Published in final edited form as:
Gastroenterology. 2013 October ; 145(4): 853–864.e9. doi:10.1053/j.gastro.2013.06.008.

Decreased Levels of miR-224 and the Passenger Strand of miR-221 Increase MBD2, Suppressing Maspin and Promoting Tumor Growth and Metastasis in Mice

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Abstract

Background and Aims—Little is known about functions of microRNA (miR) passenger strands (miR*), or their roles in tumor development or progression. We screened for miRs and miR* whose levels were altered in metastatic colorectal cancer (CRC) cells and human tumor samples, and investigated their targets and effects on cell function and tumor progression in mice.

Methods—We performed array-based profile analysis to identify miRs whose levels were increased more than 2-fold in metastatic (SW620) CRC cells, compared with non-metastatic (SW480) cells. Quantitative PCR, immunoblot, and in situ hybridization analyses were used to measure miRNA levels in CRC cell lines and human tumor samples. We used miRNA duplex mimics or inhibitors to increase and decrease levels of miRNA in CRC cells, and assessed their activities and ability to form metastatic xenograft tumors in nude mice.

Results—Levels of miR-221* and miR-224 were reduced in metastatic, compared with non-metastatic, CRC cells; levels in human tumor samples correlated inversely with tumor stage and metastasis to lymph nodes, as well as patient survival times. SW480 cells transfected with

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Author Contributions K.Y. performed all experiments; H.Z., J.F., K.X. performed some experiments; H. G. contributed reagents; M.W. and C.H. conceived the studies; K.Y., C.H., and M.W. designed studies and wrote the paper.

Competing interests' statement: The authors declare that they have no competing financial interests.

miR-221* or miR-224 inhibitors had increased motility in vitro, compared with SW480 control cells, and formed larger, more metastatic tumors following into mice. SW620 cells transfected with miR-221* or miR-224 mimics had reduced migration and motility in vitro and formed smaller tumors with fewer metastases in mice, compared with control SW620 cells. We identified the 3'UTR of *MBD2* mRNA as a target of miR-221* and 281 miR-224. MBD2 silences the gene encoding Maspin, a suppressor of metastasis. In CRC cells, we found that miR-221* and miR-224 increase the expression of Maspin through MBD2 downregulation.

Conclusion—In metastatic CRC cells, reduced levels of miR-221* and miR-224 increase levels of MBD2, thereby decreasing expression of the metastasis suppressor Maspin. Increased activities of miR-221* and miR-224 reduce growth and metastasis of CRC xenograft tumors in mice; these miRs might be developed as therapeutic reagents or biomarkers of CRC progression.

Keywords

colon cancer progression; mouse model; metastases; prognostic factor

Introduction

Colorectal cancer (CRC) is the third most commonly diagnosed cancer in males and the second in females, with over 1.2 million new cancer cases and 608,700 deaths estimated to occur yearly in the USA[1]. Various risk groups (low, intermediate, high, and metastatic) that reflect relative survival are categorized using histologic grade (Gleason score) and clinical tumor nodal metastasis (TNM) stage (local extent and/or nodal/distant metastases)[2]. According to these statistics, when detected at an early stage, most CRC patients can be categorized into the low-risk group, and 90% of these patients can be cured by surgical resection[3]. Despite significant advances in early detection, CRC is often diagnosed at an advanced stage and thereby carries a poor prognosis[4]. Development of novel prognostic biomarkers and targeted therapies may offer early diagnosis and improved treatment of this cancer.

Metastasis, which is a complex, multi-step process, has been identified to be responsible for most cancer deaths, including colorectal cancer[5]. Although metastasis is the overwhelming cause of mortality in patients with solid tumors, our understanding of its molecular and cellular determinants is limited[6]. In recent years, identification and characterization of genes and gene products that drive the metastatic process have been the focus of intensive interest. Several transcription factors have been revealed to program many of the cell-biological changes needed to execute the initial steps of the invasion–metastasis cascade[7]. Such gene sets are thus providing numerous candidate mediators of metastasis to be validated through functional and clinical studies. Much less insight, however, has been gained into the regulatory networks that establish such altered gene expression states[8]. MiRNAs, a class of small cellular RNAs acting as agents of the RNA interference pathway, are attractive candidates as upstream regulators of metastatic progression because miRNAs can post-transcriptionally regulate entire sets of genes[9].

Here, with an array-based miRNA profiling, we found that miR-221* (its function almost unknown) and miR-224 (previously linked to cancer) were down-regulated in metastatic CRC cells. Statistical analyses with human cancer tissues reveal that the expression of miR-221* and miR-224 correlated well with survival, but inversely correlated with cancer progression and recurrence. Moreover, miR-221* and miR-224 were found to inhibit migration of CRC cells *in vitro* and inhibit tumor metastasis *in vivo*. Furthermore, we demonstrated that overexpression of miR-221* and miR-224 induced the expression of maspin via direct suppression of MBD2, leading to lowered cancer metastasis. These

findings indicate that miR-221* and 224 may serve as novel CRC biomarkers and promising candidates for the development of new anti-metastasis agents.

Materials and Methods

Microarray analysis of miRNA expression

The miRNA microarray experiments were performed using Agilent's miRNA microarray system (V2), which contains 723 human and 76 human viral miRNAs catalogued in the Sanger miRNA database v 10.1 (Agilent technologies, Foster City, CA). Data was collected and normalized to non-functional small RNA internal controls. MiRNAs exhibiting a fold-change of greater than 2.0 at a false discovery rate of 10% were chosen for further study.

