Original Article

Development and validation of a novel miRNA classifier as a prognostic signature for stage II/III colorectal cancer

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Background: The TNM staging remains the gold standard for determining the prognosis of patients with colorectal cancer (CRC), which is inadequate at identifying the subset of high-risk stage II and III patients that have a high potential of developing tumor recurrence and may experience death. Emerging evidence indicates that not only microRNAs (miRNAs) play important functional role in CRC development but may serve as important disease biomarkers. In this study we aimed to develop a miRNA-based classifier as a prognostic signature for improving the clinical outcome of patients with stage II/III CRC.

Methods: We performed a systematic and comprehensive discovery step to identify differentially expressed miRNAs in CRC. We subsequently determined the prognostic relevance of these miRNAs in stage II/III patients using qRT-PCR and developed a miRNA-based classifier for predicting disease-free survival (DFS) in a clinical cohort (n=186).

Results: Based upon miRNA expression profiling studies, we identified a panel of 10 miRNAs which are consistently differentially expressed in CRC *vs.* normal tissues. By using cox proportional hazard models, we then developed 6-miRNA-classifier (miR-183, -20a, -21, -195, -139 and -20a) to predict prognosis in clinical cohort, that had significantly superior predictive performance compared to other clinicopathological factors, and could successfully identify high-risk stage II and III CRC patients with poor prognosis [hazard ratio (HR) =2.16; P=0.0048]. In a multivariate analysis, this miRNA-based classifier emerged as an independent prognostic signature for poor DFS.

Conclusions: Our miRNA-based classifier is a reliable predictive tool for determining prognosis in patents with stage II/III CRC, and might be able to identify high-risk patients that are candidates for more targeted personalized clinical management and surveillance.

Keywords: MicroRNA (miRNA); colorectal cancer (CRC); biomarker; prognosis

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Introduction

Colorectal cancer (CRC) is currently the third most common cancer worldwide, with more than one million new cases diagnosed annually. The outcomes of CRC patients in early and late stages are drastically different, with the 5-year survival rates of ~93% for stage I disease and a dismal 8% for stage IV patients. Although 60% of CRC patients with (stages I–III) present with a resectable disease at the time of diagnosis, approximately 40–50% of such patients who undergo curative surgery or another 20–30% that are post-surgically treated with adjuvant chemotherapy, eventually relapse and experience a metastatic disease and

eventual death (1-3). This clinical challenge highlights the limitation that the current golden standard of Tumor, Node, Metastasis (TNM)-based classification is inadequate at identifying the risk for tumor recurrence, which leads to potential under or over-treatment of a subset of patients with CRC.

At present, post-surgery, 5-fluorouracil (5FU)-based chemotherapy remains the standard of care treatment for some high-risk patients with stage II disease, and all patients with stage III CRC, as it helps improve survival rates by 10–20% (4,5). For stage II patients who present with specific high-risk clinical features, including advanced T stage, low differentiation grade, tumor perforation and few examined lymph nodes, are generally offered 5FU-based adjuvant treatment. Among these, ~20% of stage II patients that are deemed low-risk clinically, experience tumor relapse. On the other hand, for stage III patients, 30–40% of patients do not show any evidence for tumor recurrence in 5 years even when left untreated, while ~40% patients that receive adjuvant treatment still experience tumor recurrence and eventually die, highlighting the need for more intensive chemotherapy or the potential use of novel targeted therapies (6,7). Taken together, these data underscore the need for identification of novel and robust prognostic biomarkers that can better guide treatment decisions in CRC patients with stage II and III disease.

Although in the recent years several studies have reported potential gene-expression based prognostic biomarkers for stage II/III patients, their adoption and routine use in the clinics have been hampered due to the need for high specimen quality and the lack of consensus and difficulties with analytical approaches. In this regard, microRNAs (miRNAs) have recently emerged as promising substrates for development of prognostic biomarkers in cancers, including CRC. MiRNAs are short (18–22 nt in length) and evolutionarily conserved non-coding RNAs. Compared to mRNAs or proteins, miRNAs are relatively immune to degradation by RNAses, and hence be readily detected and accurately quantified in a variety of clinical specimens including fresh frozen and formalin-fixed paraffin-embedded (FFPE) tissues. Additionally, miRNAs have emerged as key frontiers in gene regulation due to their ability to regulate a broad range of biological processes in various human diseases, particularly cancer[8]. We and others have previously highlighted that specific miRNAs may contribute to CRC pathogenesis, and many of these may serve as biomarkers for diagnosis, prognosis and metastasis-prediction in CRC patients (8-11).

