients (\(n = 215\)). In all three series, the sample-wise increase in amounts of genes with expression levels indicating poor survival (ranging from 0 to 5 of 7 genes), was associated with increasingly poor patient survival (HR \(\geq 1.5, P \leq 0.04\), Wald test for predictive potential; Supplementary Methods).

According to the ColoGuidePro expression signature, all patients were significantly stratified to a poor and good survival group in all three series (Figure 3). In the learning series, 23\% of the patients (22 of 95) were assigned to the poor prognosis group (univariate HR = 4.0; 95\% CI, 2.2 to 7.2, \(P < 0.001\), Wald test of predictive potential). In the test and validation series, 17\% and 16\% of the patients were assigned to the poor prognosis group (13 of 77 patients, and 34 of 215 patients), respectively. Poor prognosis patients in the test series had a 5-year relapse-free survival
rate of 49%, compared to 78% for patients in the good prognosis group (univariate HR = 2.9 [1.1, 7.5]; \( P = 0.03 \)). The corresponding survival rates in the external validation series were 45% and 81%, respectively (univariate HR = 3.7 [2.0, 6.8]; \( P < 0.001 \)).

The validity of the stratification rule used for ColoGuidePro, i.e. assigning patients to a poor prognosis group when expressing three or more of the seven genes at levels associated with poor survival, was supported by evaluating the performance of the sample-wise PI’-s based on expression values and lasso-penalized multivariate regression coefficients for the seven genes (Supplementary Methods). The 20% of the patients with highest PI’-s had markedly poorer survival than the rest of the patients. The univariate HRs for the learning series, test series and validation series were 4.1 [2.2, 7.5], 2.1 [0.8, 5.5], and 2.6 [1.4, 4.7], respectively (\( P \leq 0.001, 0.1, \) and 0.003, respectively).

The gene expression signature was also an independent predictor of poor patient survival in multivariate models including tumor stage and other clinicopathological parameters. Multivariate HRs were 4.1 [2.2, 7.7], 3.2 [1.1, 9.3], and 3.1 [1.6, 5.8], in the learning series (Supplementary Table S2), test series and validation series (Table 2), respectively (\( P = <0.001, 0.03, \) and 0.001, respectively).

By analysis of patients within each tumor stage separately, prognostic stratification according to ColoGuidePro was positively validated only for stage III patients (Figure 4). In the external validation series, 21% (23 of 107) of stage III patients were assigned to the poor prognosis group. These patients had a 5-year relapse-free survival rate of 29%, compared to 76% for patients with
good prognosis (univariate HR = 4.1 [2.0, 8.2], \( P < 0.001 \), Wald test for predictive potential). This survival difference was independent of the administration of adjuvant chemotherapy (multivariate HR = 3.4 [1.7, 7.0], \( P = 0.001 \); Supplementary Figure S3). Importantly, also stage III patients above the age of 75 years (\( n = 26 \) in the validation series), who are less eligible for chemotherapeutic treatment, were correctly classified according to survival by the expression signature (survival rates were 70% and 0% for the good and poor prognosis group, respectively, \( P < 0.001 \); HR = 13.5 [2.5, 72.5]; Supplementary Figure S4). This difference was independent of adjuvant treatment (multivariate HR = 17.1 [2.9, 102.2], \( P = 0.002 \)). For stage II patients in the validation series, only 10% (11 of 108) was predicted to have poor survival, and prognostic stratification of these patients was not significant (univariate HR = 1.6 [0.3, 6.9], \( P = 0.5 \)).
Discussion

In this study, we have developed and validated ColoGuidePro, a 7-gene expression signature for prediction of prognosis for stage II and III CRC patients. This classifier is a significant predictor of poor prognosis, both in the learning series, test series, and an external validation series of patients from different countries and analyzed on different microarray platforms, with best prognostic prediction potential for stage III patients.

Prognostic value was shown in a large number of patients (totally 387 stage II and III patients). To ensure independent prediction value additional to what can be achieved by cross-validation in the learning series alone, selection of the presented 7-gene signature from the seven different signatures most commonly presented as optimal in the learning series, was aided by testing of independent samples in the test series. Accordingly, the test series is not truly independent of the model building. However, prognostic value of ColoGuidePro was shown in an additional large and independent validation series (n = 215).