MiRNA mimics and inhibitors

The mature miR-221* sequence was: ACCUGGCAUACAAUGUAGAUUU. miRIDIAN Hairpin Inhibitor hsa-miR-221* and has-miR-224 inhibitors (Cat #: IH301163-02 and IH300581-08, Dharmacon) was used to repress the expression of miR-221* in CRC cells; while miRIDIAN Mimic hsa-miR-221* and has-miR-224 (Cat #: C-301163-01 and C-300581-7, Dharmacon) was used to overexpress miR-221* in CRC cells. The mature miR-224 sequence was: CAAGUCACUAGUGGUUCCGUU. miRIDIAN Hairpin Inhibitor hsa-miR-224 (Dharmacon) was used to repress the expression of miR-224 in CRC cells; while miRIDIAN Mimic hsa-miR-224 (Dharmacon) was used to overexpress miR-224 in CRC cells. miRIDIAN microRNA Hairpin Inhibitor Negative Control #1 and miRIDIAN microRNA Mimic Negative Control #1 and were used as controls.

miRNA Real-time PCR

Total mRNAs were extracted from treated colon cancer cells and clinical samples with Trizol (Invitrogen Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. Next, mRNA from each sample was used for miRNA real-time PCR with miScript PCR Assay Kit and specific primers for miR-221* and miR-224, respectively according to the manufacturer's instructions. miRs expression was calculated relative to U44 and U48 rRNA. The expression of miR-221* and miR-224 in cell lines and tissue samples was determined by real-time PCR.

Chromatin immunoprecipitation

By using published procedures[10], immunoprecipitated DNA was quantitated by real-time quantitative PCR. Primer sets were chosen to amplify approximately 100–150 bp around the indicated region. The enrichments of MBD2 and HDAC1 at the examined regions were quantitated relative to the input amount.

In situ hybridization

In situ hybridization (ISH) was carried out on deparaffinized human colorectal tissues as previously described[11]. The sequences of the probes containing the six dispersed locked nucleic acid (LNA) modified bases with digoxigenin conjugated to the 5' end were: miR-221*(5') ATTCTACATTGTATGCCAGGT; miR-224(5') AACGGAACCACTAGTGACTTG.

Methylation assay

DNAs obtained from surgical samples were treated with bisulfite modification and examined for the methylation status of 19 CpG dinucleotides within the maspin gene promoter region as described previously[12]. The maspin gene promoter was amplified from

the bisulfite-modified DNA by two rounds of PCR using nested primers specific to the bisulfite-modified sequence of the maspin gene CpG island.

By using published procedures[12], DNAs extracted from cell lines were treated with CpGenome™ DNA Modification Kit (Intergen). Primer sequences were listed in the Supplementary Material.

Statistical Analysis

Analysis was performed with SPSS 16.0 for Windows (SPSS Inc); the Pearson χ^2 test or Fisher's exact test was used to compare qualitative variables; and quantitative variables were analyzed by the Student *t* test (One-way ANOVA for differences among 3 groups) or Pearson's correlation test. Kaplan-Meier analysis was used to determine the survival. Log-rank test was used to compare patients' survival between subgroups; the Cox regression model was used to perform multivariate analysis. Receiver operating characteristic (ROC) curve analysis was used to determine the predictive value of the parameters. $P < 0.05$ was considered statistically significant.

Results

MiR-221* and miR-224 are down-regulated in metastatic CRC cells

Using an array-based miRNA profiling (Supplementary Figure 1) and q-RT-PCR (Figure 1A), we identified and validated that miR-221* and miR-224 were markedly down-regulated in highly metastatic SW620 cells versus parental non-metastatic SW480 cells. To determine whether the under-expression of miR-221* and miR-224 was a universal phenomenon in CRC cells, the expression levels of these candidate miRNAs were then investigated in a series of CRC cell lines. Similarly, the expression levels of miR-221* and miR-224 were found to be lower in metastatic CRC cells (KM12L4a, DLD1, LoVo) than in non-metastatic ones (KM12C, HCT116, HT29) (Figure 1B and C). Importantly, the miR-221* and miR-224 expression patterns were also recapitulated in SW620 tumor xenografts. We showed that the expression levels of miR-221* and miR-224 were lower in metastasis loci of the mice than in the primary tumor sites (Figure 1D). To determine the clinical relevance of miR-221* and miR-224 in human colorectal cancer, we investigated miR-221* and miR-224 expressions by real-time PCR in normal colon tissue, primary colorectal tumor and lymph node metastasis specimens. MiR-221* and miR-224 were almost undetectable in lymph node metastasis while being highly-expressed in primary colorectal tumor samples (Figure 1E, F and Supplementary Figure 2). In addition, the expression of miR-221* was found to be down-regulated in tumors compared with normal colon tissues ($P = 0.0117$) (Fig. 1E). However, there was no significant difference in expression of miR-224 between normal colon tissues and tumors (Fig. 1F). These data further support the finding that miR-221* and miR-224 are specifically down-regulated in metastatic CRC cells.

MiR-221* and miR-224 are correlated with clinicopathologic features

We then asked whether miR-221* and miR-224 are associated with tumor stages. According to the Union of International Cancer Control (UICC) staging system, the primary CRC samples were divided into two subsets (stages I/II and stages III/IV). Importantly, our analysis showed that the expression levels of miR-221* and miR-224 were closely associated with clinical stage of CRC ($P<0.001$) (Supplementary Figure 3). Furthermore, we investigated the correlations between miR-221* and miR-224 expressions and other clinic-pathologic features. Neither miR-221* nor miR-224 expression correlated with age, sex or tumor location. However, low levels of miR-221* or miR-224 expression were correlated with poor differentiation, advanced disease stage, large tumor size, and high rates

of lymph node metastasis in patients (Supplementary Table 1), raising the possibility that these two miRNAs may serve as novel prognostic markers for advanced/metastatic colorectal cancer.

