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ORIGINAL ARTICLE

miR-150 as a potential biomarker associated with prognosis and therapeutic outcome in colorectal cancer

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The full trial protocol can be accessed from Dr HQ and via email tohl-qin@hotmail.com

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ABSTRACT

Background MicroRNAs (miRNA) have potential as prognostic biomarkers and therapeutic targets in cancer. A study was undertaken to investigate the association between miRNA expression patterns and the prognosis and therapeutic outcome of colorectal cancer (CRC).

Methods miRNA expression profiling in tumour, adenoma and normal colorectal tissues was performed to identify tumour-related miRNAs in the course of colorectal malignant changes. Quantitative reverse transcription PCR (qRT-PCR) assays were used to measure tumour-related miRNA and to assess its association with survival and response to adjuvant chemotherapy in 239 patients. In addition, to validate the findings, associations of the tumour-related miRNA with clinical characteristics of CRC were analysed in 185 patients by in situ hybridisation (ISH) analysis.

Results Only one miR-150 was found to show a decrease in expression levels in the three tissue groups (normal, adenoma and cancer tissue) in parallel with increasing carcinogenesis of the colorectal tissue. In both ISH and qRT-PCR analysis, tumour tissue had reduced levels of miR-150 expression compared with paired non-cancerous tissue, which indicated that the levels of miR-150 expression were associated with CRC. Moreover, patients whose tumours had low miR-150 expression had shorter survival and a worse response to adjuvant chemotherapy than patients whose tumours had high miRNA expression.

Conclusions The miR-150 expression status of patients with CRC is associated with survival and response to adjuvant chemotherapy. It is suggested that miR-150 should be considered as a potential biomarker associated with the prognosis and therapeutic outcome in CRC.

Significance of this study

What is already known about this subject?

- MicroRNAs (miRNA) have potential as prognostic biomarkers and therapeutic targets in cancer.

What are the new findings?

- A new potential biomarker, miR-150, was found to be helpful in diagnosing and predicting survival prognosis including response to therapy of colorectal cancer (CRC) based on miRNA profile screening and clinical confirmation.
- Tumours with low expression of miR-150 were associated with poor survival outcome and an unfavourable response to adjuvant chemotherapy in patients with CRC, independent of other clinical covariates.

How might it impact on clinical practice in the foreseeable future?

- miR-150 could be considered as a potential biomarker that is associated with the prognosis and therapeutic outcome in CRC.

INTRODUCTION

Colorectal cancer (CRC) is one of the most common malignancies in the Western world.[1] Up to 90% of patients can be cured by surgery if the disease is detected at an early stage, but unfortunately it is often diagnosed only at an advanced stage and the prognosis is therefore poor.[2 3] Chemotherapy may have significant therapeutic value in some patients. However, the response is often not satisfactory because of the limited ability to identify patients who are more likely to benefit from targeted adjuvant therapies.[4 5] There is an urgent need to find key carcinogenesis-associated molecules to stratify patients with respect to prognosis and response to therapy.

miRNAs are small endogenous non-coding RNAs that bind to partially complementary recognition sequences of target mRNAs, causing either degradation or preventing their translation.[6 7] Accumulating evidence has shown that miRNAs have crucial functions in specific cellular processes such as differentiation, morphogenesis and tumorigenesis.[6 8–11] In human cancer, miRNAs can function as oncogenes or tumour suppressor genes during tumorigenesis.[12] Gene expression profiling studies have demonstrated that miRNA expression is an excellent biomarker associated with specific tumour subtypes and clinical outcomes.[10 13 14] However, it is unclear which miRNAs as potential therapeutic and prognostic biomarkers in CRC are associated with or regulate tumour progression.

Given the prognostic and therapeutic potential for miRNAs as biomarkers in CRC, we evaluated differentially expressed miRNAs from adenoma, cancer and normal colorectal tissues to study their

potential roles in tumour formation, prognosis and response to chemotherapy in CRC. miR-150, which has shown decreasing expression levels in normal colorectal, adenoma and carcinoma tissues in parallel with increasing carcinogenesis of the colorectal tissue, was chosen for further validation and analysis.