The expression signature was an independent prognostic predictor in multivariate analyses including tumor stage in all three series. Considering each stage individually in the independent validation series, only stage III patients were stratified according to survival (HR = 4.1). Here, prognostic stratification was independent of the administration of adjuvant chemotherapy. This may be a result of the study design, as ColoGuidePro was developed from stage III patients that were not treated in an adjuvant setting. Although constituting the standard of care for stage III patients today, adjuvant treatment was administered to only approximately half (52%) of stage III
patients in the test series, which is a consecutively collected patient series. This indicates that improved rationale for selecting patients for adjuvant treatment is highly warranted also for this group of patients, strengthening the clinical potential of ColoGuidePro. Due to poorer general health conditions and the toxic side effects of chemotherapeutic treatment, older stage III patients are less commonly treated in an adjuvant setting. In Norway, such treatment is not standard for patients older than 75 years, who are individually assessed for eligibility to chemotherapy (28). Importantly, ColoGuidePro strongly stratified the small subgroup of stage III patients in the external validation series above the age of 75 years (n = 26) according to survival. Similar separation was indicated also in the test and learning series, however, the sample numbers (n = 8 and 14, respectively) were too small for this separation to be significant (data not shown). This provides strong indications that ColoGuidePro can be valuable when deciding on treatment options for older stage III patients. Due to small sample numbers, studies specifically aimed at investigating the potential of ColoGuidePro in this subgroup of patients are needed to conclude.

For stage II patients in the validation series, the results for ColoGuidePro were indiscriminant. This may be a result of the very high survival percentage in this group of patients (5-year relapse-free survival rate of 86%). This may also explain the small percentage of stage II patients in the validation series being assigned to the poor prognosis group by ColoGuidePro (10%). Recently, we have also developed and validated ColoGuideEx, a 13-gene prognostic expression signature specific for stage II CRC (29).

The external validation series used here is a collection of data from two previous studies where gene expression data have been used to develop prognostic classifiers (20, 23), and include the two major datasets with corresponding clinical information that are available from public
repositories. These samples were analyzed on gene-level microarrays (Affymetrix HG-U133 Plus2.0), whereas the two in-house datasets were analyzed by exon-level microarrays (Affymetrix GeneChip Human Exon 1.0 ST). The good performance of the 7-gene expression signature in the test and validation series, analyzed on both types of microarrays, indicates robustness. More reliable expression measures in the learning series, with large numbers of probes targeting each gene across the entire length of the expressed sequences, may have contributed to this.

The prognostic test described here is based on the expression levels of only seven genes. Previously published signatures for stage II and III CRC have typically contained a rather large number of genes, ranging from twenty to several hundred, and there has been little focus on the implications of this during development of the survival models (14-18, 20, 23). A major statistical concern regarding prognostic prediction based on gene expression profiles relates to the high-dimensionality of the data. Over-fitting of large and complex gene expression models to the limited heterogeneity represented within the learning set of tumors, compromises the independent predictive powers. This risk can be reduced by penalization of the gene expression data using parameters tuned during cross-validation (30). In this study, lasso was used for penalization and simultaneous variable selection (24, 31, 32). Recently, two promising prognostic tests based on the expression levels of a small set of genes have been reported. ColoPrint measures the expression levels of 18 genes (22), whereas Oncotype DX includes 12, seven of which are recurrence-risk genes, and five which are reference genes (21). For development of the prognostic test presented here, care has been taken not only to reduce the number of genes, but also to avoid redundancy in prognostic associations between the genes. In accordance with the
lasso model, the seven proposed genes have only weak correlations in expression (median Pearson correlation 0.1). Reduced covariation may improve the independent prognostic potential of each included gene (33).

Prospective testing of prediction models in large clinical trials provides a powerful means for assessment of their prognostic value. Currently, a phase III clinical trial is recruiting patients for assessment of the ColoPrint test in stage II CRC patients (PARSC-study) (34). Prognostic value of this test has recently been validated retrospectively in fresh frozen tissue from an independent series of stage II and III CRC patients analyzed on the same microarray platform as the training series (univariate HR = 2.5; P = 0.005) (22). These data have not been made publically available, and we have accordingly not been able to compare the performance of ColoGuidePro. Oncotype DX, a recurrence predictor for stage II CRC (35), has been developed from analyses of preselected genes (from a literature search) in more than 1,800 patients across four studies (21). This test has the advantage of being available for formalin-fixed, paraffin embedded tumor tissue. Its predictive value has been tested on patients recruited from the QUASAR-study (3), but despite reports of positive results in this initial validation study (36), more evidence is needed for a full evaluation of its value in clinical practice (37). We think it will be of great interest to evaluate both ColoGuidePro and ColoGuideEx together with these promising signatures in the same prospective study.

