HHS Public Access

Author manuscript

Gut. Author manuscript; available in PMC 2017 June 01.

Published in final edited form as:

Gut. 2016 June ; 65(6): 977–989. doi:10.1136/gutjnl-2015-309372.

THE CLINICAL AND BIOLOGICAL SIGNIFICANCE OF MIR-224 EXPRESSION IN COLORECTAL CANCER METASTASIS

Hui Ling[#1], **Karen Pickard**[#2], **Cristina Ivan**[#3], **Claudio Isella**[4,5], **Mariko Ikuo**[1,6], **Richard Mitter**[7], **Riccardo Spizzo**[1,8], **Marc Bullock**[1,2], **Cornelia Braicu**[9], **Valentina Pileczki**[9], **Kimberly Vincent**[1], **Martin Pichler**[1,10], **Verena Stiegelbauer**[10], **Gerald Hoefler**[11], **Maria I. Almeida**[1,12], **Annie Hsiao**[1], **Xinna Zhang**[3], **John Primrose**[2,13], **Graham Packham**[2], **Kevin Liu**[1], **Krishna Bojja**[1], **Roberta Gafà**[14], **Lianchun Xiao**[15], **Simona Rossi**[1], **Jian H. Song**[16], **Ivan Vannini**[17], **Francesca Fanini**[17], **Scott Kopetz**[18], **Patrick Zweidler-McKay**[19], **Xuemei Wang**[15], **Calin Ionescu**[20,21], **Alexandru Irimie**[22], **Muller Fabbri**[23,17], **Giovanni Lanza**[14], **Stanley R. Hamilton**[24], **Ioana Berindan-Neagoe**[9,25], **Enzo Medico**[4,5], **Alex Mirnezami**[#2,13], **George A. Calin**[#1,3], and **Milena S. Nicoloso**[#1,8]

[1]Department of Experimental Therapeutics, The University of Texas MD Anderson Cancer Center, Houston, TX, USA

[2]Cancer Research UK Centre, University of Southampton Cancer Sciences Division, Somers Cancer Research Building, Southampton University Hospital NHS Trust, Tremona road, Southampton, SO16 6YD, UK

[3]Center for RNA Interference and Non-Coding RNAs, The University of Texas MD Anderson Cancer Center, Houston, TX, USA

[4]University of Torino, Department of Oncology, Torino, Italy

[5]IRCC, Institute for Cancer Research and Treatment, Candiolo, Torino, Italy

[6]Laboratory of Microbiology, Graduate School of Pharmaceutical Sciences, University of Tokyo, Tokyo, Japan

[7]Bioinformatics Unit, London Research Institute, Cancer Research UK, London, UK

[8]Division of Experimental Oncology B, CRO, National Cancer Institute, Aviano, Italy

[9]Department of Functional Genomics, The Oncology Institute, Cluj-Napoca, Romania

Correspondence to: **George A. Calin**, MD, PhD, Department of Experimental Therapeutics, The University of Texas MD Anderson Cancer Center, Houston, TX 77054, USA; ; Email: gcalin@mdanderson.org; phone: +1-713-792-5461; **Milena S. Nicoloso**, MD, Division of Experimental Oncology B, CRO, National Cancer Institute, Aviano, Italy; ; Email: mnicoloso@cro.it; phone: +39-0434-659820; **Alex Mirnezami**, MD, PhD, Cancer Research UK Centre, University of Southampton Cancer Sciences Division, Somers Cancer Research Building, Southampton University Hospital NHS Trust, Tremona road, Southampton, SO16 6YD, UK; ; Email: A.H.Mirnezami@soton.ac.uk; +44-23-8079-5170..

COMPETING INTERESTS

We declare that we have no competing interests.

Contributors

AM, GAC, and MSN designed the study; MSN, HL, KP, MI, RS, MB, CB, VP, MIA, KV, VX, AH, XZ, KL, KB, JHS, SK, PZ-M, and IB-N performed the wet-lab experiments; JP, GP, RG, IV, MP, GH, FF, MF, AI, C. Ionescu, GL, SRH, IB-N, and AM obtained samples and clinical data; C. Ivan, RM, LX, HL, and XW did statistical analysis; C. Ivan, C. Isella and EM performed TCGA analysis; HL, KP, GAC, AM and MSN did data analysis and interpretation; HL, KP, MI and MSN write the initial draft; HL, GAC, MSN, AM, MB, C. Isella and EM revised the report. All authors reviewed this report and approved the final version.

[10]Division of Oncology, Medical University of Graz, Austria

[11]Institute of Pathology, Medical University of Graz, Austria

[12]INEB, Instituto de Engenharia Biomedica, University of Porto, Porto, Portugal

[13]Department of Surgery, Southampton University Hospital NHS Trust, Tremona road, Southampton, SO16 6YD, UK

[14]Section of Pathology and Molecular Diagnostics, University of Ferrara, Ferrara, Italy

[15]Division of Quantitative Science, The University of Texas M.D. Anderson Cancer Center, Houston, TX, USA

[16]Department of Genitourinary Medical Oncology, The University of Texas MD Anderson Cancer Center, Houston, TX, USA

[17]Istituto Scientifico Romagnolo per lo Studio e la Cura dei Tumori (IRST) s.r.l., IRCCS, Gene Therapy Unit, Meldola (FC), Italy

[18]Department of Gastrointestinal Medical Oncology, The University of Texas MD Anderson Cancer Center, Houston, TX, USA

[19]Department of Pediatrics, The University of Texas MD Anderson Cancer Center, Houston, TX, USA

[20]Surgical Clinic 1, Cluj County Hospital, Romania

[21]UMF Surgery Department 1, Cluj-Napoca, Romania

[22]Department of Surgical and Gynecology Oncology, University of Medicine and Pharmacy Iuliu Hatieganu, Cluj-Napoca, Romania

[23]Departments of Pediatrics, and Molecular Microbiology and Immunology, University of Southern California, Keck School of Medicine, The Saban Research Institute, Children's Center for Cancer and Blood Diseases, Children's Hospital Los Angeles, Los Angeles, CA, USA

[24]Department of Pathology, The University of Texas MD Anderson Cancer Center, Houston, TX, USA

[25]Research Center for Functional Genomics, Biomedicine and Translational Medicine, University of Medicine and Pharmacy Iuliu Hatieganu, Cluj-Napoca, Romania

[#] These authors contributed equally to this work.

Abstract

Objective—MicroRNA (miRNA) expression profile can be used as prognostic marker for human cancers. We aim to explore the significance of miRNAs in colorectal cancer (CRC) metastasis.

Design—We performed miRNA microarrays using primary CRC tissues from patients with and without metastasis, and validated selected candidates in 85 CRC samples by qRT-PCR. We tested metastatic activity of selected miRNAs, and identified miRNA targets by prediction algorithms, qRT-PCR, western blot and luciferase assays. Clinical outcomes were analyzed in six sets of CRC cases (n=449) including The Cancer Genome Atlas consortium and correlated with miR-224

status. We used the Kaplan-Meier method and log-rank test to assess the difference in survival between patients with low or high levels of miR-224 expression.

Results—MiR-224 expression increases consistently with tumor burden and microsatellite stable (MSS) status, and miR-224 enhances CRC metastasis *in vitro* and *in vivo*. We identified SMAD4 as a miR-224 target, and observed negative correlation (Spearman Rs=−0.44, p<0.0001) between SMAD4 and miR-224 expression in clinical samples. Patients with high miR-224 levels display shorter overall survival in multiple CRC cohorts (p=0.0259, 0.0137, 0.0207, 0.0181, 0.0331 and 0.0037 respectively), and shorter metastasis-free survival (hazard ratio 6.51, 95% CI 1.97-21.51, p=0.0008). In the TCGA set, combined analysis of miR-224 with SMAD4 expression enhanced correlation with survival (hazard ratio 4.12, 95% CI 1.1-15.41, p=0.0175).