METHODS

Clinical specimen collection

Fresh colorectal tissue samples were obtained from eight patients with CRC who underwent surgical resections at the Department of Surgery, The Six People's Hospital Affiliated to Shanghai Jiao Tong University from 2008 to 2009. Eight adenoma colorectal tissues were obtained from the Department of Digestive Endoscopy and samples of normal colorectal tissue were obtained from eight disease-free donors. Paraffin-embedded CRC specimens and surrounding non-tumour tissue obtained from 239 patients who underwent surgical resections from 2001 to 2007 were analysed by quantitative reverse transcription PCR (qRT-PCR) (validation cohort 1) and paraffin-embedded CRC specimens and surrounding non-tumour tissue from 185 independent patients who underwent surgical resections from 2003 to 2007 were analysed by in situ hybridisation (ISH) (validation cohort 2). Cases with familial adenomatous polyposis or human non-polyposis CRC were excluded from the study. The final date of follow-up was 30 April 2010 for the cases. A summary of the clinical characteristics of these patients is shown in table 1 in the online supplement.

The experimental methods used (including statistical analysis) are described in detail in the online supplement. The project was approved by the institutional review board of the Sixth People's Hospital Affiliated to Shanghai Jiao Tong University, China. Written informed consent was obtained from all patients and tissue sample donors, and anonymity was maintained by tracing patients through their clinical history number.

RESULTS

Search for potential miRNAs in the course of development of colorectal malignancy

Adenomas represent a precursor stage of CRC. To search for potential miRNAs in the course of the development of colorectal malignancy, we globally analysed the miRNA expression profiles of normal colorectal tissues, colorectal adenoma tissues and CRC tissues using μParaflo miRNA microarray assay (LC Sciences, Houston, Texas, USA). A comparison of miRNA expression levels in the three groups is shown in table 2 in the online supplement. We focused on only one miRNA (miR-150) because its expression decreased with the transition from normal mucosa via adenoma to CRC in parallel with increasing carcinogenesis of the colorectal tissue (figure 1A). miR-150 was therefore chosen for further validation and analysis.

Cancer miRNA array (QuantiMir System, System Biosciences, Mountain View, California, USA) was also used to calculate significant differences in the relative abundance of miRNAs in