Clinically useful prognostic tests should not require much resources in terms of expression measures and subsequent interpretation of results (38, 39). The seven genes included in ColoGuidePro have high variances in expression signals, improving the reliability of differential
expression measures. Also, stratification of patients is based on the simple principle of summarizing the number of genes with expression levels outside a threshold. This strategy resulted in similar prognostic stratification when comparing with computation of PI’-s based on multivariate regression coefficients estimated as an inherent part of the lasso survival model. This indicates that the simple stratification rule proposed here is a valid replacement for a commonly used, but more complex mathematical model. Hence, testing the performance of the proposed 7-gene expression signature should be possible for independent researchers, and we welcome such efforts.

In conclusion, we have developed and validated a clinically feasible prognostic test for stage II and III CRC, although with best prognostic prediction potential for stage III patients. This classifier is based on the expression levels of a non-redundant set of seven genes, and can be transferred to a standardized assay for validation on individual patients in a prospective study.
Grant Support

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Table 1. Characteristics of the three colorectal cancer sample series

<table>
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<tr>
<th>Characteristic</th>
<th>Learning series (n = 95)</th>
<th>Test series (n = 77)</th>
<th>Validation series(a) (n = 215)</th>
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<tbody>
<tr>
<td>Age at diagnosis (mean ± SD)</td>
<td>66 ± 11.7</td>
<td>73 ± 13.5</td>
<td>66 ± 13.3</td>
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<tr>
<td>Gender</td>
<td></td>
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<tr>
<td>Male</td>
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<td>33</td>
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</tr>
<tr>
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</tr>
<tr>
<td>III</td>
<td>43</td>
<td>33</td>
<td>107</td>
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<tr>
<td>Location</td>
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<td>Right</td>
<td>27</td>
<td>46</td>
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</tr>
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<td>77</td>
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<tr>
<td>Rectum</td>
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<td>11</td>
<td>22</td>
</tr>
<tr>
<td>Unknown</td>
<td></td>
<td></td>
<td>31</td>
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<tr>
<td>Mean follow-up, years (min; max)</td>
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<td></td>
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<tr>
<td>All patients</td>
<td>5.9 (0.3; 10)(b)</td>
<td>3.3 (0.2; 5)(c)</td>
<td>3.1 (0.04; 5.0)(c)</td>
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<tr>
<td>Patients with event</td>
<td>1.8 (0.3; 7.7)(b)</td>
<td>2.1 (0.7; 3.8)(c)</td>
<td>1.5 (0.1; 3.4)(c)</td>
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<tr>
<td>Patients with no event</td>
<td>10 (10; 10)(b)</td>
<td>3.7 (0.2; 5.0)(c)</td>
<td>3.5 (0.04; 5.0)(c)</td>
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<td>No. of events, stage II(d)</td>
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<td>13</td>
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<td>No. of events, stage III(d)</td>
<td>27</td>
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<tr>
<td>MSI-high</td>
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<td>Adjuvant chemotherapy, stage III</td>
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<tr>
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<td>ColoGuidePro</td>
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<tr>
<td>Positive predictions (true; false)</td>
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<td>6; 7</td>
<td>16; 18</td>
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<td>Negative predictions (true; false)</td>
<td>45; 28</td>
<td>51; 13</td>
<td>152; 29</td>
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GEO accession numbers GSE14333 and GSE17538. Only non-overlapping samples from stage II and III patients were included. Ten years follow-up. Five years follow-up. Relapse or death from CRC. Abbreviations: MSI, microsatellite instability; NA, not available; SD, standard deviation.
Table 2. Prognostic stratification of stage II and III colorectal cancer patients by clinical parameters and the 7-gene expression classifier ColoGuidePro

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Test series</th>
<th>Validation seriesa</th>
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<tr>
<td></td>
<td>Univariate</td>
<td>Multivariate</td>
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<tr>
<td></td>
<td>HRb [95% CI]</td>
<td>P-valuec</td>
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<td>Gender (male vs. female)</td>
<td>1.3 [0.5, 3.3]</td>
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<td>Age at diagnosis (≥70 years vs. &lt;70)</td>
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<td>Tumor stage (III vs. II)</td>
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<td>Tumor location (right vs. left and rectum)</td>
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<td>Adjuvant chemotherapy (yes vs. no)</td>
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</tr>
<tr>
<td>ColoGuidePro</td>
<td>2.9 [1.1, 7.5]</td>
<td>0.03</td>
</tr>
</tbody>
</table>

\(^a\)GEO accession numbers GSE14333 and GSE17538 (n = 215)\(^b\)HRs and corresponding 95% CI’s from univariate or multivariate Cox proportional hazards analysis as indicated. Event = relapse or death from CRC within five years. Censoring = no event or lost to follow-up within five years\(^c\)P-values from Wald test of predictive potential. Abbreviations: CI, confidence interval; HR, hazard ratio; MSI, microsatellite instability; NA, not available
Figure Legends