Conclusion—MiR-224 promotes CRC metastasis, at least in part, through the regulation of SMAD4. MiR-224 expression in primary CRC, alone or combined with its targets, may have prognostic value for CRC patient survival.

Keywords

miR-224; SMAD4; microsatellite stability; colorectal cancer; survival

INTRODUCTION

Colorectal cancer (CRC) is the second leading cause of cancer death among adults, with over 1.6 million new cancer cases and 580,350 deaths estimated to have occurred in the United States in 2013 [1]. Metastatic spread of tumor cells remains the ultimate cause of cancer-related death in most CRC cases. While most cases of localized CRC (stage I-II) are curable by surgical excision, only about 70% of stage III CRC cases with regional lymph node metastasis are curable by surgery combined with adjuvant chemotherapy. Advanced metastatic disease (stage IV), despite improved survival due to recent advances in chemotherapy and targeted agents, remains largely incurable [2],[3]. Therefore, it is of critical importance to understand the key molecular switches involved in CRC metastasis, and identify biomarkers for CRC malignancies and prognostic markers for patient survival.

MicroRNAs (miRNAs) are 18- to 25-nucleotide RNAs that control gene expression at the post-transcriptional level. Based on sequence complementarity, miRNAs bind to targeted protein-coding genes, prevalently at their 3′ untranslated region (UTR), and consequently affect mRNA stability or interfere with protein translation [4]. The functional importance of miRNAs in physiology and disease has been widely appreciated. MiRNAs are differentially expressed in normal and tumor tissues, and unique miRNA expression patterns have been characterized in many cancer types including CRC [5, 6, 7]. However, CRC metastasis-related miRNAs and their biological roles in CRC metastasis remain to be identified. In the invasive regions of primary CRC, organized structure of the tumor is lost: adhesion molecules that maintain cell–cell contact are downregulated, whereas molecules responsible for invasive and migratory behavior are upregulated [8]. These findings suggest that we could decipher the metastatic potential of tumors by analyzing miRNA expression in primary CRC specimens.

In the current study, we aimed to identify miRNAs associated with CRC metastasis, and explore their biological significance as well as diagnostic and prognostic value.

METHODS

Clinical specimens

Six independent CRC cohorts (Italy set 1, Italy set 2, UK set, Romania set, Austria set, and TCGA set) of 449 tumor samples (Table 1) and 172 non-neoplastic mucosal tissues were included in this study. Samples were obtained from University of Ferrara (Italy set 1), Istituto Scientifico Romagnolo per lo Studio e la Cura dei Tumori (IRST) s.r.l., IRCCS (Italy set 2), University of Southampton (UK set), the Oncology Institute Cluj-Napoca (Romania set), and Medical University of Graz (Austria set) respectively. Samples from patients with biopsy proven adenocarcinomas were obtained fresh at the time of surgery and snap frozen prior to being deposited. Exclusion criteria included evidence of a hereditary tumour, presence of multiple or mucinous tumours, and tumours with histologically identified extensive necrosis. Tumors were classified according to the World Health Organization pathologic classification system. Microsatellite analysis was evaluated with a fluorescence based PCR method using the five markers of the Bethesda panel (D5S346, D17S250, D2S123, BAT25 and BAT26) plus BAT40. According to the guidelines of the International Workshop of Bethesda [9], tumors were classified as MSI-H (instability at 2 or more loci), MSI-L (instability at single locus) or MSS (no instability). Patient's clinical information was registered and follow up data was recorded at checkup. All samples were obtained with patient's informed consent. The Institutional research and ethics committee approved this study.

Procedures (summarized in Figure 1)

Total RNA was isolated using TRIzol reagent (Invitrogen) for Italy set 1, Italy set 2, and Romania set, and using RNAqueous-Micro Kit for UK set. Formalin-fixed paraffin embedded samples were use for RNA extraction in Austria set. We used a custom miRNA microarray containing quadruplicates of 389 human miRNA probes for profiling as previously described [10]. Based on the microarray results, we further examined metastasis-related miRNAs using quantitative real time polymerase chain reaction (qRT-PCR) analysis with TaqMan® MiRNA assays (Life Technologies, Grand Island, NY, USA). Biological functions of miRNAs were tested using *in vitro* motility assays, and orthotopic mouse models of CRC. To identify the mRNA targets, we used a prediction algorithm, and the Human Tumor Metastasis RT2 Profiler PCR array (SA Bioscience, Frederick, MD, USA) for the screening, and qRTP-CR, western blot, luciferase assay, immunocytochemistry, and rescue experiments for validation. Clinical correlation was tested with multiple sets of CRC samples, and publically available TCGA data [11]. Other methods are shown in Supplementary methods, and primers are listed in Supplementary Table 1.

In vivo study

Six- to eight-week-old SCID mice (Charles River, UK) were anaesthetized prior to midline laparotomy and exteriorization of the caecum. A 1:1 suspension of cells and Matrigel was injected submucosally into the caecal wall under magnified vision, raising a bleb on the

caecum. For each animal, 5×10^6 cells stably overexpressing GFP tagged miR-224 or control were implanted orthotopically, with the entire experiment conducted in duplicate. Primary tumors grew in all animals. When showing signs of disease or more than 10% weight loss, mice were humanely culled, and colon, liver and lungs were harvested. Excised tissue was paraffin embedded, and stained with haematoxylin and eosin.

Statistical analysis

Differences between groups were analyzed using Student t-test (2-tailed), assuming equal or unequal variance determined by the F-test of equality of variances. Graphics represent the mean ± standard deviation, unless otherwise stated. The Spearman correlation coefficients were computed to assess the correlation between expression level of miR-224 and its target genes in clinical samples. For survival analysis, we divided patients into low/high groups using as cut-off the value that optimally separated the patients, and used the Kaplan-Meier method to estimate the survival curves, and the log-rank test for the comparison. A p value <0.05 was considered statistically significant.

RESULTS

Identification of metastasis-related miRNAs in colorectal cancer

We performed miRNA microarray using primary CRCs from patients with or without metastasis at diagnosis (Italy set 1; n=4 and n=8, respectively), and in cell lines derived from a primary CRC lesion (SW480) or its metastatic dissemination to the lymph node (SW620). According to the microarray data (Supplementary Figure 1A **and** Supplementary Table 2) 11 miRNAs significantly correlated with the presence of metastasis, and were tested by qRT-PCR in a larger cohort of samples (Italy set 1, comprising 85 primary CRC tumors and 25 matched adjacent non-neoplastic colon mucosae). Four miRNAs (miR-141, miR-181b, miR-221, and miR-224; Table 2) showed higher expression in primary CRCs with metastatic dissemination (stage III-IV) compared to early stages (stage I-II), as well as in the CRC metastasis-derived SW620 compared to primary CRC-derived SW480 cell line. Among these, miR-181b, miR-221 and miR-224 were significantly increased in neoplastic CRC tissue compared to normal mucosa. Higher levels of miR-224 in advanced stages, and in tumor versus normal tissue were supported by analysis from multiple cohorts of primary CRC, an independent dataset from The Cancer Genome Atlas (TCGA) consortium, and cell lines with different metastatic features (Table 1, Figure 2A, Supplementary Figure 1B, and Supplementary Figure 2).