MiRa-221* and miR-224 could be used as prognostic factors of CRC

To evaluate the potential of miR-221* and miR-224 as prognostic indicators, Kaplan-Meier survival test was performed. It was shown that low levels of miR-221* or miR-224 expression were inversely associated with overall survivals (OS) (P <0.001) (Figure 2A) and disease-free survivals (DFS) (P <0.001) (Figure 2B). Univariate and multivariate analyses further revealed that disease stage, presence of lymph node metastasis, miR-221*, miR-224, and the combination of miR-221* and miR-224 were independent risk factors for OS and DFS (Supplementary Table 2). Thus, examining the levels of miR-221*, miR-224 individually or in combination could be clinically useful for predicting cancer disease stages.

To determine whether the combination of miR-221* and miR-224 was a more accurate prognostic factor in predicting the outcome of CRC, patients were classified into four groups according to their miR-221* and miR-224 densities: group I (n = 35), high miR-221* and miR-224 density; group II (n = 27), high miR-221* but low miR-224; group III (n = 24), low miR-221* but high miR-224 density; and group IV (n = 22), low miR-221* and miR-224 density. Differences in both OS (P < 0.001) and DFS (P < 0.001) were significant among the four groups. The 5-year OS and DFS rates were 94% and 93%, respectively, for group I but 9% and 0%, respectively, for group IV (Figure 2C). Moreover, clinic-pathologic factors showing significance by multivariate survival analysis (Supplementary Table 2) and the combination of miR-221* and miR-224 were adopted, and their predictive values were then studied by receiver operating characteristic (ROC) analysis. All of the adopted factors predicted death and recurrence precisely (P < 0.05 for all); however, ROC analysis indicated that the predictive value of the combination of miR-221* and miR-224 was the best for both death and recurrence (Figure 2D). Taken together, miR-221* and miR-224 may both be negative regulators for colorectal cancer metastasis, and this study is the first to reveal that passenger strand miRNAs can be a significant player in inhibiting tumor progression.

Up-regulation of miR-221* and miR-224 reduces tumor size and inhibits metastasis in mice

To evaluate the therapeutic potential of miR-221* and miR-224 *in vivo*, miR-221* inhibitor and miR-224 inhibitor (synthetic RNA duplex, Dharmacon, see methods) were transfected into SW480-GFP cells, respectively; while, miR-221* mimic and miR-224 mimic were transfected into SW620-GFP cells, respectively. The cells were then injected subcutaneously into nude mice. Mice received the SW480 cells with miR-221* inhibitor or miR-224 inhibitor showed increased tumor sizes and reduced survival times; while mice received the SW620 cells with miR-221* mimic or miR-224 mimic exhibited decreased tumor sizes and prolonged survival times (Figure 3A, and Supplementary Figure 4A, C). Moreover, we performed orthotopic injections with the CRC cells expressing inhibitors or mimics[13], and observed similar effects of miR-221* and miR-224 on cancer growth and survivals: injection of miR-221* or miR-224 mimic bearing cells repressed tumor extended survivals in mice, whereas injection of miR-221* or miR-224 inhibitor increased tumor sizes and decreased survivals (Figure 3B, and Supplementary Figure 4B, D). To determine the effect of miR-221* and miR-224 on metastasis, we enumerated the CRC cell number in sera from the orthotopic injection mice as well as the metastasis number in their major metastatic organs (lung, spleen, liver, and kidney). MiR-221* or miR-224 under-expression markedly increased the metastatic CRC cell number in serum of the mice (Figure 3C), and the metastasis number in liver of orthotopic SW480 tumor-bearing mice (Figure 3D), as well as in other organs (data not shown). In contrast, miR-221* or miR-224 overexpression suppressed the metastasis in SW620 tumor-bearing mice. To account for any manipulation

errors in these animal models, we evaluated the effect of miR-221* and miR-224 on late stage CRC metastasis in tail vein injection mice model. As expected, miR-221* inhibitor or miR-224 inhibitor induced a pronounced increase of lung metastasis number and a sharp decrease of survival in treated-SW480 injected mice, while miR-221* mimic or miR-224 mimic decreased the lung metastasis number and prolonged the survival time in treated-SW620 injected mice (Figure 3E, F and Supplementary Figure 4E). Moreover, we demonstrated that down-regulation of miR-221* and miR-224 could also promote the lymphogenous metastasis (Supplementary Figure 5). Taken together, these results strongly indicate that miR-221* and miR-224 may have therapeutic efficacy against established CRC tumors, as well as late stage metastasis.

Up-regulation of miR-221* and miR-224 inhibits metastasis in vitro

To gain the molecular mechanism underlying tumor inhibition, we then sought to determine whether miR-221* and miR-224 could regulate metastasis *in vitro*. We performed standard matrigel invasion and transwell migration assays[14] and found that both miR-221* mimic and miR-224 mimic decreased cell invasion and migration; while their inhibitors increased the invasion and migration after 12 h treatment (Figure 4A and B). These data suggest that miR-221* and miR-224 may inhibit cell invasiveness and motility, which are mechanistic determinants for tumor metastasis. To further determine the mechanism of tumor metastasis inhibition, we measured the tumor growth rate and cell cycle in these genetically-altered SW620 and SW480 cells. We found that cell cycle and growth rate were not significantly changed following treatment with miR-221* and miR-224 inhibitors as well as their mimics (Figure 4C and D). These results indicate that miR-221* and miR-224 may primarily exert their anti-metastatic activity by limiting cell mobility and reducing cancerous spread, probably with limited effects on cancer growth speed.

