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

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BACKGROUND & 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* with levels that 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 with levels that were increased more than 2-fold in metastatic (SW620) CRC cells compared with nonmetastatic (SW480) cells. Quantitative polymerase chain reaction 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 nonmetastatic 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 miR-221* or miR-224 inhibitors had increased motility in vitro compared with SW480 control cells and formed larger, more metastatic tumors when injected 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′ untranslated region of *MBD2* messenger RNA as a target of miR-221* and 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 down-regulation. **CONCLUSIONS: 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.

Colorectal cancer (CRC) is the third most commonly diagnosed cancer in men and the second in women, with more than 1.2 million new cases of cancer and 608,700 deaths estimated to occur yearly in the United States.[1] Various risk groups (low, intermediate, high, and metastatic) that reflect relative survival are categorized using histological grade (Gleason score) and clinical TNM stage (local extent and/or nodal/distant metastases).[2] According to these statistics, when detected at an early stage, most patients with CRC 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, multistep process, has been identified to be responsible for most deaths from cancer, including CRC.[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 intense 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] MicroRNAs (miRNAs), a class of small cellular RNAs acting as agents of the RNA interference pathway, are attractive candidates

Abbreviations used in this paper: CRC, colorectal cancer; DAC, 5-aza-2′-deoxycytidine; DFS, disease-free survival; LNM, lymph node metastasis; miRNA, microRNA; mRNA, messenger RNA; OS, overall survival; ROC, receiver operating characteristic; RT-PCR, reverse-transcription polymerase chain reaction; TSA, trichostatin A; UTR, untranslated region.

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as upstream regulators of metastatic progression because miRNAs can posttranscriptionally 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 were 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 showed 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 miR-224 may serve as novel CRC biomarkers and promising candidates for the development of new antimetastasis 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 version 10.1 (Agilent Technologies, Foster City, CA). Data were collected and normalized to nonfunctional small RNA internal controls. miRNAs exhibiting a fold change of >2.0 at a false discovery rate of 10% were chosen for further study.

miRNA Mimics and Inhibitors

The mature miR-221* sequence was ACCUGGCAUAC AAUGUAGAUUU. miRIDIAN Hairpin Inhibitor hsa-miR-221* (catalog no. IH301163-02; Dharmacon, Pittsburgh, PA) was used to repress the expression of miR-221* in CRC cells, and miRIDIAN Mimic hsa-miR-221* (catalog no. C-301163-01; Dharmacon) was used to overexpress miR-221* in CRC cells. The mature miR-224 sequence was CAAGUCACUAGUGGUUCCGUU. miRIDIAN Hairpin Inhibitor hsa-miR-224 (catalog no. IH300581-08; Dharmacon) was used to repress the expression of miR-224 in CRC cells, and miRIDIAN Mimic hsa-miR-224 (catalog no. C-300581-7; Dharmacon) was used to overexpress miR-224 in CRC cells. miRIDIAN microRNA Hairpin Inhibitor Negative Control #1 and miRIDIAN microRNA Mimic Negative Control #1 were used as controls.

miRNA Real-Time Polymerase Chain Reaction

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

Chromatin Immunoprecipitation

Using published procedures,[10] immunoprecipitated DNA was quantitated by real-time quantitative PCR. Primer sets were chosen to amplify approximately 100 to 150 base pairs 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 was performed on deparaffinized human colorectal tissues as previously described.[11] The sequences of the probes containing the 6 dispersed locked nucleic acid modified bases with digoxigenin conjugated to the 5′ end were as follows: 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 2 rounds of PCR using nested primers specific to the bisulfite-modified sequence of the maspin gene CpG island.

Using published procedures,[12] DNAs extracted from cell lines were treated with the CpGenome DNA Modification Kit (Intergen, Burlington, MA). Primer sequences are listed in Supplementary Materials and Methods.

Statistical Analysis

Analysis was performed using SPSS 16.0 for Windows (SPSS Inc, Chicago, IL). Pearson χ^2 test or Fisher exact test was used to compare qualitative variables, and quantitative variables were analyzed using Student *t* test (one-way analysis of variance for differences among 3 groups) or Pearson correlation test. Kaplan–Meier analysis was used to determine survival. The log-rank test was used to compare patients' survival between subgroups, and 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. $P < .05$ 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 quantitative reverse-transcription (RT)-PCR (Figure 1*A*), we identified and validated that miR-221* and miR-224 were markedly down-regulated in highly metastatic SW620 cells versus parental nonmetastatic SW480 cells. To determine whether the underexpression 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 nonmetastatic ones (KM12C, HCT116, HT29) (Figure 1*B* and *C*). Importantly, the miR-221* and miR-224