Association of miR-224 expression with microsatellite status and tumor site

Unlike miR-181b and miR-221, miR-224 levels were significantly higher in microsatellite stable (MSS) samples compared to microsatellite instability-high (MSI-H) tumors, concordant with the notion that MSI-H CRCs are less aggressive and less prone to metastatic spread than MSS tumors [12] (Figure 2B). Also consistent with the reported association of CRC localization with microsatellite status [13], miR-224 levels were significantly lower in right colon tumors than those occurring in the left colon and rectum (Figure 2B). These findings were validated in TCGA dataset comprising 143 samples (Figure 2C), and Italy set 2 comprising 67 samples (Figure 2D). Concomitantly, *in situ* hybridization showed stronger

miR-224 staining in epithelial cells of MSS CRCs compared with normal and MSI-H tumor samples (fold change = 3.6) (Figure 2E and Supplementary Figure 3).

Pro-metastatic activity of miR-224 *in vitro* and *in vivo*

We selected five miRNAs (the four miRNAs from primary screening, miR-141, miR-181b, miR-221, and miR-224, as well as miR-222 that contains identical seed sequences with miR-221) for evaluation with transwell-based assays (migration, haptotaxis, and invasion) using HCT116 cells. Cell motility increased consistently only upon overexpression of miR-224 and miR-141 by either transient or stable transfection (Figure 3A, Supplementary Figure 4, and Supplementary Figure 5A). Next, we investigated *in vivo* activity of miR-224 using orthotopic CRC SCID mouse model. Direct caecal implantation of HCT116 cells stably over-expressing miR-224 (n=4) resulted, at 5 weeks post-procedure, in a greater number and size of metastatic tumor deposits in the liver and lungs compared to control cells **(**Figures 3B and 3C**)**. Liver replacement was almost complete in some mice implanted with HCT116-miR-224, suggesting a rapid metastatic and growth process. Similarly, stable expression of miR-224 (75-fold induction) with a GFP co-expression plasmid construct in RKO, a CRC cell line with low miR-224 expression (Supplementary Figure 1B), promoted both *in vitro* cell motility (Supplementary Figure 5B**)** and *in vivo* tumor metastasis to the liver in the orthotopic SCID mouse model (Figure 3D**)**. Although RKO lung metastases were not as overt as those with HCT116 cells, by immunostaining with anti-GFP antibodies, we observed a significantly greater number of miR-224-RKO cells disseminated to the lung compared to control RKO cells (Figure 3E). The metastatic phenotype observed using two cell models supports the pro-metastatic function of miR-224 in CRC.

SMAD4 and CDH1 as miR-224 targets

We performed a PCR array comprising 84 metastasis-related genes on SW480 cells. Among the 13 genes that were reduced more than half by miR-224 overexpression (Supplementary Table 3), only CDH1 and SMAD4 were predicted miR-224 targets by the miRGEN database (http://diana.pcbi.upenn.edu/cgi-bin/miRGen/v3/Targets.cgi) (Figure 4A). Overexpression of miR-224 decreased the mRNA and protein expression of CDH1 and SMAD4, both upon transient and stable miRNA transduction (Supplementary Figure 6 and Figure 4B, respectively). Inversely, knockdown of miR-224 in HCT116 and AAC1/82 increased SMAD4 protein expression (Figure 4C). Next, we generated luciferase constructs containing SMAD4 and CDH1 3′ UTR sequences (pGL3-CDH1, pGL3-SMAD4 constructs A and B, and their respective miR-224 target mutants) [14]. Co-transfection with synthetic miR-224 precursor reduced the luciferase activity of pGL3-CDH1, but not the mutant construct with the predicted interaction sequence deleted (Figure 4D). The reporter activity of pGL3-SMAD4 construct A, which was reduced to half by miR-224 overexpression, reverted after deletion of binding site 1 but not of binding site 2 (Figure 4D). On the contrary, pGL3-SMAD4 construct B, containing binding site 3 was not affected by miR-224 (Figure 4D). Independent experiments with point mutants of the binding sites consistently showed that binding site 1 is the interaction site for miR-224 action (Supplementary Figure 7). As a further proof, in the HCT116 cells transfected with GFP-miR construct, we observed a mutually exclusive expression pattern of GFP (indicating miR-224 expression) and SMAD4 (Figure 4E).

Inverse correlation of miR-224 and SMAD4 in clinical samples

We moved on to measure SMAD4 and CDH1 protein levels in stage I-II CRC samples with low miR-224 levels and in stage IV samples with high miR-224 levels selected from the Italian CRC set 1. Consistent with *in vitro* findings, we detected an inverse correlation between miR-224 levels and SMAD4 protein expression (Figure 5A). To exclude the possibility that SMAD4 expression arises from tissues other than the epithelial cells, we performed laser capture microdissection (LCM) in normal colon and CRC tissues, and observed a reciprocal expression of miR-224 and SMAD4 mRNA (Figure 5B). However, we did not find an inverse correlation between CDH1 and miR-224 (**data not shown**). Similarly, in the TCGA consortium data (n=133) we found an inverse correlation between miR-224 and SMAD4 (Spearman Rs=−0.44, p<0.0001), but not with CDH1 (Spearman Rs=0.32, p=0.0002) (Supplementary Figure 8).

SMAD4 as effector of miR-224 in promoting cell motility

We next investigated whether SMAD4 is the mediator of miR-224's pro-metastatic effect. Similar to what occurred after miR-224 transfection, silencing of SMAD4 increased HCT116 cell motility (Figure 5C). Moreover, exogenous SMAD4 expression with a miR-224-resistant SMAD4 construct (SMAD4 without its 3'UTR containing the putative miR-224 target site) abrogated miR-224's ability to promote cell migration and invasion (Figure 5D). Taken together, the phenocopy and the rescue experiment support the hypothesis that SMAD4 is a key effector of miR-224's pro-metastatic capacity.

Association of miR-224 with CRC patient survival

To determine the clinical significance, we performed patient survival analysis in five CRC cohorts with available follow-up information (Table 1). In all the cohorts, patients with high miR-224 expression had shorter overall survival compared to those with low miR-224 levels with p=0.0259 in TCGA dataset (n=143), p=0.0137 in Italy set 1 (n=54), p=0.0207 in Italy set 2 (n=68), p=0.0181 in UK set (n=41), and the p=0.0331 in Romania set (n=38) (Figure 6A-6E **and** Table 3). Multivariate analysis showed a trend of correlation but did not reach statistical significance, possibly due to small group size after stage separation. To examine if miR-224 association with survival depends on stage, we used an Austrian sample cohort comprising 74 colon tumors, with majority of them in stage III and IV. In the multivariate analysis, miR-224 showed prognostic value on overall survival (HR 2.36, 95% CI 1.32-4.21; p=0.0037) independent of tumor stage (Figure 6F and Table 3). In addition, in the TCGA dataset, combined analysis of miR-224 with SMAD4 expression increased the separation of the survival curves obtained by either gene alone, and patients with miR-224 (high)/SMAD4 (low) had shorter survival compared to those with miR-224 (low)/SMAD4 (high) (HR 4.12, 95% CI 1.1-15.41; p=0.0175) (Figure 6G **and** Supplementary Figure 9). Interestingly, although we did not observe an inverse correlation for miR-224 and CDH expression, and CDH1 alone did not predict patient survival, combined analysis with CDH1 greatly improved the prediction power of miR-224 for patient overall survival (p=0.0009) (Supplementary Figure 10). Furthermore, in the UK set (where complete clinical information was available), patients with high miR-224 expression in primary CRC had shorter metastasis-free survival than those with low miR-224 expression (HR 6.51, 95% CI

1.97-21.51; p=0.0008) (Figure 6H). Notably, miR-224 showed higher sensitivity and specificity (Area UnderCurve=0.739) for metastasis-free survival than for overall survival in the receiver-operating characteristic (ROC) analysis (Supplementary Figure 11).