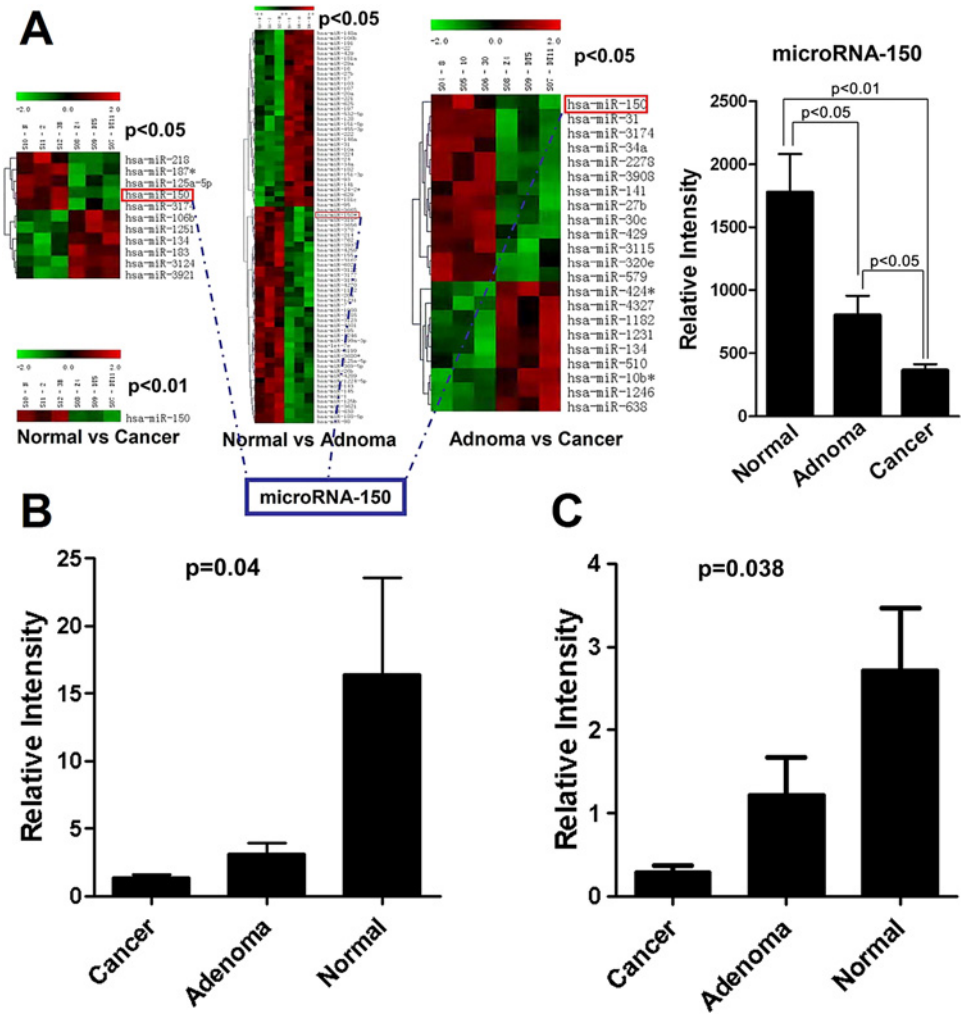

Figure 1 Expression levels of miR-150 in colorectal cancer (CRC), adenoma and normal colorectal tissue. (A) μParaflo miRNA microarray assay and (B) cancer miRNA array assay showed that the expression of miR-150 levels in normal colorectal tissue, adenoma tissue and CRC tissue decreased with the progression of carcinogenesis from normal colorectal tissue to adenoma tissue to CRC ($p<0.05$, one-way ANOVA). (C) A validation experiment was carried out using quantitative reverse transcription PCR (qRT-PCR) which also showed that the expression of miR-150 decreased with the transition from normal mucosa via adenoma tissue to CRC tissue ($p=0.038$, one-way ANOVA analysis).

normal colorectal tissue, colorectal adenoma tissue and CRC tissue and the same expression pattern of miR-150 was found (p=0.04, one-way ANOVA analysis; figure 1B).

To confirm the changes in expression of miR-150 in CRC, validation experiments were carried out by qRT-PCR and a similar expression pattern was confirmed (p=0.038, one-way ANOVA analysis; figure 1C).

Low miR-150 expression and prognosis

We reasoned that miR-150 might act as a tumour suppressor gene and, if so, the silencing of miR-150 would be a frequent event in tumours. We therefore measured miR-150 expression by qRT-PCR in tumour and non-tumour tissues obtained from 239 patients with CRC. Analysis showed that there was a significant reduction in miR-150 expression in tumour tissue compared with non-tumour tissue in patients with low levels of miR-150 expression (p<0.001) but not in those with high levels of miR-150 expression (p=0.07; figure 2A).

To analyse the association of miR-150 expression with clinicopathological characteristics in patients with CRC, we used median tumour:non-tumour (T:N) expression ratios to dichotomise CRC cases where low miR-150 expression was classified as the lower 50th percentile and high miR-150 expression was classified as the upper 50th percentile. This high–low cut-off was used universally throughout the study. The median factor change in miR-150 expression in tumours compared with non-tumours was 6.85 in patients with high levels of miR-150 expression and 0.11 in patients with low levels of miR-150 expression, which suggests that silencing of miR-150 expression is associated only with low miR-150 expression. Using the high–low cut-off, we found that low T:N expression ratios for miR-150 were associated with reduced survival (p=0.042, Kaplan–Meier test, figure 2B). This association could be due to miR-150 expression levels in the tumour tissue, the surrounding non-tumour tissue, or a combination of the two. To investigate these possibilities, we analysed the association of miR-150 expression in tumours and paired non-tumour tissues separately. Low miR-150 expression levels in tumours (based on the upper 50th percentile) were associated with reduced survival (p=0.027, Kaplan–Meier test, figure 2C). No significant association between survival and miR-150 expression was observed in non-tumour tissue (p=0.167, Kaplan–Meier test, figure 2D).

To determine whether the prognostic value of miR-150 expression was independent of other risk factors associated with the clinical outcome of CRC, multivariate analysis was performed using the Cox proportional hazard model. The risk variables examined included miR-150 expression levels, age of patients, differentiation, lymph node metastasis, surgical-pathological staging, tumour location, history of schistosomiasis and tumour size. These factors are generally known to significantly affect the outcome of CRC. In univariate analysis, low expression of miR-150 in tumours (HR 0.52; 95% CI 0.31 to 0.85; p=0.009),