MBD2 is a target of miR-221* and miR-224

To determine the targets of miR-221* and miR-224, we screened a large number of potential target proteins in database library to identify promising miRNA binding seed sequences within 3' UTR. Using bioinformatic analyses (microRNA.org, Targetscan, microRNASeq), we found that MBD2 is a candidate target for both miR-221* and miR-224 (Figure 5A). To verify this analysis, MBD2 3' UTR, containing miR-221* or miR-224 binding site, were cloned downstream of the luciferase open reading frame. On the other hand, MBD2 3' UTR, which contains mutated miR-221* or miR-224 binding site, were also introduced into the luciferase construct. The luciferase constructs were transfected into SW480 and SW620 cells. Next, we cotransfected miR-221* mimic and miR-224 mimic to SW620 cells, respectively; and we also cotransfected miR-221* inhibitor and miR-224 inhibitor into SW480 cells, respectively. qRT-PCR analysis confirmed that the cotransfection altered the expression of these miRNAs (Figure 5B), which in turn significantly affected luciferase expression (Figure 5C). We observed a consistent reduction of luciferase expression in cells transfected with miRNA binding site mutant plasmids (Figure 5C). Using western blotting and qRT-PCR analyses, we also found that over-expression of miR-221* or miR-224 significantly decreased MBD2 protein and mRNA expression in SW620 cells; while, knockdown of miR-221* and miR-224 up-regulated MBD2 protein and mRNA expression in SW480 cells (Figure 5D and E). To further determine the significance of miR-221* and miR-224 expression, we used several additional CRC cells and confirmed that MBD2 mRNA and protein levels were inversely correlated with miR-221* and miR-224 expression (Supplementary Figure 6A and B). To gain the insight of our experimental data in clinical indications, we further examined the expression of miR-221*, miR-224 and MBD2 in primary tumor and metastasis samples of CRC patients. We observed that CRC lymph node metastasis (LNM) samples exhibited up-regulated MBD2 expression and down-regulated miR-221* and miR-224 expression, compared with the primary tumor tissues from the same

patients (Supplementary Figure 7). To further corroborate these findings, in situ hybridization analysis was performed on CRC LNM samples and primary CRC tissues, followed by immunohistochemistry for MBD2 (Figure 5F). LNM samples showed high levels of MBD2 and undetectable levels of miR-221* and miR-224, whereas primary CRC cells showed relatively lower levels of MBD2 and readily-detectable miR-221* and miR-224 expression. Collectively, these observations from cell lines and patient lymph node tissues suggest that MBD2 may be an important direct target of miR-221* and miR-224. Moreover, we demonstrated that miR-221* and miR-224 have an additive role in controlling MBD2 transcription, as shown by the expression of MBD2 in primary tumor tissues of CRC patients which were divided into subgroups as in Figure 2C (Supplementary Figure 8). Of note, our study is the first to suggest that MBD2 may be a direct tumor metastasis target.

MiR-221* and miR-224 increased the expression of maspin through MBD2 down-regulation

To convincingly validate our data and further illustrate the anti-metastasis mechanism of miR-221* and miR-224 via MBD2, we examined the expression of metastasis-related proteins. Maspin, a recently characterized metastasis suppressor, was found to be negatively associated with MBD2 (Figure 6A), while some pro-metastasis proteins were found to be positively correlated with MBD2 (data not shown). Maspin expression levels were significantly altered by knockdown or overexpression of MBD2, indicating that maspin might be directly regulated by MBD2 (Figure 6B). To evaluate the clinical relevance of maspin expression, we found that CRC lymph node metastasis (LNM) samples exhibited down-regulated maspin expression, compared with the primary tumor tissues from the same patients (Supplementary Figure 7). As predicted, the levels of maspin correlated with miR-221* and miR-224 expression, but inversely correlated with MBD2 expression in patients.

To determine the mechanism by which MBD2 regulates maspin expression, we evaluated the mRNA expression level of maspin. Maspin mRNA expression was shown to be significantly down-regulated by MBD2 overexpression in SW480 cells, while elevated by MBD2 inhibition in SW620 cells (Figure 6C). Since MBD2 was known as a transcription repressor which causes gene silencing through promoter methylation[15], we further asked whether the under-expression of maspin was caused by epigenetic modifications. Using a methylation-specific PCR (MSP) method, we evaluated the promoter status of maspin gene. Maspin promoter methylation was found to be induced by MBD2 over-expression in SW480 cells, and suppressed by MBD2 inhibition in SW620 cells (Figure 6D). Furthermore, a total of 19 CpG cites located between nucleotides −194 and +105 of the maspin promoter was examined by bisulfite sequencing. Consistent with the differential expressions of MBD2, dense cytosine methylation was observed in the CpG islands of the maspin promoter procured from lymph node metastasis specimens; on the other hand, only sporadic cytosine methylation was identified in the CpG islands of the maspin promoter procured from primary colorectal tumor specimens (Figure 6E). To mechanistically characterize the epigenetic patterns, we used a demethylating agent, 5-aza-2'-deoxycytidine (DAC) and histone deacetylase inhibitor, trichostatin A (TSA) alone or together and evaluated the methylation. We found that demethylation of the maspin gene promoter restored maspin mRNA expression by all these treatments (Figure 6C and D), indicating that the maspin gene might be regulated by both MBD2 and HDACs. However, we cannot exclude the possibility that MBD2 might indirectly bind to an unmethylated maspin gene promoter through HDAC1.

MBD2 down-regulates maspin in association with HDAC1

To further understand the epigenetic mechanism, we performed ChIP assays in SW620 cells and confirmed that HDAC1 and MBD2 could bind to maspin gene (Figure 7A). We then

applied methylation and acetylation inhibitor as indicated to SW620 cells and found that both MBD2-maspin and HDAC1-maspin interactions could be disrupted by MBD2 inhibition, indicating that MBD2 may be a bona fide target for miR-221* and miR-224 involved in these interactions (Figure 7B). However, DAC could only disrupt MBD2-maspin interaction and had no effect on HDAC1-maspin interaction, indicating that MBD2-maspin interaction required the hypermethylated status of maspin gene, while HDAC1-maspin interaction did not. Moreover, MBD2 and HDAC1 have been reported to be able to interact with each other[15]. To further strengthen the significance of epigenetic regulation and elaborate the concrete mechanism, we confirmed this interaction in CRC cells (Figure 7C). Collectively, our data indicate that MBD2 regulates metastasis pathway in association with HDAC1 by epigenetically inhibiting maspin via direct binding.