DISCUSSION

The role of miR-224 during CRC initiation and progression remains controversial [15, 16, 17, 18, 19, 20, 21]: MiR-224 is overexpressed in inflammatory bowel disease-associated CRC [21], it promotes CRC tumor growth in mice by repressing PHLPP1 and PHLPP2 [19], and ectopic miR-224 expression decreases the chemoradiosensitivity of CRC [22], all of which suggests an oncogenic function. Yet, reports that methotrexate-resistant colon cancer cells express lower miR-224 [23], and that miR-224 suppresses tumor growth and metastasis *in vivo* [20] suggest that an opposite role in CRC may be true. The latter study also showed lower miR-224 expression in metastatic CRC cell lines versus non-metastatic cell lines, and in metastatic tumors in the lung versus primary CRC tumors [20].

In the current study we analyzed miR-224 expression in primary tumor samples, and both our *in vitro* and *in vivo* data strongly support a pro-metastatic rather than anti-metastatic function of miR-224 in CRC. This pro-metastatic influence is also reflected in outcome data from multiple international patient cohorts, which demonstrated that elevated miR-224 expression is associated with advanced disease stages, and impaired survival in CRC in a manner consistent with previous reports [19].

Interestingly, among the clinical parameters we have analyzed (including tumor stage and tumor site), microsatellite status was the most significant factor associated with miR-224 expression in the 85 Italian CRC samples and the 143 TCGA dataset cases. MSS CRCs, which account for 80% of all CRC cases, are characterized by their unstable chromosomal status, and an aggressive clinical course characterized by early metastasis and poor prognosis [24]. Although a previous report identified that miR-224 expression in CRCs with proficient DNA mismatch repair is more than twice that of CRC with defective DNA mismatch repair [25], this association between miR-224 expression and MSS status has not previously been examined in depth. MSS is identified by the absence of multiple microsatellite instability marks, and because no direct test for MSS status currently exists, quantitating miR-224 expression in CRC specimen may offer a promising avenue for the development of future diagnostic applications.

SMAD4 protein is a transcription factor required for synergistic transcriptional activity in response to TGF-beta. Loss of SMAD4 in CRC has been shown to switch TGF-beta function from tumor suppression to the promotion of tumorigenicity and metastasis [26]. Furthermore, loss of SMAD4 function due to 18q genomic deletion, or protein inactivation or suppression, has been associated with advancing tumor stage and metastatic status in CRC [27, 28]. However, CRC tumors with or without 18q21 allelic imbalance showed no difference in SMAD4 levels, suggesting additional mechanisms of SMAD4 regulation may also apply [29]. Furthermore, while 18q21 allelic imbalance and SMAD4 mutations did not perform well as prognostic markers, the absolute level of SMAD4 protein expression was found to have prognostic utility in CRC [29].

SMAD4 has been identified as a miR-224 target in several disease models [17, 30, 31] including CRC [18]. Here we provide multiply validated evidence that SMAD4 is a miR-224 target using in-silico target prediction analysis, overexpression and knockdown cell models, luciferase assays, and expression correlation in clinical samples. Furthermore, we have established that the pro-metastatic activity of SMAD4 is in part mediated by miR-224 using phenocopy and rescue experiments. We also identified CDH1 as a miR-224 target, by target prediction, qRT-PCR, Western blot, and luciferase assay; however, CDH1 mRNA shows positive, instead of inverse, correlation with miR-224 levels in clinical samples. Despite this discrepancy, high miR-224 and low CDH1 show a strikingly better prediction for CRC patient overall survival than either gene alone, suggesting that miR-224-CDH1 regulation may also play a role in CRC pathogenesis.

We are aware of the limitations of the present study. Firstly, the PCR array we used for screening does not cover all the possible miR-224 targets. It is possible that miR-224 promotes metastasis by mediating multiple targets rather than SMAD4 alone. Secondly, although we used multiple independent sets of CRC samples, larger cohorts are required to validate the association between miR-224 expression and patient survival, and the prognostic advantage of combining the expression levels of miR-224 and its mRNA targets. Thirdly, survival analysis in the current study may be confounded by the treatment received by patients. In future retrospective and prospective biomarker analysis, the potential impact of therapy on outcome should also be considered.

Despite these limitations, our study has a number of potential implications for clinical practice. Firstly, the association between miR-224 and MSS status suggests there may be potential for the development of a specific diagnostic marker based on miR-224 expression. Secondly, miR-224 expression alone or in combination with its target genes (SMAD4, CDH1, or possibly other targets) may serve as prognostic marker in CRC. The recent finding that CRC patients with MSS status and loss of SMAD4 expression had significantly worse survival [32] highlights the promise of this approach. Third, high miR-224 expression in advanced CRCs and its pro-metastatic consequences suggest that this miRNA could be an ideal candidate for targeted therapeutic interventions, particularly in tumors that express low levels of SMAD4. The recent success of miravirsen (an LNA anti-miR-122) in a clinical trial for treating HCV infection [33] indicates technical plausibility of anti-miR-224 treatment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

FUNDING

Dr Calin is The Alan M. Gewirtz Leukemia & Lymphoma Society Scholar. Work in Dr. Calin’s laboratory is supported in part by the NIH/NCI grants 1UH2TR00943-01 and 1 R01 CA182905-01, the UT MD Anderson Cancer Center SPORE in Melanoma grant from NCI (P50 CA093459), Aim at Melanoma Foundation and the Miriam and Jim Mulva research funds, the Brain SPORE (2P50CA127001), the Center for radiation Oncology Research Project, the Center for Cancer Epigenetics Pilot project, a 2014 Knowledge GAP MDACC grant, a CLL Moonshot pilot project, the UT MD Anderson Cancer Center Duncan Family Institute for Cancer Prevention and Risk Assessment, a SINF grant in colon cancer, the Laura and John Arnold Foundation, the RGK Foundation and

the Estate of C. G. Johnson, Jr,. Dr Ling is an Odyssey Fellow, and his work is supported in part by the Odyssey Program at The University of Texas MD Anderson Cancer Center. Dr. Pichler is supported by an Erwin-Schroedinger Scholarship of the Austrian Science Funds (project no. J3389-B23). Dr. Almeida is supported as a fellow by the Foundation for Science and Technology, Portugal (SFRH/BPD/91011/2012). Dr. Hamilton is the recipient of the Frederick F. Becker Distinguished University Chair in Cancer Research from The University of Texas. Work in Dr. Fabbri's lab is supported in part by award number P30CA014089 from the National Cancer Institute. Work in Dr. Medico's laboratory is supported in part by funds from AIRC (IG n. 12944 and 5×1000 project n. 9970), FPRC and Regione Piemonte (E-LAB). Dr. Isella is a University of Torino Fellow, supported in part by Regione Piemonte (E-LAB). Dr. Pickard and Dr. Mirnezami are supported by grant funding from Wessex Medical Research and Cancer Research UK (C28503/A10013). Work in Dr Nicoloso's lab is supported in part by funds from AIRC (MFAG n. 13589), 5×1000 to CRO and Marie Curie Actions (CIG n. 303877).