However, since the clinical usefulness of miRNAs in predicting the prognosis of stage II/III CRC patients remains unclear, we envisaged the present study to address this important gap in knowledge. Accordingly, we performed a systematic and comprehensive identification of CRC-specific miRNAs that are differentially expressed (DE) in stage II/III CRCs, followed by determining their combinatorial efficiency in predicting disease free survival by analyzing their expression in multiple, independent cohorts of patients with CRC.

We present the following article in accordance with the STROBE reporting checklist (available at http://dx.doi.org/10.21037/atm-20-1751).

Methods

Patients and tumor specimens

Clinical specimens analyzed for the miRNA classifier (n=186) were obtained from patients enrolled between 2008 and 2012 at the Shanghai Tenth People's Hospital, Shanghai, China. Patients were staged according to the American Joint Committee on Cancer (AJCC) staging guidelines. Detailed patient information is listed in *Table 1*. All CRC patients were followed up for survival for at least up to 5 years after surgery. Patients treated with radiotherapy or chemotherapy before surgery were excluded from this study. Personal or family history of polyposis or Lynch syndrome, personal history of inflammatory bowel disease, R1 or R2 resections (microscopic or macroscopic neoplastic involvement of surgical margins, respectively), and cases with lack of available FFPE tissues were also excluded from this study. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013) and was approved by the institutional review boards of Shanghai Tenth People's Hospital, School of Medicine, Tongji University (ID: KN84-01). Informed consent was taken from all the patients.

Biomarker screening phase

We performed an initial biomarker discovery phase wherein we performed an extensive published literature survey for all miRNA expression profiling studies published between 2006 and 2014. We included studies that exclusively performed direct comparison for miRNA expression profiles between normal and CRC tissues. MiRNAs were ranked according to several criteria as described previously (12):

Table 1 Clinicopathological characteristics of patients in the clinical validation cohort

Variables	Clinical cohort			
	Number of patients	Low risk	High risk	P
Age, years				0.079
>71	94	53	41	
≤71	92	40	52	
Gender				0.086
Female	96	49	47	
Male	90	44	46	
Tumor location				0.003**
Proximal	50	16	34	
Distal	136	77	59	
Tumor size				0.380
Small	94	44	50	
Large	92	49	43	
Lymph node metastasis				0.092
Negative	118	60	58	
Positive	68	33	35	
Histological type				0.057
Well/moderate	158	109	49	
Poor	27	18	9	
Serum CEA				0.380
Low	84	42	42	
High	84	46	38	

Pearson chi-squared testing was used to compare the correlation between triple-miRNA based classifier and clinical variables. **, P<0.01. CEA, carcinoembryonic antigen.

(I) each miRNA was consistently reported as DE; (II) the direction of expression change (up- or down-regulation) was consistent across all studies; (III) the frequency of miRNA expression alteration was consistent and reported in multiple studies.

Quantitative MiRNA expression analysis

The miRNA expression analysis was performed using QuantStudio6 Flex Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). All miRNA TaqMan probes were purchased from Ambion (Austin, TX, USA). The qRT-PCR assays were conducted using TaqMan MicroRNA Reverse Transcription Kit and TaqMan Universal PCR Master Mix kit (Applied Biosystems, Foster City, CA, USA) according to manufacturer's instructions. The relative expression of miRNA was determined by $2^{-\Delta\Delta Ct}$ method using miR-16 as a normalizer, as described previously (9,13-15).