In summary, our studies have revealed novel metastasis negative regulators miR-221* and miR-224 in colorectal cancer by a combination of analyses in patient samples, cell lines and mice models. MiR-224 has been shown to affect CRC methotrexate resistance *in vitro*[16], consistent with our findings for repressing tumor growth. Previous reports have generally shown that miRNA star forms have functions similar to those to their counterpart miR strands[17], while we found that miR221* has a novel function in relation to its counterpart miR-221. Mechanistically, we identified that a passenger strand may modify metastatic repressor maspin through molecular interactions and epigenetic regulations of MBD2 (Supplementary Figure 9). This information may benefit the development of novel therapeutic targets or diagnostic markers for colorectal cancer.

Discussion

In this study, we found that the expression levels of miR-221* and miR-224 negatively correlated with metastatic abilities of CRC cells both *in vitro and in vivo*. Statistical analyses further revealed that miR-221* and miR-224 could serve as promising prognostic factors for both severity and recurrence of CRC patients. Our study showed that miR-221* and miR-224 could synergistically target the same protein MBD2 in CRC cells. Thus, we hypothesized that their combination, which had greater inhibitory effect on their target, might be a more accurate prognostic factor. Hence our studies are consistent with the current notion that multiple miRNAs can share a target to synergistically repress one gene. Statistical analyses with a number of clinical CRC samples further confirmed that the predictive value of the combination of miR-221* and miR-224 was the best, suggesting that this combination could be used as a prognostic biomarker of CRC.

MiR-224 is aberrantly expressed in several human neoplasms[18–20] and is characterized by contradictory properties, since it can promote[18] or inhibit[19] cancer cell growth, depending on the malignancy types. Accumulating evidence has demonstrated that miR-224 is up-regulated in CRC compared with normal colorectal tissue[21, 22]. However, the relative expression levels of miR-224 between primary colorectal tumor and its LNM tissue or among different stages of CRC are still unclear. Here, we found that miR-224 expression levels decreased as CRC progressed from early stage tumor to late stage tumor and LNM, which finally leads to metastasis and death. On the other hand, the role of miRNA* in tumor metastasis is totally undefined. Although several studies have reported that the function of miRNA* in cancer cells may be similar to their counterpart strand[17, 23], we demonstrated here that miR-221* possessed metastasis-suppressive ability, distinct from its counterpart miR-221[24–27] (Supplementary Figure 10).

In the present study, expression levels of miR-221* and miR-224 were shown to significantly modulate tumor sizes, metastatic cell numbers, and survival times in nude mouse models. However, *in vitro* experiments revealed that miR-221* and miR-224 only

modulated invasion and migration, while having no impact on cell viability and cell-cycle progression of CRC cells. This inconsistency might have resulted from the anti-angiogenic effect of maspin, which was shown to be a downstream target of miR-221* and miR-224[28]. The effect of maspin has mostly been studied in the endothelial cells. *In vitro*, maspin acts directly on cultured endothelial cells to impede their migration towards basic fibroblast growth factor and vascular endothelial growth factor and to limit mitogenesis and tube formation, while *in vivo*, it blocks neovascularization[28]. Our results showed that maspin did not affect mitogenesis of CRC cells *in vitro*. However, maspin could be synthesized in CRC cells and function on endothelial cells *in vivo* because it is a secreted protein[29].

To our knowledge, the present study, for the first time, revealed a novel role of miR-221* and miR-224 in CRC metastasis. A new anti-metastatic mechanism mediated by these miRNAs was further identified as epigenetic regulation of metastatic promoter and inhibitor by targeting MBD2. Our studies identified that miR-221* and miR-224 may be potential prognostic predictors and therapeutic targets for CRC.

Acknowledgments

We thank S. Rolling of UND imaging core for help with confocal imaging.

Declaration of all sources of funding: This project was supported by National 973 Basic Research Program of China (2013CB911300, 2011CB910703), the National Science and Technology Major Project (2012ZX09501001-003) and Chinese NSFC (81172173, 81225015) to C.H.; Flight Attendant Medical Research Institute (FAMRI, Grant #103007), NIH AI101973-01, and AI097532-01A1 to M.W. The funding agencies have no role in the design of the study and analysis of the data.

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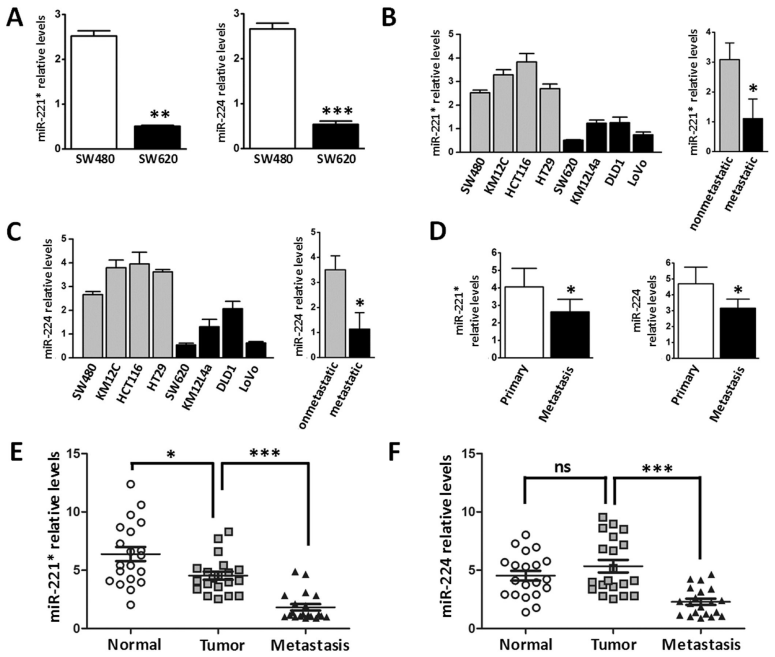