Abbreviations

miRNA	MicroRNA
CRC	colorectal cancer
qRT-PCR	quantitative real time polymerase chain reaction
MSS	microsatellite stable
MSI	microsatellite instability
MSI-H	microsatellite instability-high
UTR	untranslated region
TCGA	The Cancer Genome Atlas
LCM	laser capture microdissection
ROC	receiver-operating characteristic

REFERENCES

1. Siegel R, Naishadham D, Jemal A. Cancer statistics, 2013. CA Cancer J Clin. 2013; 63:11–30. [PubMed: 23335087]
2. Markowitz SD, Bertagnolli MM. Molecular origins of cancer: Molecular basis of colorectal cancer. N Engl J Med. 2009; 361:2449–60. [PubMed: 20018966]
3. Lindmark G, Gerdin B, Pahlman L, et al. Prognostic predictors in colorectal cancer. Dis Colon Rectum. 1994; 37:1219–27. [PubMed: 7995147]
4. He L, Hannon GJ. MicroRNAs: small RNAs with a big role in gene regulation. Nat Rev Genet. 2004; 5:522–31. [PubMed: 15211354]
5. Slaby O, Svoboda M, Michalek J, et al. MicroRNAs in colorectal cancer: translation of molecular biology into clinical application. Mol Cancer. 2009; 8:102. [PubMed: 19912656]
6. Bandres E, Cubedo E, Agirre X, et al. Identification by Real-time PCR of 13 mature microRNAs differentially expressed in colorectal cancer and non-tumoral tissues. Mol Cancer. 2006; 5:29. [PubMed: 16854228]
7. Ma Y, Zhang P, Yang J, et al. Candidate microRNA biomarkers in human colorectal cancer: systematic review profiling studies and experimental validation. Int J Cancer. 2012; 130:2077–87. [PubMed: 21671476]
8. Brabletz T, Hlubek F, Spaderna S, et al. Invasion and metastasis in colorectal cancer: epithelial-mesenchymal transition, mesenchymal-epithelial transition, stem cells and beta-catenin. Cells Tissues Organs. 2005; 179:56–65. [PubMed: 15942193]
9. Boland CR, Thibodeau SN, Hamilton SR, et al. A National Cancer Institute Workshop on Microsatellite Instability for cancer detection and familial predisposition: development of international criteria for the determination of microsatellite instability in colorectal cancer. Cancer Res. 1998; 58:5248–57. [PubMed: 9823339]

10. Liu CG, Calin GA, Meloon B, et al. An oligonucleotide microchip for genome-wide microRNA profiling in human and mouse tissues. Proc Natl Acad Sci U S A. 2004; 101:9740–4. [PubMed: 15210942]
11. Comprehensive molecular characterization of human colon and rectal cancer. Nature. 2012; 487:330–7. [PubMed: 22810696]
12. Popat S, Hubner R, Houlston RS. Systematic review of microsatellite instability and colorectal cancer prognosis. J Clin Oncol. 2005; 23:609–18. [PubMed: 15659508]
13. Thibodeau SN, Bren G, Schaid D. Microsatellite instability in cancer of the proximal colon. Science. 1993; 260:816–9. [PubMed: 8484122]
14. Cimmino A, Calin GA, Fabbri M, et al. miR-15 and miR-16 induce apoptosis by targeting BCL2. Proc Natl Acad Sci U S A. 2005; 102:13944–9. [PubMed: 16166262]
15. Adamopoulos PG, Kontos CK, Rapti SM, et al. miR-224 overexpression is a strong and independent prognosticator of short-term relapse and poor overall survival in colorectal adenocarcinoma. Int J Oncol. 2015; 46:849–59. [PubMed: 25420464]
16. Ke TW, Hsu HL, Wu YH, et al. MicroRNA-224 suppresses colorectal cancer cell migration by targeting Cdc42. Dis Markers. 2014; 2014:617150. [PubMed: 24817781]
17. Wang Y, Ren J, Gao Y, et al. MicroRNA-224 targets SMAD family member 4 to promote cell proliferation and negatively influence patient survival. PLoS One. 2013; 8:e68744. [PubMed: 23922662]
18. Zhang GJ, Zhou H, Xiao HX, et al. Up-regulation of miR-224 promotes cancer cell proliferation and invasion and predicts relapse of colorectal cancer. Cancer Cell Int. 2013; 13:104. [PubMed: 24152489]
19. Liao WT, Li TT, Wang ZG, et al. microRNA-224 Promotes Cell Proliferation and Tumor Growth in Human Colorectal Cancer by Repressing PHLPP1 and PHLPP2. Clin Cancer Res. 2013; 19:4662–72. [PubMed: 23846336]
20. Yuan K, Xie K, Fox J, et al. Decreased Levels of miR-224 and the Passenger Strand of miR-221 Increase MBD2, Suppressing Maspin and Promoting Colorectal Tumor Growth and Metastasis in Mice. Gastroenterology. 2013; 145:853–64. e9. [PubMed: 23770133]
21. Olaru AV, Yamanaka S, Vazquez C, et al. MicroRNA-224 negatively regulates p21 expression during late neoplastic progression in inflammatory bowel disease. Inflamm Bowel Dis. 2013; 19:471–80. [PubMed: 23399735]
22. Salendo J, Spitzner M, Kramer F, et al. Identification of a microRNA expression signature for chemoradiosensitivity of colorectal cancer cells, involving miRNAs-320a, -224, -132 and let7g. Radiother Oncol. 2013
23. Mencia N, Selga E, Noe V, et al. Underexpression of miR-224 in methotrexate resistant human colon cancer cells. Biochem Pharmacol. 2011; 82:1572–82. [PubMed: 21864507]
24. Hemminki A, Mecklin JP, Jarvinen H, et al. Microsatellite instability is a favorable prognostic indicator in patients with colorectal cancer receiving chemotherapy. Gastroenterology. 2000; 119:921–8. [PubMed: 11040179]
25. Oberg AL, French AJ, Sarver AL, et al. miRNA expression in colon polyps provides evidence for a multihit model of colon cancer. PLoS One. 2011; 6:e20465. [PubMed: 21694772]
26. Zhang B, Halder SK, Kashikar ND, et al. Antimetastatic role of Smad4 signaling in colorectal cancer. Gastroenterology. 2010; 138:969–80. e1–3. [PubMed: 19909744]
27. Miyaki M, Iijima T, Konishi M, et al. Higher frequency of Smad4 gene mutation in human colorectal cancer with distant metastasis. Oncogene. 1999; 18:3098–103. [PubMed: 10340381]
28. Xie W, Rimm DL, Lin Y, et al. Loss of Smad signaling in human colorectal cancer is associated with advanced disease and poor prognosis. Cancer J. 2003; 9:302–12. [PubMed: 12967141]
29. Alazzouzi H, Alhopuro P, Salovaara R, et al. SMAD4 as a prognostic marker in colorectal cancer. Clin Cancer Res. 2005; 11:2606–11. [PubMed: 15814640]
30. Yao G, Yin M, Lian J, et al. MicroRNA-224 is involved in transforming growth factor-beta-mediated mouse granulosa cell proliferation and granulosa cell function by targeting Smad4. Mol Endocrinol. 2010; 24:540–51. [PubMed: 20118412]

31. Lichner Z, Mejia-Guerrero S, Ignacak M, et al. Pleiotropic action of renal cell carcinoma-dysregulated miRNAs on hypoxia-related signaling pathways. Am J Pathol. 2012; 180:1675–87. [PubMed: 22326755]

32. Roth AD, Delorenzi M, Tejpar S, et al. Integrated analysis of molecular and clinical prognostic factors in stage II/III colon cancer. J Natl Cancer Inst. 2012; 104:1635–46. [PubMed: 23104212]

33. Janssen HL, Reesink HW, Lawitz EJ, et al. Treatment of HCV infection by targeting microRNA. N Engl J Med. 2013; 368:1685–94. [PubMed: 23534542]

SUMMARY

What is already known about this subject?