Figure 1. Workflow for development of a prognostic gene expression signature. From the learning series of 95 stage II and III CRC samples, a filtered gene expression dataset was used as input for survival modeling. From 1,000 iterations of lasso penalized multivariate modeling, seven models were reported as optimal for survival prediction more than 50 times (middle bar plot). For each of the gene expression signatures, patients were dichotomized to good and poor prognosis groups according to all the possible stepwise increases in amounts of genes being expressed at levels associated with poor prognosis (lower panel). All 28 possible stratifications (pale and dark blue boxes in heatmap) were tested for univariate associations with patient survival, yielding significant associations for 22 (dark blue). HR, hazard ratio

Figure 2. Statistical characteristics of genes in the identified prognostic expression signature. The seven genes in the expression signature (blue) had A) low \( P \)-values from univariate Cox proportional hazards analyses (median \( P = 0.02 \), Wald test for predictive potential), and B) high variances in gene expression signals (median 2.4, log2-scale), compared to the remaining 3,091 genes included for survival modeling (grey; median \( P = 0.3 \); median variance 0.3). C) The Pearson correlations of expression signals between the seven genes in the signature were generally weak (absolute values ranging from 0.006 to 0.55).
Figure 3. Survival curves for stage II and III CRC patients in the three independent series stratified by the 7-gene expression signature. A) Patients in the learning series assigned to the poor prognosis group had a 10-year relapse-free survival rate of 9%, significantly poorer than the 62% survival rate for patients in the good prognosis group. The corresponding 5-year survival rates for patients in B) the test series and C) external validation series, were 49% compared to 78%, and 45% compared to 81%, respectively. All survival curves were calculated by Kaplan-Meier statistics and compared by log-rank tests. Relapse or death from CRC within the ten or five years of follow up was regarded events, and patients with no events within the indicated period of follow-up were censored.

Figure 4. Survival curves in each stage for patients in the three independent series. Stage II (left) and III (right) CRC patients were individually stratified according to the 7-gene expression signature. In the learning series, A) 19% of stage II patients (HR = 6.6 [2.7, 16.1]) and B) 28% of stage III patients (HR = 2.6 [1.2, 5.7]) were assigned to the poor prognosis group. In the test series, C) 16% of stage II patients (HR = 3.3 [0.8, 13.3]) and D) 18% of stage III patients (HR = 2.3 [0.6 to 8.8]) were assigned to the poor prognosis group. In the external validation series, only E) 10% of stage II patients were assigned to the poor prognosis group (HR = 1.6 [0.3, 6.9]), compared to F) 21% of stage III patients (HR = 4.1 [2.0, 8.2]). All survival curves were calculated by Kaplan-Meier statistics and compared by log-rank tests. Relapse or death from CRC within the ten or five years of follow up was regarded events, and patients with no events within the indicated period of follow-up were censored. HR, hazard ratio.
Figure 1

3,098 genes
(variance > 0.2; P-value univariate Cox < 0.5)

Lasso-penalized multivariate Cox proportional hazards analyses
(1,000 iterations)

Analysis iterations

No. of genes in optimal expression signature

Poor prognosis:
Expression < 60th percentile (for genes with HR > 1)
Expression < 20th percentile (for genes with HR < 1)

Prognostic stratification of patients in the learning series (n = 95):
Patients assigned to poor prognosis groups according to the amounts of genes with expression levels associated with poor prognosis:

1-gene signature
2-gene signature
7-gene signature
8-gene signature
9-gene signature
11-gene signature
12-gene signature

Univariate Cox proportional hazards analyses:
- $P < 0.05$
- $P > 0.05$
- Not applicable
Figure 2
Figure 3

A Relapse-free survival (learning series, n = 95)

B Relapse-free survival (test series, n = 77)

C Relapse-free survival (validation series, n = 215)
Figure 4

**Stage II**

**Learning series**

A. Relapse-free survival

- 74%; n = 42
- P < 0.001

B. Relapse-free survival

- 45%; n = 31
- P = 0.02

**Test series**

C. Relapse-free survival

- 80%; n = 37
- P = 0.07

D. Relapse-free survival

- 73%; n = 27
- P = 0.2

**Validation series**

E. Relapse-free survival

- 87%; n = 97
- P = 0.5

F. Relapse-free survival

- 76%; n = 84
- P < 0.001

- 29%; n = 23
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