MicroRNA-224 promotes cell proliferation and tumor growth in human colorectal cancer by repressing PHLPP1 and PHLPP2

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Running title: miR-224 target PHLPP1 and PHLPP2 in CRC

Keywords: miR-224, colorectal cancer, PHLPP1, PHLPP2, proliferation

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.
**Statement of Translational Relevance:**

Dysregulation of miR-224 has been implicated in the pathogenesis of hepatocellular carcinoma and breast cancer. However, the biological functions of miR-224 in the development and progression of colorectal cancer (CRC) remains unknown. This study revealed that miR-224 is overexpressed in CRC. High level of miR-224 expression is associated with aggressive phenotype of CRC and is an independent prognostic factor for poor survival of patients with CRC. Overexpression of miR-224 enhances the proliferation and tumorigenicity of CRC cells both in vitro and in vivo. Additionally, overexpression of miR-224 leads to downregulation of p21Cip1 and p27Kip1, and upregulation of cyclin D1 via activation of AKT/FOXO3a signaling. Moreover, both PHLPP1 and PHLPP2, antagonists of PI3K/AKT signaling, were confirmed as bona fide targets of miR-224. Our data highlight an important role for miR-224 in the pathogenesis of CRC, and miR-224 could be considered as a potential prognostic marker or therapeutic target for CRC.
ABSTRACT

Purpose: To investigate the clinicopathologic significance, role and mechanism of action of miR-224 in colorectal cancer (CRC).

Experimental Design: Real-time PCR was used to quantify miR-224 expression. The association of miR-224 with the clinicopathologic features and survival was evaluated in 110 CRC patients. The role of miR-224 in CRC was investigated using in vitro and in vivo assays. Luciferase reporter assays were conducted to confirm target gene associations.

Results: MiR-224 was overexpressed in CRC. High level expression of miR-224 was significantly associated with an aggressive phenotype and poor prognosis. Overexpression of miR-224 promoted CRC cell proliferation in vitro and tumor growth in vivo. Specifically, miR-224 accelerated the G1/S-phase transition through activation of AKT/FOXO3a signaling, downregulation of p21Cip1 and p27Kip1, and upregulation of cyclin D1. Moreover, both PHLPP1 and PHLPP2, antagonists of PI3K/AKT signaling, were confirmed as bona fide targets of miR-224. MiR-224 directly targeted the 3’ untranslated regions (3’-UTR) of the PHLPP1 and PHLPP2 mRNAs and repressed their expression.

Conclusion: This study reveals functional and mechanistic links between miRNA-224 and the tumor suppressors PHLPP1 and PHLPP2 in the pathogenesis of CRC. MiR-224 not only plays important roles in the regulation of cell proliferation and tumor growth in CRC, but also has potential as a prognostic marker or therapeutic target for CRC.
INTRODUCTION

Colorectal cancer (CRC) is one of the most common causes of cancer-related deaths worldwide (1). Although progress has been made in diagnostic and therapeutic strategies, the clinical outcome and prognosis of CRC patients with advanced stage disease still remains poor (2). Thus, understanding the molecular mechanisms which regulate the initiation and progression of CRC represents a pivotal step in the discovery and exploration of novel molecular targets, which may help to generate more effective therapies. Similarly to many other solid tumors, the initiation of CRC is a multi-gene and multi-step process. Activation of onco-proteins or inactivation of tumor suppressor genes affects multiple aspects of CRC tumorigenesis, such as cell proliferation, apoptosis, invasion and metastasis (3). However, although numerous mutations are well recognized to be associated with the development of CRC, the other genetic and epigenetic alterations responsible for this disease remain largely unknown.

MicroRNAs (miRNAs) are a class of small regulatory RNA molecules that repress protein translation through binding to the 3' untranslated region (UTR) of their target mRNAs in a sequence-specific manner. MiRNAs are highly conserved across species and participate in the regulation of a variety of basic biological processes such as development, cellular differentiation, proliferation, apoptosis and metabolism (4, 5). In recent years, miRNAs have been proven to play important roles in the pathogenesis of human cancer. Several miRNA expression profiling studies have been conducted in CRC, and a series of miRNAs (including miR-20, miR-21, miR-17-5p, miR-15b, miR-181b and miR-191) have been demonstrated to be valuable biomarkers associated with tumor progression and the clinical outcome of CRC (6-8). In addition, deregulation of miRNAs such as miR-21, miR-34a, miR-135 and miR-200 has been shown to modulate the cell viability, proliferation, invasion and metastasis of CRC cells (9-12). Nevertheless, the role of
miRNAs in the regulation of CRC-associated genes, and thus the role of miRNAs in the pathogenesis of CRC, remains elusive. Therefore, further extensive investigations are required to identify miRNAs that are associated with or involved in the initiation and progression of CRC.