- Metastasis is the main cause of cancer-related death in colorectal cancer (CRC) cases.
- Reports on miR-224 involvement in CRC metastasis are inconsistent, with some studies supporting pro-metastatic function of miR-224, and others reporting its anti-metastatic function.
- A comprehensive and systemic analysis addressing significance of miR-224 in CRC metastasis is absent.

What are the new findings?

- Expression levels of miR-141, miR-181b, miR-221, and miR-224 are significantly increased in primary CRC with metastatic dissemination compared to early stage diseases. Among these, miR-224 shows higher expression in microsatellite stable (MSS) CRCs compared to those with microsatellite instability (MSI).
- Ectopic overexpression of miR-224 increases CRC cell motility *in vitro*, and promotes CRC metastasis to lung and liver in two orthotopic CRC mouse models.
- SMAD4 mediates miR-224's pro-metastatic effect, and shows inverse correlation with miR-224 in CRC tumor samples.
- High miR-224 expression in primary CRC tumors is associated with shorter overall and metastasis-free survival of patients in multiple CRC cohorts.

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

- Our data strongly support that miR-224 is an activator for CRC metastasis via targeting SMAD4, and suggest that miR-224, alone or combination with SMAD4, may be an independent prognostic marker for CRC patient survival.

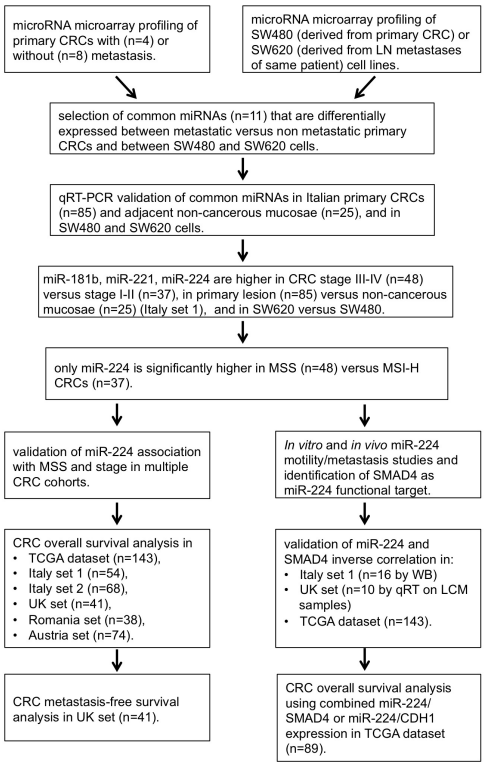

Figure 1. A schematic description of the workflow in this study

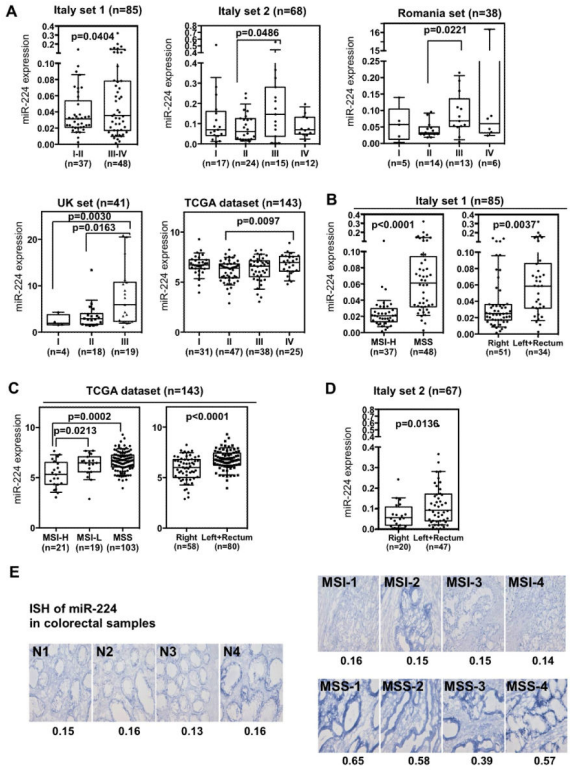

Figure 2. miR-224 expression in primary CRC samples

(A) Higher miR-224 expression in advanced stages from multiple CRC cohorts, as determined by Taqman qRT-PCR or obtained from TCGA dataset. **(B)** Differential miR-224 expression in the Italian set 1 of CRC samples subdivided by MSS/MSI status or tumor site. **(C, D)** Validation of miR-224 association with MSS/MSI status and tumor site with TCGA dataset and Italian set 2. **(E)** *In situ* hybridization of the miR-224 in normal mucosae (N 1-4), MSI-H (MSI 1-4) and MSS CRC samples (MSS 1-4). MiR-224 expression was normalized by U6. Data in **A-D** are presented as box-whisker plots showing the five statistics (lower whisker is the 10th percentile, lower box part is the 25th percentile, solid line in box is the median, upper box part is 75th percentile, and upper whisker is 90th percentile).

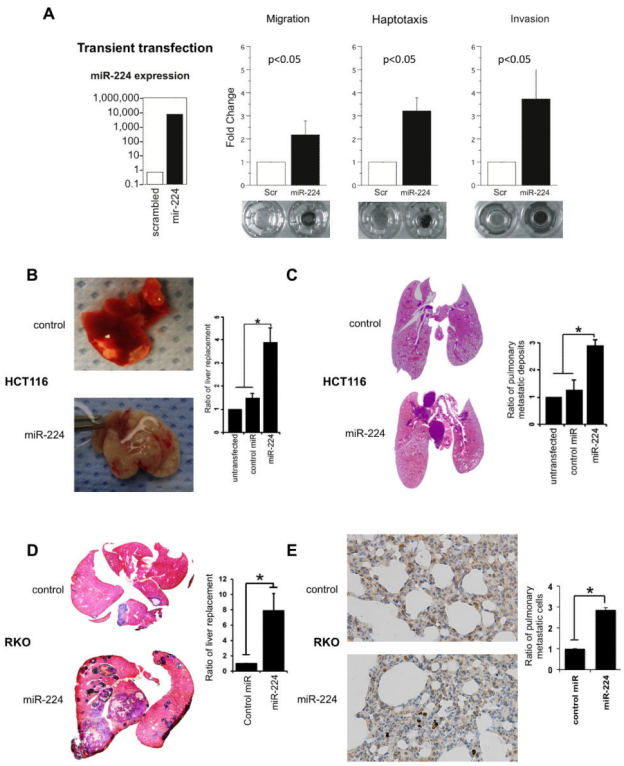

Figure 3. MiR-224 promotes CRC metastasis

(A) Transwell-based motility assays in HCT116 cells transiently transfected with miRNA mimics. Motility is expressed as fold change compared to control cells. Data are presented as mean ± SD of three independent experiments each in triplicate. A representative image is shown under the bar chart for each treatment condition. **(B-D)** Ectopic miR-224 expression promotes CRC metastasis to the liver and lung in both HCT116 (**B** and **C,** n=4 in each group) and RKO (**D** and **E,** n=6 in each group) cell systems. Orthotopic SCID mouse models were established with CRC cells stably transfected with miR-224 or control (0.5 million cells). The number of pulmonary deposits and degree of liver replacement was calculated using imageJ software. Results are presented as relative to untransfected cells. *, p<0.05. **(E)** Lung immunostaining with antibody against GFP, which is coexpressed by miR ctrl or miR-224 constructs, to detect CRC cell infiltrates.