Statistical analysis

All statistical analyses were performed using Medcalc version 12.3, SPSS version 13.0 or GraphPad Prism version 6.0. We conducted receiver operating characteristic (ROC) curves and calculated the area under the ROC curves (AUC) to evaluate the predictive power of candidate miRNAs for prognosticating CRC patients. For the disease-free

survival (DFS) analysis, we defined the probability that patients remained free of tumor recurrence or death as the first event. Data were analyzed from the date of surgery to the time of the first event or the date on which data were censored, according to the Kaplan-Meier method, and the curves were compared using the log-rank test. To develop a miRNA panel and determining patient survival, we used Cox's proportional hazard regression models and obtained a risk score derived from this prediction model. We categorized patients into high-risk and low-risk group based on median cutoff value. Furthermore, we calculated estimate hazard ratios (HRs) for each miRNA, clinic-pathological variables and combination model, based on univariate and multivariate Cox proportional hazard regression models. All data were expressed as mean ± standard deviation (SD). We compared two groups using the two-sided χ^2 test for categorical variables. All P values were two-sided, and those less than 0.05 were considered statistically significant.

Results

Systematic discovery and identification of potential miRNAs for prognosis prediction in stage II and III CRC patients

During the past decade, several miRNAs with a prognostic potential for CRC patients have been identified, but majority of them have failed to validate across different studies. In order to avoid bias in selection of prognostic candidate miRNAs reported in previous studies, we initially performed a comprehensive and systematic literature review to identify most frequently and consistently reported miRNAs that are DE between CRC and normal tissues (*Figure 1*). Performing an exhaustive search of miRNA profiling studies, we identified a panel of 60 up-regulated and 41 down-regulated miRNAs that showed consistent data and were reported in at least 3 independent studies (Table S1). In order to narrow down this list further, we thereafter selected DE-miRNAs consistently reported in ≥10 studies and gathered 10 miRNAs (miR-20a, -31, -183, -182, -21, -17, -145, -139, -195 and -215) which were significantly DE in CRCs, implicating their important role in the development of this disease, and their potential relevance in determining the clinical outcome of stage II/III CRC patients.

Development of a prognostic miRNA classifier to predict survival in stage II/III patients

We subsequently enrolled a clinical cohort of 186 patients to determine the optimal miRNA combinations for survival prediction. Accordingly, we measured expression level of each miRNA in CRC tissues, and the expression data was normalized by Z score transformation, allowing the comparison of different cohort independent of the original signal intensities. Cox proportional hazard models were used to build a prognostic classifier. Of note, we adopted the back-step elimination algorithm to exclude non-significant confounders and thereafter identified miR-183, miR-21, miR-20a, miR-139 and miR-195 was the optimal combination for survival prediction. We then derived a formula to calculate the risk score for their risk of disease recurrence for every patient based on their individual six miRNA expression levels, where the risk score = (–1.2681 × miR-139) + (–0.8916 × miR-145) + (0.7084 × miR-183) + (1.4509 × miR-195) + (0.9662 × miR-20a) + (–1.5493 × miR-21).

Performance evaluation of the miRNA-classifier in a clinical cohort of stage II/III CRC patients

We performed ROC analysis to evaluate the prediction accuracy of individual miRNAs and 6-miRNA classifier between DFS and recurrence/death. As shown in *Table 2*, our 6-miRNA-classifier (AUC: 0.705) significantly improved prediction ability of individual miRNA (AUC range, 0.530–0.643). Furthermore, compared to known clinicopathological risk factors, such as poor differentiation, lymph node metastasis, and tumor location, our newly developed miRNA-based classifier revealed the highest predictive accuracy (AUC =0.715; *Figure 1B*).

When we assessed the distribution of each patient's survival status and risk scores generated by this miRNA-based classifier, patients with lower risk scores showed better outcomes *vs.* those with higher risk scores (*Figure 1C*), highlighting its high predictive accuracy (*Figure 2A*). Based on cutoff value (the median value of all patients' risk scores), we divided patients into high-risk group and low-risk group and noted that high-risk group had worse prognosis compared to patients in the low-risk group (HR =2.16, P=0.0048; *Figure 2B*). Furthermore, we observed high risk scores have strong tendency in association with proximal tumor (P=0.003), lymph node metastasis (P=0.092) and poor differentiation (P=0.057, *Table 1*). In the univariate analysis, this 6-miRNA-classifier emerged as the strongest predictor of DFS (HR =2.1604, P=0.0059) compared to other clinicopathological variables such as serum CEA (HR =1.8134, P=0.0384), lymph node metastasis (HR =1.5021, P=0.1227) and tumor differentiation (HR =1.0408,