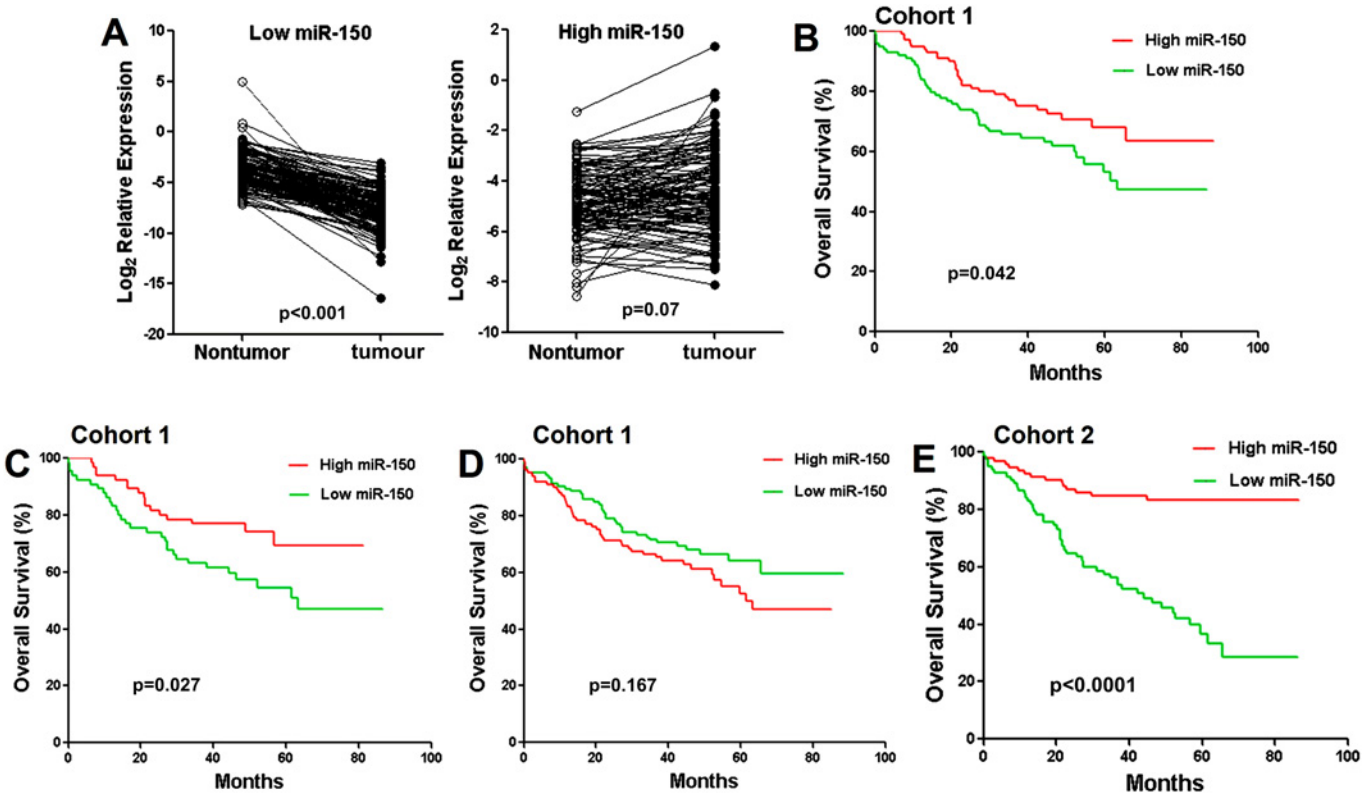

Figure 2 Association between miR-150 expression in tumours and overall survival in 239 patients with colorectal cancer (CRC) using quantitative reverse transcription PCR (qRT-PCR) (cohort 1) and in 185 patients with CRC using in situ hybridisation (cohort 2). (A) Relative levels of miR-150 expression in paired tumour and non-tumour samples from patients in cohort 1 according to miR-150 status using paired t tests. Data are $\log_2$ relative expression levels normalised to values in disease-free samples from eight control subjects. (B) Expression levels of miR-150 were measured by qRT-PCR. High expression is based on the upper 50th percentile. Using the high–low cut-off, low tumour:non-tumour (T:N) expression ratios for miR-150 were associated with reduced survival (p=0.042, Kaplan–Meier test) in cohort 1. (C) Low expression levels in tumours (based on the upper 50th percentile) for miR-150 were associated with reduced survival in cohort 1 (p=0.027, Kaplan–Meier test). (D) No significant association between miR-150 expression and survival in non-tumour tissue was observed in cohort 1 (p=0.167, Kaplan–Meier test). (E) Low miR-150 expression in tumours was significantly associated with shorter survival in CRC patients in cohort 2 (p=0.007, Kaplan–Meier test).

TNM staging (HR 2.64; 95% CI 1.69 to 4.13; p<0.001) and lymph node metastasis (HR 2.57; 95% CI 1.65 to 3.99; p<0.001) were significantly associated with survival, while age, gender, tumour location, differentiation, history of schistosomiasis and tumour size were not (see table 3 in online supplement). In the final multivariate Cox regression model including miR-150 expression, TNM staging, lymph node metastasis, distant metastasis, tumour size, age, gender, differentiation, tumour location and history of schistosomiasis, low miR-150 expression in tumours was associated with a poor survival prognosis (HR 0.57; 95% CI 0.33 to 0.97; p=0.037) independent of other clinical covariates (see table 3 in online supplement).