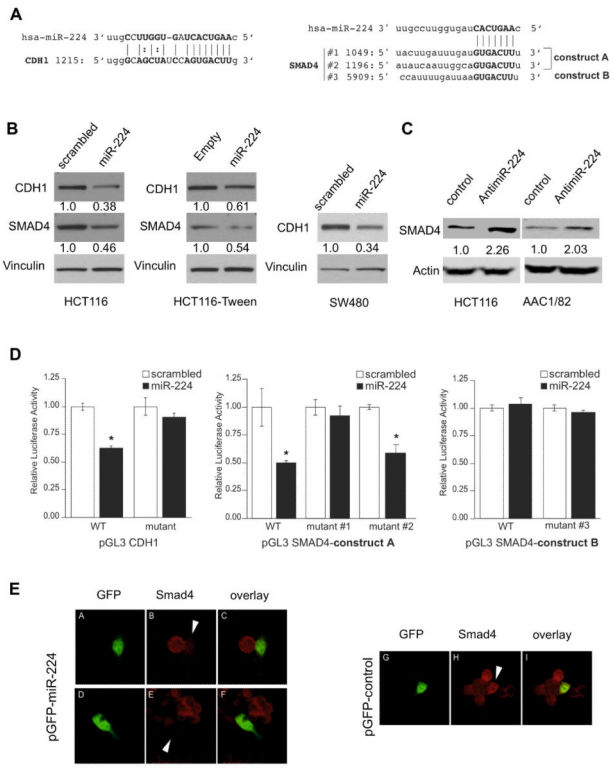

Figure 4. MiR-224 regulates SMAD4 and CDH1 expression

(A) Predicted miR-224 interaction sites with 3'UTR of CDH1 (left), and SMAD4 (right). Two SMAD4 constructs were produced as indicated (construct A, containing sites 1 and 2, and construct B, containing site 3). **(B)** MiR-224 reduces SMAD4 and CDH1 protein expression. SMAD4 was never detected at the protein level in SW480 cells. **(C)** Anti-miR-224 treatment increases the protein expression levels of SMAD4. Western blot bands were quantified by image J and the number shows the relative expression. **(D)** Luciferase activity of pGL3-CDH1 and SMAD4 constructs containing miR-224 predicted binding sites (see **A** for annotation) after scrambled or miR-224 transfection in HCT116 cells. One representative experiment is shown (presented as mean ± SD) out of at least three independent experiments performed in quadruplicates. *, $p<0.05$. **(E)** Immunocytochemistry for GFP-miR-224 (green) and SMAD4 (red) in stably transfected HCT116 cells.

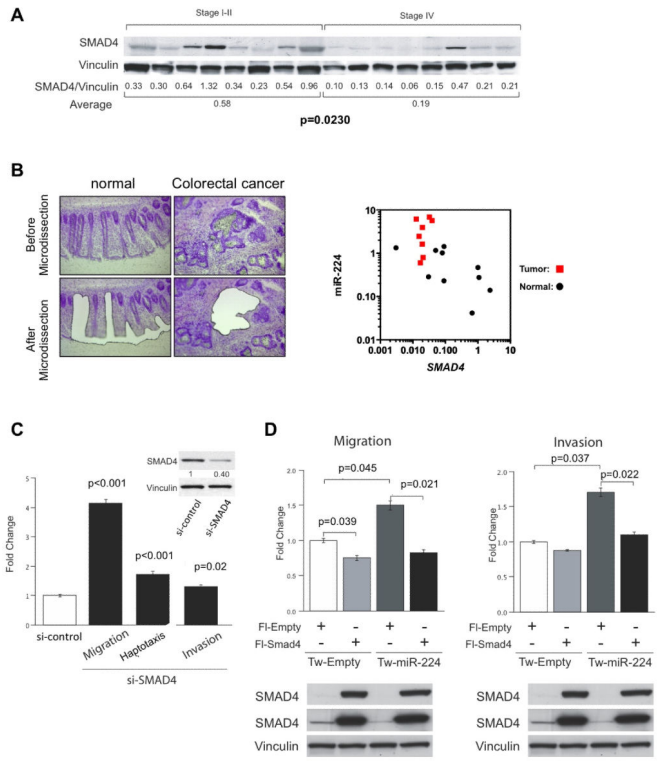

Figure 5. Inverse correlation of miR-224 and SMAD4 in clinical samples, and rescue experiment

(A) SMAD4 protein expression in Italian cohort 1 CRC samples with different stages. **(B)** Inverse expression profile of miR-224 and SMAD4 protein in epithelial cells of normal tissues and CRC samples by LCM. **(C)** SMAD4 siRNA enhanced CRC cell motility, similar with that observed in miR-224 overexpressing experiments. **(D)** Enforced SMAD4 expression abrogated miR-224's promoting effect on HCT116 cell motility. The experiments were performed twice in triplicate. One representative experiment is shown as mean ± SD.

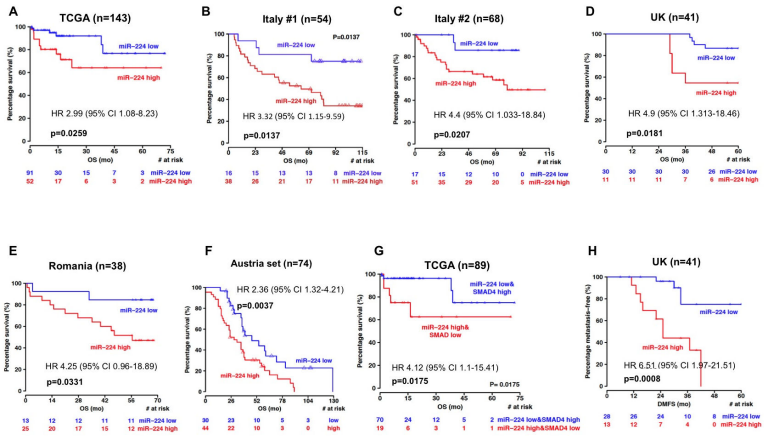

Figure 6. MiR-224 and its target gene SMAD4 as prognostic markers in CRC patients

(A-F) Association of miR-224 expression in primary CRC with overall survival in multiple CRC cohorts. **(G)** Combined expression of miR-224 and SMAD4 improved the separation curve of patient overall survival. **(H)** Metastasis-free survival analysis of miR-224 association in the UK set. A statistically determined optimal cut-off was used to stratify the patient groups, and the log-rank test was used for survival analysis.