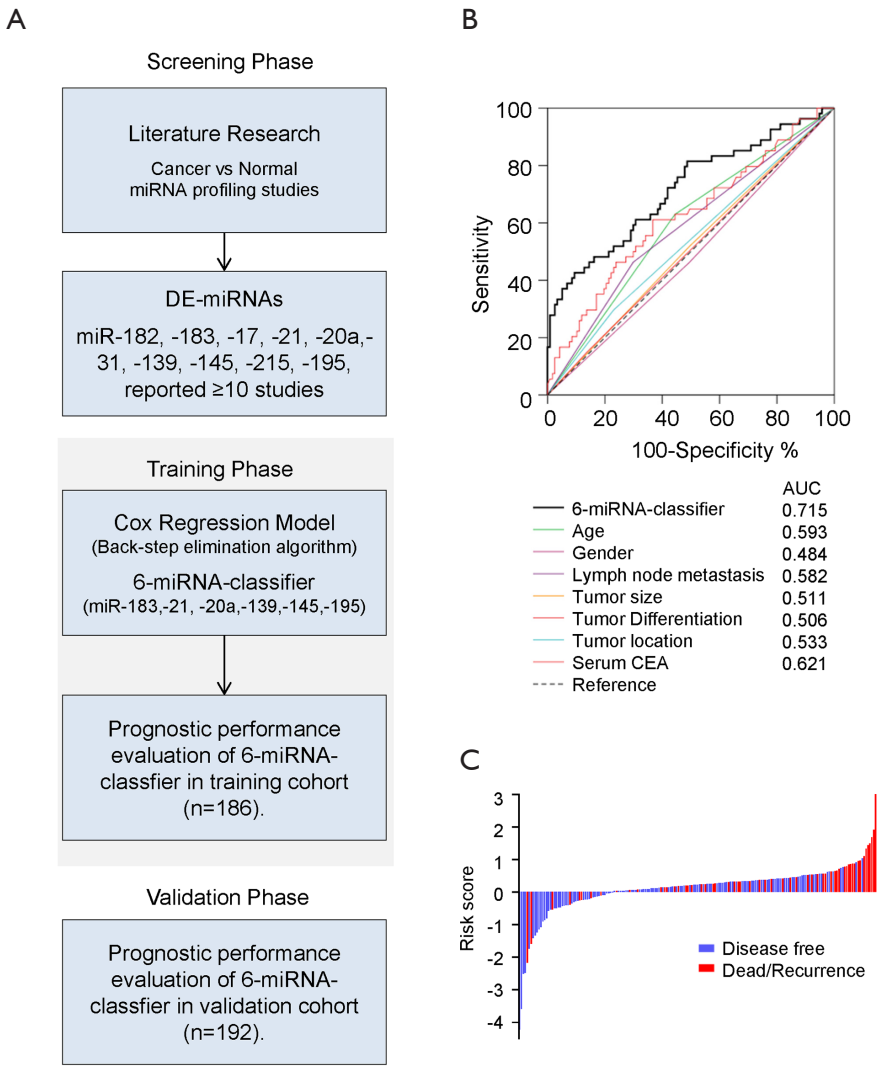

Figure 1 Overview of the study design and receiver operating characteristic (ROC) curves for the comparison of the prognostic accuracy of miRNA-classifier and clinicopathological variables in clinical cohort. (A) The overview of the study design. We performed a systematic and comprehensive discovery step to identify differentially expressed miRNAs in colorectal cancer (CRC). We subsequently determined the prognostic relevance of these miRNAs in stage II/III patients using qRT-PCR and developed a miRNA-based classifier for predicting disease-free survival (DFS) in a clinical cohort (n=186), which was later validated in an independent cohort (n=192). (B) The ROC analysis was used for the discrimination between disease free and recurrence or death cases. (C) The distribution of each patient's risk scores and survival status (recurrence or death). AUC, area under curve; DE, differentially expressed.