Herein, we report that upregulation of miR-224 in CRC is associated with an aggressive phenotype and poor patient prognosis. Further investigations revealed that miR-224 directly targeted the 3′-UTRs of PH domain leucine-rich-repeats protein phosphatase 1 (PHLPP1) and PHLPP2, which function as tumor suppressors in CRC, to suppress the expression of these genes, which in turn promoted the proliferation and tumorigenicity of CRC cells.
MATERIALS AND METHODS

Tissue specimens and cell cultures

For the use of clinical materials for research purposes, prior approval was obtained from the Southern Medical University Institutional Board (Guangzhou, China). All samples were collected and analyzed with the prior written informed consent of the patients.

230 patients with histologically-diagnosed with colorectal columnar adenocarcinoma and undergoing surgical treatment were recruited for this study from the Department of General Surgery, Nanfang Hospital. Exclusion criteria included smoking, psychiatric disease, cerebrovascular disease, abnormal electrolytes, anaemia or hypertension. 110 cases of archived CRC tissue samples were collected between Mar 2005 and Oct 2007. 24.5% (27/110) of patients with rectal cancer received neo-adjuvant therapy and 78.2% (86/110) of patients received post-operative adjuvant therapy. 43 pairs of CRC biopsies and their matched adjacent normal tissues, and 77 cases of fresh CRC biopsies were collected between Jan 2011 and May 2013. All the tissue biopsies were freshly frozen in liquid nitrogen and stored at -80°C until further use. The medical records of the patients were reviewed to collect the following clinicopathologic information: age, gender, pathologic stage, Dukes’ stage, T stage, lymph node metastases, and distant metastasis. Survival data was available for the cohort of 110 patients. The median follow-up time was 56.7 months (range, 2-87 months).

Two CRC cell lines SW620 and HCT116 were purchased from ATCC Cell Biology Collection and were maintained in Department of Pathology, Southern Medical University. Cells were cultured in RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS, HyClone, Logan, UT) and 1% penicillin-streptomycin (Invitrogen, Carlsbad, CA) at 37°C with 5% CO2.
RNA Extraction and Real-time Quantitative PCR

For miRNA quantification, total miRNA was extracted from the cells and tissues using the mirVana miRNA Isolation Kit (Ambion, Austin, TX, USA) according to the manufacturer’s instructions, and then cDNA was synthesized from 5 ng of total RNA using the Taqman miRNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA). The expression levels of miR-224 were quantified using the miRNA-specific TaqMan MiRNA Assay Kit (Applied Biosystems). The relative miR-224 expression levels after normalization to U6 small nuclear RNA were calculated using $2^{\Delta \Delta Ct}$.

Real-time Quantitative PCR for target genes was performed as previously described (13). Primer sequences were summarized in Supplementary Table S1.

Plasmids and transfection

To generate a miR-224 expression vector, a 281 bp genomic fragment covering the region coding for pri-miR-224 and its up-and downstream regions was PCR amplified and cloned into the pLvthm vector (Addgene). The full-length PHLPP1 3′-UTR is 1012 bp long and the PHLPP2 3′-UTR is 3883 bp long. The miR-224 binding site in the PHLPP1 3′-UTR is located at 5917 to 5924 bp, and 7540 to 7546 bp in the PHLPP2 3′-UTR. The region of the human PHLPP1 3′ UTR from 5857 to 6318 bp and PHLPP2 3′-UTR from 7525 to 7835 bp were generated by PCR amplification and subcloned into the SacI/XmaI sites of the pGL3-basic luciferase reporter plasmid (Promega). The primers used to generate these constructs are listed in Supplementary Table S2. The miR-224 mimics, negative control and anti-miR-224 inhibitors were purchased from Genecopoeia (Genecopoeia Co. Ltd, Guangzhou, Guangdong, China) and transfected into CRC cells using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer’s
Western blotting

Protein lysates were prepared, subjected to SDS/PAGE, transferred onto PVDF membranes and blotted according to standard methods using anti-PHLPP1 (ab71972), anti-PHLPP2 (ab71973, Abcam, Cambridge, MA, USA), anti-p21 (#2947), anti-p27 (#3688), anti-cyclinD1 (#2978), anti-p-AKT (#4056), anti-AKT (#9272) antibodies (Cell Signaling Technology, Danvers, MA, USA), anti-p-FOXO3a (BS5019) or anti-FOXO3a (BS5520) antibodies (Bioworld Technology, MA, USA). Anti-α-Tubulin monoclonal antibody (T6199, Sigma, Saint Louis, MO, USA) was used as a loading control.