Figure 1.

miR-221* and miR-224 are repressed in metastatic cancer cell lines and human cancer tissues. (A) Real-time RT-PCR of miR-221* and miR-224 in SW480 and SW620 cells. (B, C) Real-time RT-PCR of miR-221* and miR-224 with RNA extracts isolated from different CRC cells. (D) Real-time RT-PCR of miR-221* and miR-224 with RNA extracts isolated from orthotopic CRC mouse model. RNA samples were prepared with primary tumor tissue and lung metastasis tissue, respectively (n=6). (E, F) Real-time RT-PCR of miR-221* and miR-224 on 20 groups of normal colon, colorectal cancer and lymph-node metastasis tissues (each group of normal, cancer and metastasis tissues was collected on the same patient) (One-way ANOVA). Average values and SDs were calculated from triplicate samples. *$P <$ 0.05; **$P <$ 0.01; ***$P <$ 0.001.

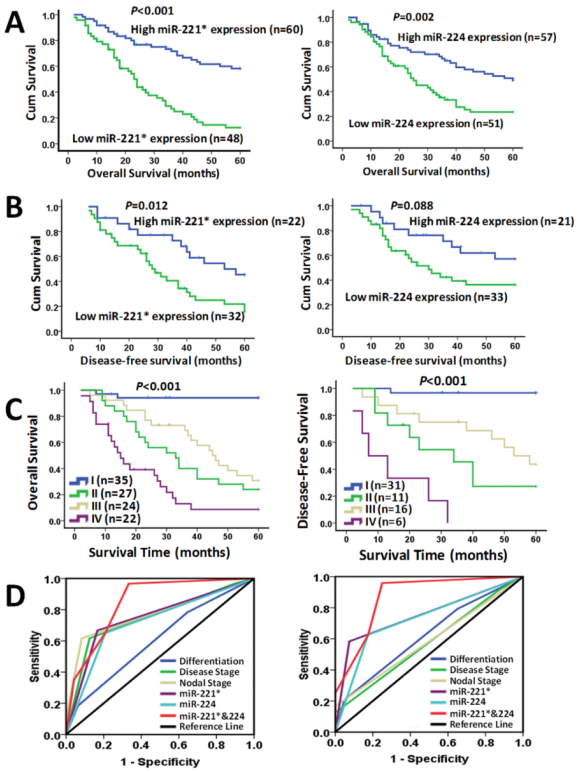


Figure 2.

Prognostic value of miR-221* and miR-224. (A, B) Cumulative overall survival and disease-free survival curves of patients with high or low miR-221* and miR-224 expression levels. (C) Cumulative overall and disease-free survival curves of the combination of miR-221* and miR-224. Patients were classified into four groups according to their tumoral miR-221* and miR-224 expressions: group I (n=35), both high expression; group II (n=27), high miR-221* but low miR-224 expression; group III (n=24), high miR-224 but low miR-221* expression; group IV (n=22), both low expression. (D) All factors adopted in ROC analysis predicted death and recurrence precisely during follow-up ($P < 0.05$ for all). The predictive value of the combination of miR-221* and miR-224 was the best one.

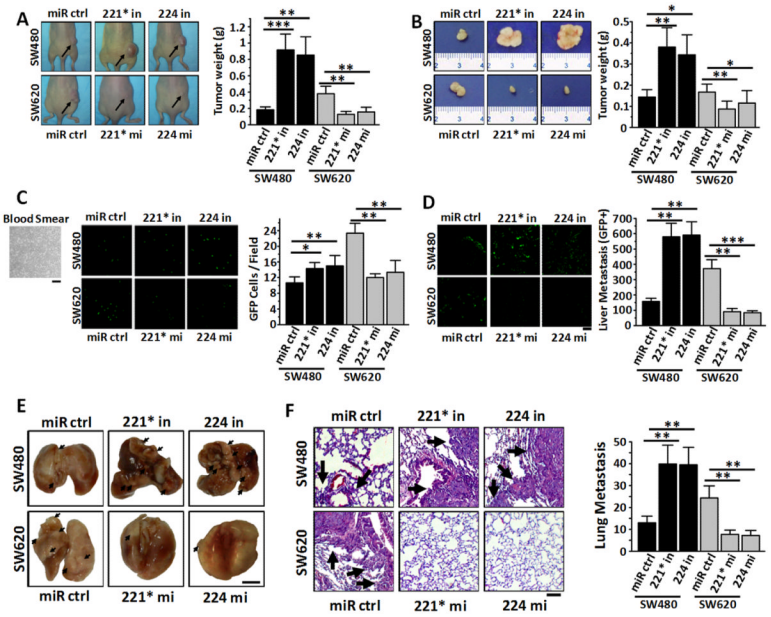
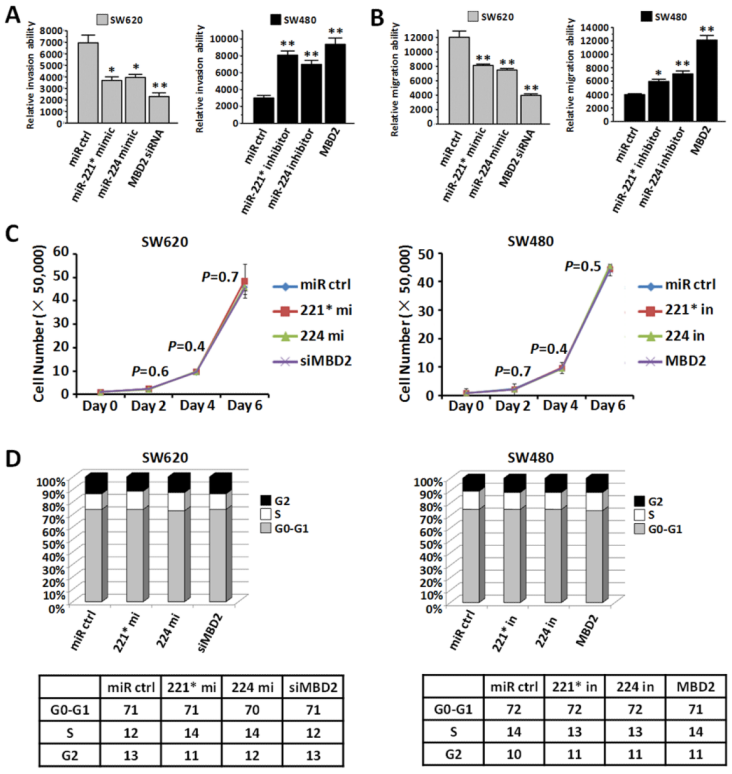


Figure 3.