P=0.9125). Consistently, multivariate analysis revealed that this 6-miRNA classifier was an independent prognostic factor in stage II/III CRC patients (HR =2.5727, P=0.0015, *Table 3*).

When stratified by tumor stage, this miRNA-classifier still demonstrated clinically and statistically significant predictive power. As depicted in *Figure 2B,C*, the AUC of miRNA-classifier is 0.73 and 0.67 in stage II and stage III respectively. In consistent, stage II patients with higher *vs.* lower risk scores had poor prognosis (HR =2.09, P=0.049). When we analyzed the subset of stage III patients separately, the 6-miRNA-classifier also showed to be a highly predictive prognostic indicator, wherein patients in the high-risk group were more likely to have a poor outcome

Table 2 The area under a ROC curve (AUC) of individual miRNAs and 6-miRNA classifier for disease free survival analysis in the clinical validation cohort

MiRNAs	Clinical cohort		
	AUC	SE	95% CI
miR-139	0.643	0.0465	0.570–0.712
miR-145	0.585	0.0490	0.511–0.657
miR-183	0.625	0.0437	0.551–0.695
miR-195	0.575	0.0502	0.501–0.647
miR-20a	0.626	0.0447	0.553–0.696
miR-21	0.530	0.0456	0.455–0.603
miR-17	0.608	0.0460	0.533–0.678
miR-182	0.633	0.0438	0.560–0.703
miR-215	0.542	0.0473	0.467–0.615
miR-31	0.559	0.0460	0.485–0.632
6-miRNA classifier	0.705	0.0429	0.634–0.770

ROC, receiver operating characteristic; SE, standard error; CI, confidence interval.

vs. those with low-risk (HR =2.26, P=0.041; *Figure 2D*). Notably, the high-risk stage II group yielded similar survival curves as stage III patients (HR =0.91, P=0.7463; *Figure 2E*), suggesting our classifier is able to identify high risk stage II group which has same prognosis as stage III group. Collectively, these results indicate that our newly developed 6-miRNA-classifier could successfully segregate high *vs.* low-risk patients with stage II/III disease. Which highlighting that our 6-miRNA based classifier is indeed a promising and reliable prognostic tool for identifying high-risk stage II and stage III patients, which has important implications for their clinical management.

Discussion

In this study, we have firstly performed a systematic discovery step, followed by development and validation of a novel prognostic tool based on a miRNA-classifier aimed at improving the predictive potential for the clinical outcomes of stage II/III CRC patients following surgery. Based upon a logical discovery, clinical validation steps, we provide data that our triple-miRNA based classifier was able to successfully discriminate high *vs.* low-risk CRC patients with a better predictive performance compared to the currently used TNM classification based clinicopathological variables used for determining therapeutic decision-making in stage II/III patients with CRC.

Although several studies have recently suggested that assessment of gene or protein expression changes may be used for prognostication of stage II/III CRC patients, methodological standardization including tissue handling, RNA or DNA processing, and lack of stability of these analytes have hampered the adoption of these biomarkers in routine clinical settings. In contrast, measurement of expression alterations of miRNAs offers several distinct advantages for their clinical use as biomarkers as these short non-coding RNA genes are highly stable in a variety of clinical specimens, have important functional role in regulating gene expression of key cancer-related genes, and their expression can be very accurately measured using simple PCR-based analytical tools. In view of these salient features of miRNAs, in this study we aimed to develop a miRNA-based predictive model for improved prognostication of stage II/III CRC patients.