MTT assay, colony formation assay, soft-agar colony formation assay, flow cytometry and Immunohistochemistry (IHC)

The MTT assay, colony formation assay, soft-agar colony formation assay, flow cytometry and IHC were performed according to previously described methods (13). Further details are provided in the Supplementary Materials and Methods section.

Luciferase assays

Cells were seeded in triplicate in 24-well plates (1 × 10^5/well) and cultured for 24 h. The pGL3-luciferase reporter gene plasmids pGL3.0-PHLPP1-3′-UTR, pGL3-PHLPP2-3′-UTR or the control-luciferase plasmid were co-transfected into the cells with the control pRL-TK Renilla plasmid (Promega) using Lipofectamine 2000 Reagent (Invitrogen). Luciferase and Renilla activities were assayed 48 h after transfection using the Dual Luciferase Reporter Assay Kit (Promega) following the manufacturer’s instructions. All experiments were performed at least three times and the data are presented as mean ±
CRC xenograft model in nude mice

Animal experiments were performed as previously described (13). For details are provided in the Supplementary Materials and Methods.

Statistical analysis

All statistical analyses were performed using SPSS13.0 for Windows. The two-tailed paired Student’s t-test was used for analyzing two groups. The Mann-Whitney U test and Spearman’s correlation analyses were used to analyze the relationship between miR-224 expression and the clinicopathologic features of CRC. Survival curves were plotted by the Kaplan–Meier method and compared with the log-rank test. The significance of various survival-related variables was assessed using a multivariate Cox regression model. $P < 0.05$ was considered statistically significant.
RESULTS

MiR-224 is overexpressed in CRC

The expression of miR-224 was detected in 43 pairs of CRC biopsies and their matched adjacent normal tissues, and 77 cases of fresh CRC biopsies by real-time PCR revealed that miR-224 was significantly overexpressed in 76.7% (33/43) of the CRC tissue samples examined (T/N > 2 fold) compared to the matched adjacent normal tissues from the same patient, with up to 68-fold increases observed in the CRC tissue samples (Fig. 1A). Students’ t-test revealed that the miR-224 expression levels were significantly lower in the adjacent normal tissues than the CRC tissue samples ($P = 0.004$, Fig. 1B). In addition, miR-224 was expressed at relatively low levels in tumors with an early T classification (T1 and T2), and markedly increased in T3 tumors and further elevated in T4 tumors (Fig. 1C).

High levels of miR-224 expression are associated with progression and poor prognosis in CRC

To further investigate the clinicopathological and prognostic significance of miR-224 expression in CRC patients, the levels of miR-224 were quantified in a cohort of 107 CRC tissue samples using real-time PCR. The median relative expression level of miR-224 in all 110 CRC samples was chosen as the cut-off point for separating tumors with low expression of miR-224 and high expression of miR-224. Mann-Whitney U tests and Spearman’s correlation analysis showed that low expression of miR-224 was significantly associated with pathologic stage, Dukes’ stage, T classification and distant metastasis in CRC ($P < 0.05$, Table 1 and Supplementary Table S3). However, there were no significant correlations between the miR-224 expression level and age, gender, histology or N classification in CRC. Kaplan-Meier survival analysis revealed that patients with low miR-224 expression levels had a better clinical outcome (Fig. 1D). Multivariate survival
analysis indicated that the miR-224 expression level, T stage and pathologic stage were independent prognostic factors for outcome in patients with CRC (Supplementary Table S4).