Enforced expression of miR-221* and miR-224 suppresses tumor growth and metastasis in nude mouse models. (A) Whole body photos of subcutaneous CRC tumor-bearing mice *(left)*. Tumor weight of subcutaneous CRC tumors *(right)*. (B) Tumor mass images of orthotopic CRC tumors *(left)*. Tumor weight of orthotopic CRC tumors *(right)*. (C) Fluorescence images (scale bar, 1 mm) of fresh blood isolated from orthotopic CRC tumor-bearing mice for the presence of GFP-labeled tumor cells *(left)*. Quantification of GFP-labeled tumor cells *(right)*. (D) Fluorescence images of liver metastases of orthotopic CRC tumor-bearing mice *(left)*. Quantification of liver metastases *(right)*. (E) Bright field imaging (scale bar, 2 mm) of the lungs from SW480 and SW620 tumor-bearing mice treated with miR-221* and miR-224 mimics or miR-221* and miR-224 inhibitors, at 4 weeks after tail vein injection. Arrows indicate lung metastases. (F) H&E staining of the lungs from tail vein injected mice *(left)*. Quantification of lung metastases *(right)*. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$. Data are representative of three experiments in triplicate. Image data are representative for each group (n=7 orthotopic group or n=6 tail vein injection group).



	miR ctrl	221* mi	224 mi	siMBD2
G0-G1	71	71	70	71
S	12	14	14	12
G2	13	11	12	13

	miR ctrl	221* in	224 in	MBD2
G0-G1	72	72	72	71
S	14	13	13	14
G2	10	11	11	11

Figure 4.

miR-221* and miR-224 inhibit tumor metastasis by down-regulating cell migration and motility without inhibiting tumor cell growth. (A) Invasion assay showing the invasion ability of SW620 cells transfected with either miR-221* mimic, miR-224 mimic, or MBD2 siRNA, and SW480 cells transfected with either miR-221* inhibitor, or miR-224 inhibitor, or pMyc-MBD2 plasmid. (B) Migration assay showing the migration ability of SW620 and SW480 cells treated as in A. (C) Growth curves of SW620 cells and SW480 cells treated as in A. (D) Flow cytometric distributions of SW620 and SW480 cells treated as in A. *$P <$ 0.05; **$P <$ 0.01. All above data are representative of three experiments in triplicate.

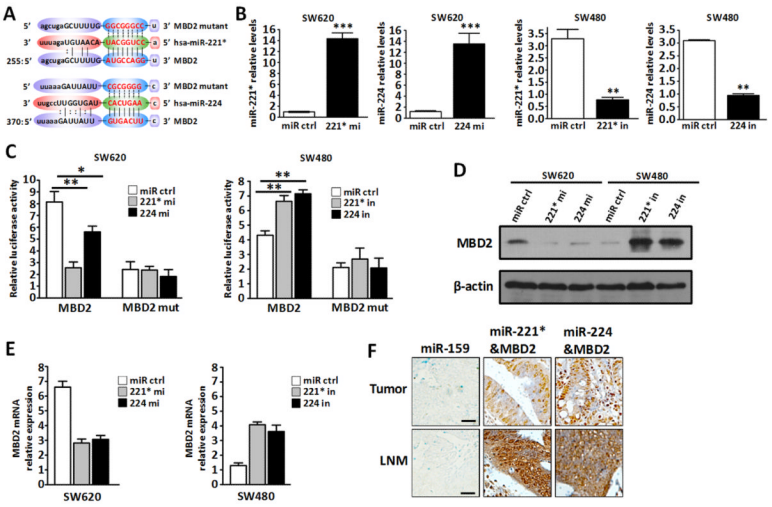


Figure 5.

MBD2 is a target of miR-221* and miR-224. (A) MBD2 3'UTRs contain one predicted miR-221* binding site and one predicted miR-224 binding site. The figure shows predicted duplex formations between MBD2 3′UTR (bottom) and miR-221* or miR-224 (middle). The sites of target mutagenesis (top) are indicated in red. (B) Real-time RT-PCR of miR-221* in SW620 and SW480 cells after enforced expression or down-regulation of miR-221* and 224. (C) pGL3-MBD2 luciferase constructs, containing a wild-type (left side of the histograms) or mutated (right side of the histograms) MBD2 3'UTRs, were transfected into SW620 and SW480 cells. Next, SW620 cells were co-transfected with miR-221* or miR-224 mimic, while SW480 cells were co-transfected with miR-221* or miR-224 inhibitor. Relative repression of firefly luciferase expression was standardized to a transfection control. (D) Western blot showing MBD2 expression in SW620 and SW480 cells after miR-221* or miR-224 overexpression or down-regulation. β-actin was used for loading control. (E) qRT-PCR of MBD2 mRNA in SW620 and SW480 cells after miR-221* or miR-224 overexpression or down-regulation. All above data are representative of three experiments in triplicate. (F) Colorectal cancer, and LNM tissues were analyzed for miR-221* and miR-224 (blue) expression by in situ hybridization (ISH), followed by immunohistochemistry for MBD2 (brown). Arrows indicate that the cancer cells expressing miR-221* and miR-224 were distinct from those cells expressing MBD2. MiR-159, which is not expressed in colon, was used as negative control. Scale bars, 25μm. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