In order to identify prognosis-related miRNAs for stage II/III CRC patients, we first selected robustly and DE miRNAs between cancer and normal tissues. We hypothesized that aberrant expression of these miRNAs may directly correlate with prognosis in stage II/III patients. Based upon a discovery step involving systematic literature review for miRNA expression profiling studies, we identified several DE miRNAs such as miR-145 (16-18), miR-21 (19,20), miR-17 (21) and miR-20a (22). We subsequently measured expression level of 10 candidate miRNAs, which

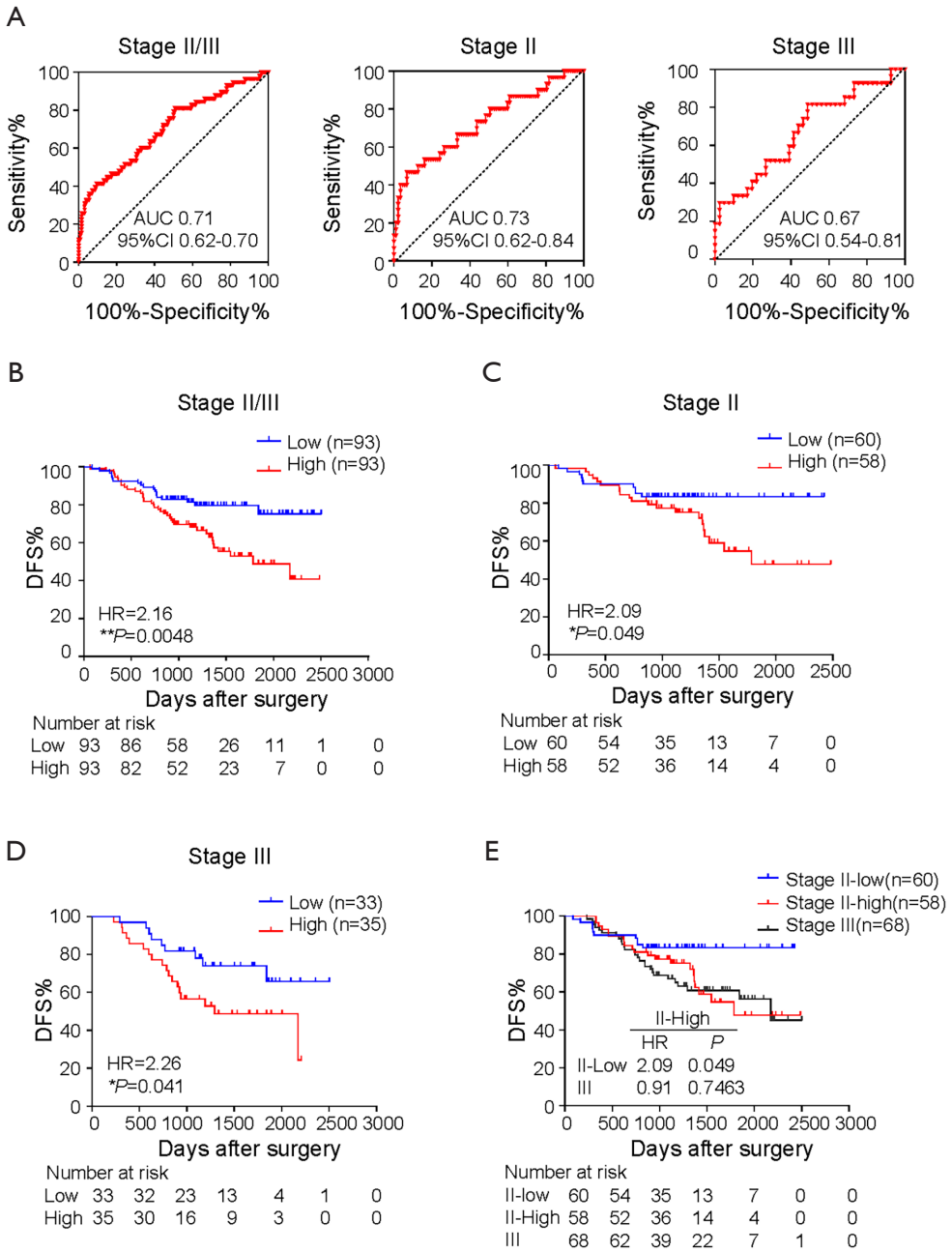

Figure 2 The prediction performance of 6-miRNA-classifier in the clinical cohort. (A) The predictive power of 6-miRNA-classifier was demonstrated in stage II/III patients by ROC analysis. (B,C,D,E) All the patients were divided into low and high-risk group based on risk scores calculated from 6-miRNA-classifier. The Kaplan-Meier analysis was used to estimate the prognosis of low and high-risk group in stage II and stage III. *, P<0.05; **, P<0.01. HR, hazard ratio; ROC, receiver operating characteristic; AUC, area under curve; DFS, disease-free survival.