Expression of miR-150 in colorectal epithelial cells

Although we found that low expression of miR-150 in tumours was associated with an adverse survival outcome, these experiments did not identify the cells within a tumour that expressed miR-150. To identify these cells we used ISH to visualise miR-150 expression in tumour and corresponding non-tumour tissue. miR-150 is expressed at lower levels in colorectal epithelial cells in human tumour tissue compared with normal tissue (figure 3), which is consistent with a role for miR-150 underexpression within tumour cells during colorectal carcinogenesis.

To further validate the association between miR-150 expression and prognosis, we also detected miR-150 expression by ISH analysis in tumour and non-tumour tissues obtained from patients in cohort 2. Consistent with cohort 1, miR-150 expression was more abundant in non-tumour tissue but a significant reduction was observed in tumour tissue. Moreover, reduced miR-150 expression in tumour tissue was significantly associated with shorter survival (p<0.001, Kaplan–Meier test, figure 2E). In univariate analysis, low expression of miR-150 in tumour tissue (HR 0.10; 95% CI 0.05 to 0.19; p<0.001), TNM staging (HR 2.88; 95% CI 1.74 to 4.76; p<0.001) and lymph node metastasis (HR 2.88; 95% CI 1.75 to 4.74; p<0.001) were significantly associated with survival, while age, gender, tumour location, differentiation, history of schistosomiasis and tumour size were not. In the final multivariate Cox regression model including miR-150 expression, TNM staging, lymph node metastasis, distant metastasis, tumour size, age, gender, differentiation, tumour location and history of schistosomiasis, low miR-150 expression in tumour tissue was associated with a poor survival prognosis (HR 0.11; 95% CI 0.06 to 0.22; p<0.001) independent of other clinical covariates (see table 3 in online supplement). The results were also consistent with those of cohort 1.

Expression levels of miR-150 and therapeutic outcome

Analysis of the response to adjuvant therapy includes only patients with TNM stage II and III disease because those with TNM stage I have an excellent survival prognosis regardless of