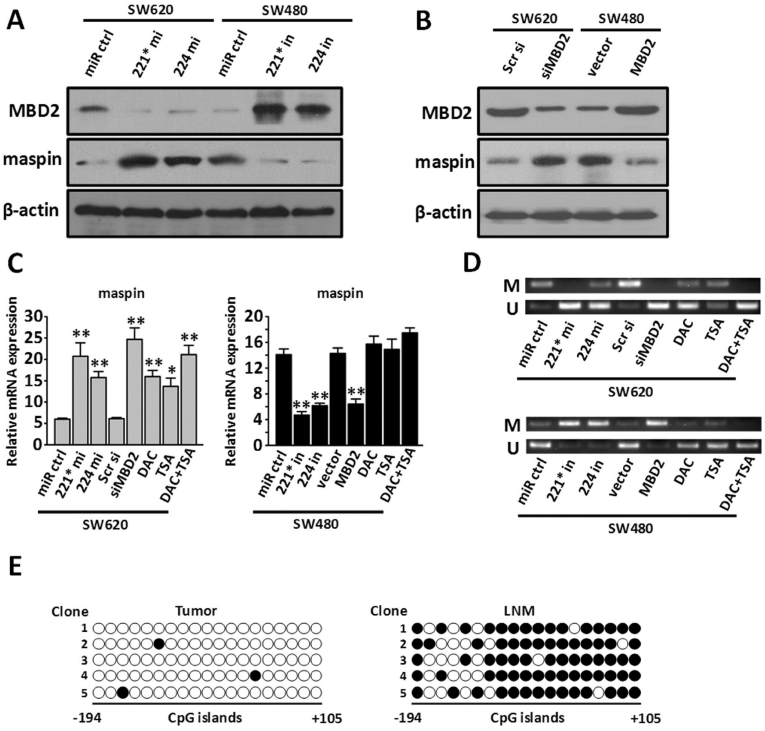


Figure 6.

MiR-221* and miR-224 increased the expression of maspin through MBD2 down-regulation. (A) Western blot showing MBD2 and maspin expression in SW620 and SW480 cells after miR-221* or miR-224 overexpression or down-regulation. β-actin was used for loading control. (B) Levels of MBD2 and maspin were assessed by western blot in SW620 and SW480 cells after MBD2 down-regulation or overexpression. β-actin was used for loading control. (C) miR-221*, miR-224 and MBD2 were overexpressed or down-regulated in SW620 and SW480 cells. Moreover, SW620 and SW480 cells were treated with either DAC (5μM) for 48 hours or TSA (0.5μM) for 24 hours or both (DAC for 24 hours, followed by TSA for additional 24 hours). Levels of maspin mRNA were assessed in treated SW620 and SW480 cells by qRT-PCR. (D) SW620 and SW480 cells were treated as in C. Genomic DNA extracted from the cells was treated with bisulfite and then subjected to methylation-specific PCR using the methylated DNA- (M) and unmethylated DNA- (U) specific primer sets. (E) PCR products were sequenced for the 19 CpG sites located between nucleotides −194 and +105 of the maspin promoter. The horizontal dots represent CpG islands while the vertical dots represent the individual 5 clones sequenced. Each black dot represents a methylated cytosine residue within the CpG islands. All above data are representative of three experiments in triplicate.

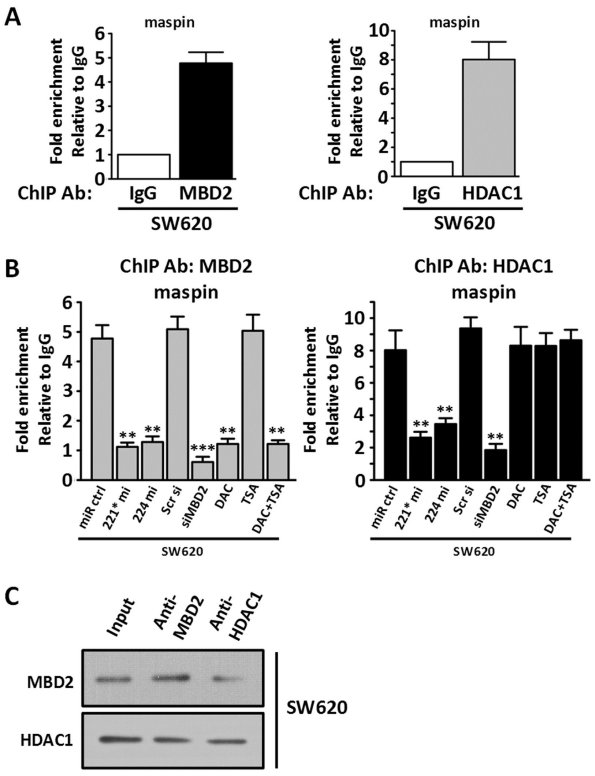


Figure 7.

MBD2 down-regulates maspin in association with HDAC1. (A) ChIP assay of MBD2 and HDAC1 on the promoter of maspin gene in SW620 cells. Unspecific IgG antibody was used as negative controls. Quantification of binding was represented as fold change to control IgG. (B) miR-221*, miR-224 and MBD2 were overexpressed or down-regulated in SW620 cells. Moreover, SW620 cells were treated with either DAC (5μM) for 48 hours or TSA (0.5μM) for 24 hours or both (DAC for 24 hours, followed by TSA for additional 24hours). ChIP assay of MBD2 or HDAC1 on the promoter of maspin gene was then performed. (C) MBD2 and HDAC1 were detected by western blot in nuclear extract (Input) and in complexes that were immuno-precipitated with anti-MBD2 and anti-HDAC1 antibodies. All above data are representative of three experiments in triplicate.