**Overexpression of miR-224 enhances the proliferation and tumorigenicity of CRC cells in vitro and in vivo**

We transfected the CRC cell lines SW620 and HCT116 with hsa-miR-224 mimic oligonucleotides and examined the effects on cellular proliferation. MTT and colony formation assays revealed that overexpression of miR-224 significantly increased the growth rate of both CRC cell lines, compared to negative control (NC)-transfected cells (Supplementary Fig.S1 A and B). Moreover, the expression of Ki-67, a well-known marker of proliferation, was dramatically increased in miR-224-overexpressing SW620 and HCT116 cells compared with NC-transfected cells (Supplementary Fig.S1 C). Furthermore, the BrdUrd incorporation assay demonstrated that a higher percentage of miR-224-overexpressing SW620 and HCT116 cells contained newly synthesized DNA, compared with NC control cells (Supplementary Fig.S1 D).

We next examined the effect of miR-224 on the tumorigenicity of CRC cells using an anchorage-independent growth assay. Overexpression of miR-224 significantly enhanced the anchorage-independent growth ability of SW620 and HCT116 cells, as indicated by increased colony number and size (Fig. 2A). To test whether miR-224 could promote the growth of CRC tumors in vivo, we engineered SW620 and HCT116 cells to stably overexpress miR-224. These stably miR-224-overexpressing and control cells were subcutaneously inoculated into nude mice. As shown in Fig. 2B, the tumors in the SW620/miR-224 group grew more rapidly than the tumors in the SW620/Vector group ($P=0.004$). Similar results were obtained using HCT116 cells ($P<0.001$, Fig. 2D).
IHC staining confirmed that the tumors of the control group displayed much lower Ki-67 indexes than the tumors from the miR-224-overexpressing group (Fig. 2C and E).

**Upregulation of miR-224 accelerates CRC cell cycle progression and regulated cell cycle factors: AKT/FOXO3a activation in CRC cells was involved**

We determined the cell cycle distribution of miR-224-overexpressing SW620 and HCT116 cells by flow cytometry, to explore the possible mechanism by which miR-224 regulates CRC cell proliferation. A significant decrease in the percentage of cells in the G1/G0 phase and increase in the percentage of cells in the S phase were observed in miR-224-overexpressing cells (Fig. 3A). The expression levels of a number of critical cell cycle regulators were also detected. As expected, p21Cip1 and p27Kip1 were strikingly downregulated, while cyclin D1 was significantly upregulated at both the protein and mRNA levels in miR-224-overexpressing cells (Fig. 3B and C). It has been well documented that the expression of p27 Kip1 and cyclin D1 can be transcriptionally regulated by FOXO3a and, in turn, the transcriptional activity of FOXO3a is modulated by AKT phosphorylation (14). Thus, we hypothesized that upregulation of miR-224 may activate AKT/FOXO3a signaling. As shown in Fig. 3B and C, the phosphorylation levels of both FOXO3a and AKT increased in miR-224-overexpressing CRC cells. In addition, FOXO3a activity was strongly repressed by overexpression of miR-224, as demonstrated by a FOXO-luciferase reporter gene (Fig. 3C).

**Inhibition of miR-224 reduces the growth of CRC cells**

To confirm the effects of miR-224 on the proliferation of CRC cells, endogenous miR-224 was inhibited by ectopically expressing a miR-224 inhibitor in SW620 and HCT116 cells. As shown in Supplementary Fig.S2, inhibition of miR-224 evidently
increased the mRNA expression levels of both p21Cip1 and p27Kip1, and decreased the level of cyclin D1 mRNA in both cell lines. In addition, inhibition of miR-224 dramatically increased the percentage of cells in the G0/G1 phase and decreased the percentage of cells in the S phase, indicating that silencing of miR-224 induced G1/S arrest in CRC cells (Fig. 4A). Moreover, MTT assays (Fig. 4B) and the colony formation assay (Fig. 4C) confirmed that inhibition of miR-224 reduced the growth rate of SW620 and HCT116 CRC cells, compared to NC-transfected cells.