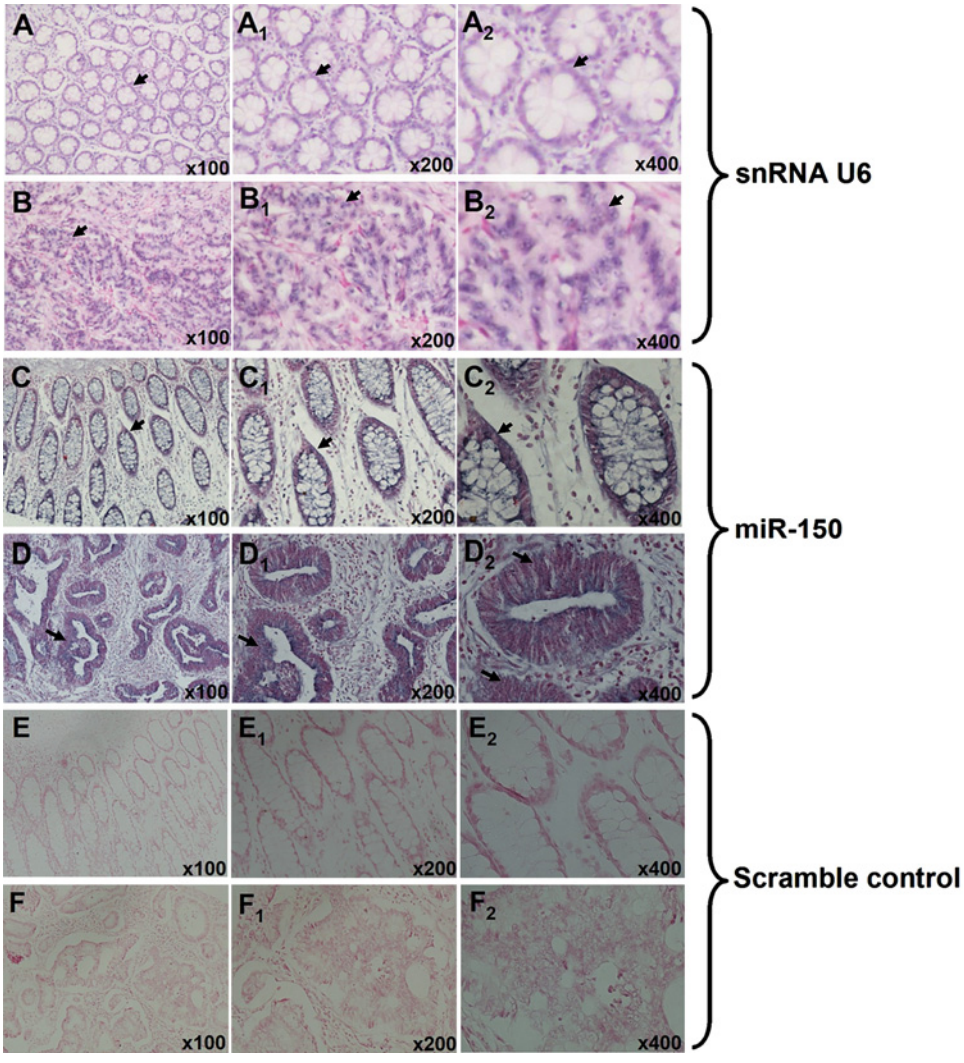

Figure 3 Expression of miR-150 in colorectal epithelial cells using in situ hybridisation (ISH) analysis. Tissue sections were incubated with a full length DIG-labelled LNA probe to miR-150. The positive staining of epithelium cells was expressed as blue-violet. The snRNA U6 ISH signal is mainly expressed in the cytoplasm and nucleus in all colonic epithelial cells of (A) normal colorectal tissue and (B) colorectal cancer tissue. Higher magnifications show that colonic epithelial cells in normal (A_1/A_2) and tumour tissues (B_1/B_2) express significant amounts of miR-150. (C) Colonic epithelial cells in human tumour tissue express lower levels of miR-150 than adjacent non-tumour tissue. Higher magnifications show that colonic epithelial cells in tumour tissue express no significant amounts of miR-150 (C_1/C_2). (D/D_1/D_2) Non-tumour tissue shows significant expression of miR-150 at the same magnification. The scramble control probe shows no significant staining at low or high magnification in non-tumour (E/E_1/E_2) and tumour (F/F_1/F_2) tissue, as expected.