were reported to be differentially altered in colorectal *vs.* normal tissues at least 10 or more studies. We thereafter derived predictive models by using Cox's regression model, and identified a 6-miRNA-classifier consisting of miR-183, miR-145, miR-20a, miR-21, miR-195 and miR-139, which was significantly superior in its prognostic accuracy

Table 3 Univariate and multivariate association for the 6-miRNA-classifier and other clinicopathological characteristics with disease-free survival

Clinical cohort	Univariate analysis			Multivariate analysis		
	HR	95% CI	P	HR	95% CI	P
Age (>71 *vs.* ≤71 years)	1.6310	0.9636–2.7604	0.0684	–	–	–
Gender (female *vs.* male)	0.8628	0.5139–1.4484	0.5765	–	–	–
Tumor location (proximal *vs.* distal)	1.3471	0.7782–2.3316	0.2872	–	–	–
Tumor size (large *vs.* small)	1.0200	0.9000–1.1560	0.7568	–	–	–
Lymph node metastasis (pos *vs.* neg)	1.5021	0.8961–2.5179	0.1227	–	–	–
Differentiation (poor *vs.* well/mod)	1.0408	0.5104–2.1224	0.9125	–	–	–
Serum CEA (high *vs.* low)	1.8134	1.0324–3.1853	0.0384*	1.9074	1.0854–3.3519	0.0248*
6-miRNA-classifier (high *vs.* low)	2.1604	1.2479–3.7401	0.0059**	2.5727	1.4333–4.6179	0.0015**

*, P<0.05; **, P<0.01. CEA, carcinoembryonic antigen; HR, hazard ratio; CI, confidence interval.

compared to the expression of individual miRNAs.

The biological function of these identified miRNAs selected for our classifier has been investigated previously. MiR-183 is a member of miR-183 cluster, which is comprised of miR-183, -182 and 96. The miR-183 family is reported to be highly expressed in CRC and exerts its oncogenic activity via inhibition of several tumor suppressor genes (23). Furthermore, miR-183 was reported to be associated with poor prognosis in CRC patients (24). MiR-195, is a tumor suppressor, since its overexpression results in the inhibition of proliferation and metastasis in various cancers (25-28). MiR-195 is downregulated in CRC and its reduced expression associates with poor prognosis (29,30). MiR-139 was shown to be down-regulated in a stage-dependent manner in CRC, and regulates the expression of several oncogenes such as NOTCH1 (31), IGF1R (32,33), MAPK, NF-κB, and STAT3 (34). MiR-145 (35,36) and miR-21 (37) are well-known miRNAs which function as tumor suppressor and onco-miR in CRC. MiR-20a was also reported to promote tumor development through suppression of GABBR1 (38). Considering the functional role as well as the clinical significance of these miRNAs in the development of cancer, it is rational to evaluate their expression in a miR-classifier for predicting prognosis of CRC patients.

With regards to potential limitations, our current study is retrospective in nature, and our results must be validated in future, prospective, multi-center clinical trials. In addition, some of the clinical parameters such as vascular invasion or number of analyzed lymph nodes were not consistently recorded or evaluated in our retrospective cohorts, which may be easier to address in a future well-defined patient cohort.

In conclusion, we provide compelling evidence that our newly developed miRNA-based prognostic classifier tool can effectively stratify patients with stage II/III CRCs into high and low risk groups based upon clinical outcomes, thereby adding significant prognostic value to the currently used clinicopathological risk factors used for such purposes. If validated in future studies, such a miRNA classifier potentially offers tremendous clinical value in directing personalized treatment regimens and clinical management of patients with stage II/III CRC.

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Footnote

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Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013) and was approved by the institutional review boards of Shanghai Tenth People's Hospital, School of Medicine, Tongji University (ID: KN84-01). Informed consent was taken from all the patients.

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