Table 1

Main characteristics of patients and tissue samples

	Italy set 1 (n=85)	Italy set 2 (n=68)	UK set (n=41)	Romania set (n=38)	Austria set (n=74)	TCGA set (n=143)
	Normal v. tumor (25 normal; 85 tumor)	Normal v. tumor (n=64, paired)		Normal v. tumor (n=23, paired)	Normal v. tumor (n=60, paired)	
	Stage association (n=85)	Stage association (n=68)	Stage association (n=41)	Stage association (n=38)		Stage association (n=141)
Role of cohort in present study	Site association (n=85)	Site association (n=67)				Site association (n=136)
	Microsatellite status association (n=85)					Microsatellite status association (n=143)
	Survival analysis (n=54)	Survival analysis (n=68)	Survival analysis (n=41)	Survival analysis (n=38)	Survival analysis (n=74)	Survival analysis (n=143)
Median age (range) years	73 (42-93)	67(41-88)	70 (38-94)	69.5 (38-81)	63 (38-80)	68 (34-89)
Sex						
Male	40 (47%)	40 (59%)	20 (49%)	25 (66%)	46(62%)	75 (52%)
Female	45 (53%)	28 (41%)	21 (51%)	13 (34%)	28 (38%)	68 (48%)
Tumor location						
Right	51 (60%)	20 (29%)	14 (34%)	10 (26%)	27 (36%)	58 (40%)
Left	23 (27%)	27 (40%)	27 (66%)	13 (34%)	47 (64%)	37 (26%)
Rectum	11 (13%)	20 (29%)		9 (24%)		43 (30%)
Not known		1 (2%)		6 (16%)		5 (3%)
Tumor stage						
I	3 (4%)	17 (25%)	4 (10%)	5 (13%)	0 (0%)	31 (22%)
II	33 (39%)	24 (35%)	18 (44%)	14 (37%)	7 (9%)	47 (33%)
III	20 (23%)	15 (22%)	19 (46%)	13 (34%)	23 (31%)	38 (27%)
IV	29 (34%)	12 (18%)		6 (16%)	44 (59%)	25 (17%)
not known						2 (1%)
Microsatellite status						
MSI-H	37 (44%)					21 (15%)
MSI-L	0 (0%)					19 (13%)
MSS	48 (56%)					103 (72%)

All data are n (%) unless stated otherwise

Table 2

Differentially expressed miRNAs in primary CRCs from patients or CRC cell lines

	Tum/Norm	p-value	MSS/MSI	p-value	III-IV/I-II	p-value	SW620/SW480
miR-141	**0.74**	**0.0000**	1.18	0.1411	**1.36**	**0.0065**	4.90
miR-181b	**1.28**	**0.0015**	0.93	0.5580	**1.44**	**0.0025**	4.51
miR-191	0.91	0.1283	1.22	0.0931	**1.28**	**0.0338**	0.52
miR-200a	**0.80**	**0.0370**	0.92	0.5013	1.25	0.0656	NOT AVAILABLE
miR-200b	**0.81**	**0.0019**	1.09	0.4497	1.23	0.0835	25.76
miR-203	**1.57**	**0.0020**	1.20	0.4218	**1.92**	**0.0024**	0.19
miR-215	**0.27**	**0.0000**	0.74	0.3673	1.15	0.6852	50.20
miR-221	**3.59**	**0.0000**	0.82	0.3669	**1.73**	**0.0057**	4.04
miR-222	**1.45**	**0.0044**	**0.62**	**0.0215**	**1.80**	**0.0023**	NOT AVAILABLE
miR-224	**3.76**	**0.0000**	**2.99**	**0.0000**	**1.54**	**0.0404**	1.49
miR-425	1.01	0.9484	0.98	0.8111	1.21	0.0863	NOT AVAILABLE

(Red: ratio larger than 1; Green: ratio lower than 1; Bold: difference significant ($p < 0.05$))

Table 3

Association of clinical parameters or gene expression with colorectal cancer patient survival

		UNIVARIATE		MULTIVARIATE	
Cohort	**Variable**	**HR (95%CI)**	**p-value (log-rank)**	**HR (95%CI)**	**p-value(wald)**
TCGA	Age (<median vs >median)	1.02(0.37,2.83)	0.9614		
	Gender (Male vs Female)	1.32(0.49,3.55)	0.579		
	Tumor location (Left vs Right)	0.92(0.32,2.68)	0.8846		
	Tumor stage(III-IV vs I-II)	7.87(1.77,35.07)	0.0014 *	8.37(1.82,38.51)	0.0063 *
	Microsatellite status (MSS vs MSI)	2.896(0.66,12.75)	0.1597		
	miR-224(High vs Low)	2.99(1.08,8.23)	0.0259 *	2.88(0.97,8.56)	0.0571
Italian set 1	Age (<median vs >median)	0.81(0.39,1.71)	0.5817		
	Gender (Male vs Female)	1.56(0.72,3.39)	0.2598		
	Tumor location (Left vs Right)	0.74(0.35,1.57)	0.4284		
	Tumor Stage(Stage III-IV vs Stage I-II)	2.85(1.28,6.35)	0.0073	2.44(1.09, 5.48)	0.0302 *
	MSI_status (MSS vs MSI)	2.04(0.87,4.81)	0.0951		
	miR-224(High vs Low)	3.32(1.15,9.59)	0.0137	2.77(0.95, 8.105)	0.063
Italian set 2	Age (<median vs >median)	0.74(0.32,1.69)	0.4757		
	Gender (Male vs Female)	2.2(0.9,5.36)	0.0744		
	Tumor location (Left vs Right)	1.68(0.73,3.83)	0.2148		
	Tumor stage(III-IV vs I-II)	4.04(1.7,9.57)	0.0006 *	3.89(1.63,9.26)	0.0022 *
	miR-224(High vs Low)	4.41(1.03,18.84)	0.0207 *	4.14(0.96,17.76)	0.056
Romania	Age (<median vs >median)	1.16(0.42,3.2)	0.7733		
	Gender (Male vs Female)	2.44(0.69,8.66)	0.154		
	Tumor location (Left vs Right)	1.3(0.44,3.9)	0.633		
	Tumor stage(III-IV vs I-II)	9.33(2.1,41.54)	0.0004 *	7.47(1.52,36.65)	0.0132 *
	miR-224(High vs Low)	4.25(0.96,18.89)	0.0331 *	1.76(0.36,8.64)	0.4831
Austria	Age (<median vs >median)	0.88(0.51,1.49)	0.6239		
	Gender (Male vs Female)	0.82(0.48,1.43)	0.4909		
	Tumor location (Left vs Right)	0.62(0.36,1.06)	0.0802		
	Tumor Stage(Stage III-IV vs Stage II)	2.89(1.02,8.15)	0.037 *	3.41(1.18,9.83)	0.023 *
	miR-224(High vs Low	2.14(1.21,3.77)	0.0059 *	2.36(1.32,4.21)	0.0037 *
UK (Overall survival)	Age (<median vs >median)	1.15(0.31,4.3)	0.8322		
	Gender (Male vs Female)	0.8(0.21,2.98)	0.7399		
	Tumor location (Left vs Right)	0.98(0.24,3.92)	0.9781		
	Tumor stage(III-IV vs I-II)	2.7(0.68,10.83)	0.1428		
	miR-224(High vs Low)	4.92(1.31,18.46)	0.0181 *		

		UNIVARIATE		MULTIVARIATE	
Cohort	**Variable**	**HR (95%CI)**	**p-value (log-rank)**	**HR (95%CI)**	**p-value(wald)**
UK (Metastasis free survival)	Age (<median vs >median)	1.54(0.5,4.72)	0.446		
	Gender (Male vs Female)	0.75(0.25,2.24)	0.6074		
	Tumor location (Left vs Right)	0.6(0.16,2.18)	0.4326		
	Tumor stage(III-IV vs I-II)	2.92(0.93,9.21)	0.0558		
	miR-224(High vs Low)	6.51(1.97,21.51)	0.0008 *		

* Statistically significant with p less than 0.05; HR, hazard ratio; CI: confidence interval