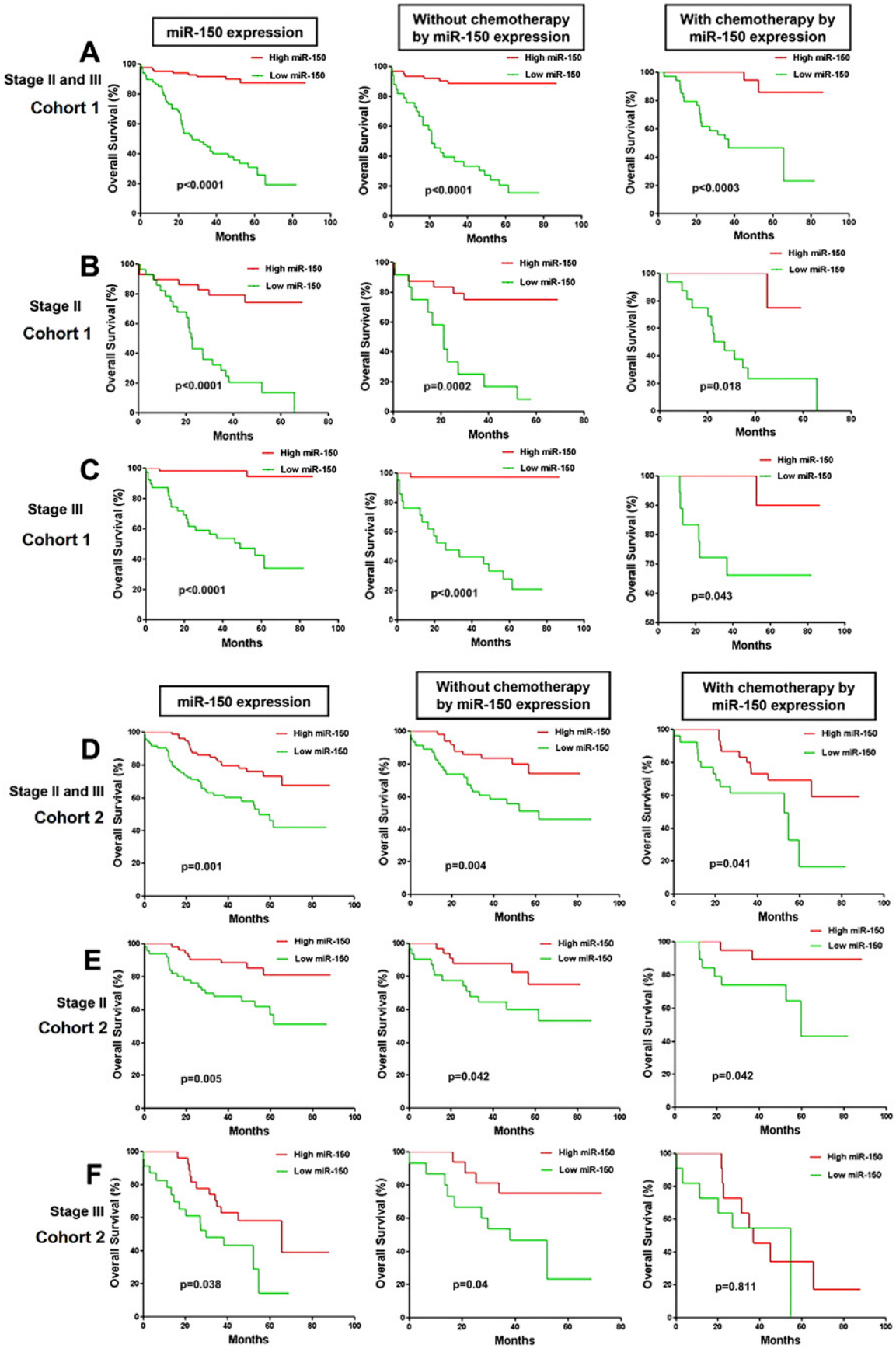

Figure 4 Association of miR-150 expression levels with chemotherapy outcome in patients with TNM stage II or III colorectal cancer (CRC) using quantitative reverse transcription PCR (cohort 1) and in situ hybridisation (cohort 2). (A) For the 195 patients with stage II or III CRC in cohort 1, low miR-150 expression was associated with poor survival for those who received chemotherapy (p=0.041, Kaplan–Meier log rank test). (B) Among the 126 patients with stage II CRC in cohort 1, low miR-150 expression was significantly associated with poor survival among those who received

treatment, and treatment of patients with stage IV disease is palliative. We analysed associations between miR-150 expression and therapeutic outcomes in patients with stage II and III CRC treated with adjuvant chemotherapy in cohort 1. The chemotherapy regimens were primarily fluorouracil-based, with or without leucovorin, levamisole or cisplatin. Kaplan–Meier analysis demonstrated that low miR-150 expression was associated with a worse prognosis in patients with either stage II (p=0.005) or stage III (p=0.038) CRC in cohort 1 (figure 4), further indicating its potential as a prognostic biomarker. For individuals who received adjuvant therapy, low miR-150 expression was associated with a poor therapeutic outcome in patients with stage II or III CRC (p=0.041, Kaplan–Meier log rank) or in those with stage II cancer alone in cohort 2 (p=0.042, Kaplan–Meier log rank; figure 4). Multivariate Cox regression showed that low miR-150 expression predicted a worse prognosis (HR 0.48; 95% CI 0.25 to 0.95; p=0.034) and treatment with adjuvant chemotherapy was also associated with worse survival (HR 0.44; 95% CI 0.20 to 0.93; p=0.032) independent of other clinical covariates in cohort 1 (see table 4 in online supplement). These results were also validated in cohort 2. Kaplan–Meier analysis showed that low miR-150 expression was associated with a worse prognosis in patients with stage II (p<0.001) or stage III disease (p<0.001) in cohort 2 (figure 4). For patients who received adjuvant therapy, low miR-150 expression was associated with a poor therapeutic outcome in patients with stage II or III cancer (p=0.0003, Kaplan–Meier log rank), or in patients with stage II cancer alone (p=0.018, Kaplan–Meier log rank) or those with stage III cancer alone in cohort 2 (p=0.043, Kaplan–Meier log rank; figure 4). Multivariate Cox regression showed that low miR-150 expression predicted worse prognosis (HR 0.18; 95% CI 0.05 to 0.25; p<0.001) and treatment with adjuvant chemotherapy was also associated with worse survival (HR 0.38; 95% CI 0.19 to 0.79; p=0.009), independent of other clinical covariates in cohort 2 (see table 4 in online supplement). Therefore, miR-150 expression emerged as an independent predictor of the response to adjuvant chemotherapy.

DISCUSSION

CRC remains one of the leading causes of death, so finding new molecular targets for its diagnosis, prognosis and treatment has the potential to improve the clinical strategy and outcome of this disease.[2 15] In this study we compared the miRNA profiles in CRC, adenoma and normal colorectal tissues using two screening methods (μParaflo miRNA microarray and cancer miRNA array). Only miR-150 showed decreased expression levels in normal colorectal tissue, adenoma tissue and CRC tissue in parallel with increasing carcinogenesis of the colorectal tissue. The expression pattern of miR-150 was also validated in two cohorts, which suggests that predictable and systematic changes in miRNA expression patterns may occur during tumorigenesis and may be representative of sporadic CRC.

The expression of miR-150 was significantly downregulated in tumour samples compared with paired samples of non-tumour tissue, although it is uncertain whether these changes in the miR-150 expression pattern were merely associated with CRC or were causal to the histological progression to cancer. Our data are consistent with published studies that provide evidence for changes in miR-150 expression promoting tumour formation. The miR-150 gene is on chromosome 19q13 and expressed at low levels in most solid tumours.[16 17] Downregulation of miR-150 expression acts as an anti-apoptotic factor in human diffuse gastric cancer[16] and adrenocorticotrophic hormone-secreting pituitary tumours.[17] Overexpression of miR-150 inhibits tumour cell growth in vitro and inhibits tumour growth in animal models through direct downregulation of DKC1 and AKT2, reduction of phosphorylated AKTser473/4 and an increase in tumour suppressors such as Bim and p53, leading to telomerase activation and immortalisation of cancer cells.[18] Chang *et al* showed that miR-150 is downregulated by c-Myc and that it may function as a tumour suppressor. Injection of mouse lymphoma cell lines into mice expressing miR-150 produced fewer tumour cells in vivo.[19] Taken together, these findings also support a causal role for changes in miR-150 expression during tumorigenesis.

Adenomas represent a precursor stage of carcinoma. They express low levels of miR-150. If decreased miR-150 expression promotes progression to CRC, decreased expression in adenomas may be an early cellular event in tumour development. Promoting miR-150 activity may help to prevent tumour development in populations at high risk of CRC, such as individuals with familial adenomatous polyposis.[20]

In this study we also demonstrated an association between miR-150 expression levels and the prognosis or therapeutic outcome of CRC. A robust association between low miR-150 expression in tumours and poor survival was confirmed in 239 patients with CRC by qRT-PCR (cohort 1) and in 185 patients with CRC using ISH analysis (cohort 2). In both cohorts the association was independent of other clinical covariates, indicating that miR-150 expression may be a useful prognosis biomarker to help identify patients at higher risk of terminal CRC. Moreover, we demonstrated that low miR-150 expression in tumours was associated with an unfavourable response to adjuvant chemotherapy in both cohorts. This association might help to predict the likely success of treatment in individuals whose miR-150 expression status is known and to identify patients who are candidates for more aggressive initial therapies. Thus, low expression of miR-150 in tumours precedes the progression, therapeutic response and subsequent death due to cancer. All of these are consistent with a role for miR-150 in colorectal carcinogenesis and progression.

In conclusion, a potential biomarker, miR-150, was found to be helpful for diagnosing and predicting survival prognosis including response to treatment of CRC, based on miRNA profile screening and clinical confirmation. Tumours with a low expression of miR-150 are associated with poor survival and an

(Continued)

chemotherapy (p=0.042, Kaplan–Meier log rank test). (C) For all 69 patients with TNM stage III CRC in cohort 1, the association between high miR-150 expression and prognosis was not statistically significant in patients who received chemotherapy (p=0.811, Kaplan–Meier log rank test). (A) For the 157 patients with stage II or III CRC in cohort 2, low miR-150 expression was associated with poor survival for those who received chemotherapy (p=0.0003, Kaplan–Meier log rank test). (B) Among the 99 patients with stage II CRC in cohort 2, low miR-150 expression was significantly associated with poor survival among those who received chemotherapy (p=0.018, Kaplan–Meier log rank test). (C) For all 58 patients with TNM stage III CRC in cohort 2, low miR-150 expression was significantly associated with poor survival among those who received chemotherapy (p=0.043, Kaplan–Meier log rank test).

unfavourable response to adjuvant chemotherapy in patients with CRC, independent of other clinical covariates.

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Competing interests None.

Patient consent Obtained.

Ethics approval The institutional review board of the Sixth People's Hospital Affiliated to Shanghai Jiao Tong University, China.

Contributors HQ was the principal investigator and supervised the implementation of the study. YM and PZ wrote the protocols and, with FW and JY, analysed the data and interpreted the findings. HZ, JP and WL were responsible for the collection of clinical data. YM and HQ gave input into the study implementation and contributed to data monitoring. All authors had full access to the primary data and the final analysis and all have seen and approved the final version of the manuscript.

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