MiR-224 directly targets the tumor suppressors PHLPP1 and PHLPP2 in CRC cells

Recently, the PHLPP phosphatase family members PHLPP1 and PHLPP2 have been identified to directly dephosphorylate and serve as negative regulators of AKT (15). This prompted us to investigate whether the modulation of AKT/FOXO3a signaling activity by miR-224 were due to regulation of PHLPP1 and PHLPP2. The three bioinformatic algorithms (miRBase, Pictar and TargetScan) were utilized to predict miR-224 target sites. Gene Ontology enrichment analysis demonstrated that PHLPP1 and PHLPP2 were theoretical target genes of miR-224 (Fig. 5A). Western blotting analyses showed that the protein levels of both PHLPP1 and PHLPP2 were dramatically downregulated in miR-224-overexpressing cells, whereas PHLPP1 and PHLPP2 were upregulated after inhibition of miR-224 in both cell lines (Fig. 5B). RNA-immunoprecipitation (RIP) analysis revealed that overexpression of miR-224 increased the recruitment of PHLPP1 mRNA and PHLPP2 mRNA to miRNP complexes (Fig. 5C). To further analyze the relationship between miR-224 and PHLPP1 and PHLPP2, the PHLPP1 or PHLPP2 3′UTR fragments containing the wild type or mutant miR-224 binding sites were subcloned into the pGL3.0-Basic luciferase reporter vector. As shown in Fig. 5D, a dose-dependent reduction in both wild-type PHLPP1 and PHLPP2 reporter gene luciferase activity was
observed upon overexpression of miR-224 in both CRC cell lines, while inhibition of miR-224 increased wild-type \textit{PHLPP1} and \textit{PHLPP2} luciferase activity, compared to NC-transfected cells. However, when miR-224-binding sites in the 3’UTRs were mutated, we observed a dramatic relief of silencing of \textit{PHLPP1} and \textit{PHLPP2} genes, suggesting that these are probably true miR-224 target sites (Fig. 5D).

**Repression of PHLPP1 and PHLPP2 play essential roles in miR-224-induced proliferation of CRC cells**

Further analyses revealed that overexpression of PHLPP1 or PHLPP2 dramatically abrogated the miR-224-mediated regulation of AKT and FOXO3a phosphorylation (Fig. 6A and B) and modulation of the cell-cycle regulators p21, p27 and cyclin D1 (Supplementary Fig.S3). Additionally, flow cytometry analysis demonstrated that overexpression of PHLPP1 or PHLPP2 in miR-224-overexpressing CRC cancer cells decreased the percentage of cells in S phase (Fig. 6C). MTT assays indicated that overexpression of PHLPP1 or PHLPP2 significantly reduced the growth rate of miR-224-overexpressing SW620 and HCT116 cells (Fig. 6D).

We further investigated the expression levels of miR-224, PHLPP1 and PHLPP2 in clinical CRC samples. Analyses of the 43 paired CRC samples and matched adjacent normal tissues in which miR-224 was upregulated revealed that miR-224 expression correlated negatively with \textit{PHLPP1} and \textit{PHLPP2} mRNA expression (Supplementary Fig.S4). We also measured the expression of PHLPP1 and PHLPP2 in the xenograft tumors formed by SW620/Vector cells, SW620/miR-244 cells, HCT116/Vector cells and HCT116/miR-244 cells. Real-time PCR and IHC demonstrated that PHLPP1 and PHLPP2 mRNA and protein were expressed at lower levels in the tumors generated from miR-224 overexpressing cells, compared to the vector control cells (Supplementary Fig.S5).
DISCUSSION

MiRNAs are a large family of gene regulators that negatively regulate their target mRNAs in a sequence-specific manner (16). miRNAs may also function as tumour suppressors or oncogenes (17). Recent evidence has shown that miRNAs play essential roles in multiple biological processes related to cancer, including cell differentiation, proliferation, tumorigenesis, angiogenesis, invasion and metastasis (18-20). Upregulation of miR-224 has only been observed in a few tumor types, such as hepatocellular carcinoma, pancreatic ductal carcinoma, breast and renal cancer (21-24). Importantly, in hepatocellular carcinoma, p65/NFkappaB has been shown to act as a direct transcriptional regulator of miR-224 expression, which links upregulation of miR-224 with cell migration and invasion (21). Additionally, miR-224 can promote cell invasion and the expression of metastasis-related genes by targeting the Raf kinase inhibitor protein (RKIP) in human breast cancer cells (22). However, it was uncertain whether dysregulation of miR-224 was associated with the progression of CRC. In the current study, we found that miR-224 was upregulated in CRC tissues, and high miR-224 expression levels were found to be significantly associated with aggressive characteristics and poor patient prognosis in CRC. Additionally, ectopic overexpression of miR-224 promoted cell proliferation, proliferation and tumorigenesis in CRC cells. Thus, our data are consistent with previously published studies which associated changes in miR-224 expression with tumor formation and progression (21, 22), implicating miR-224 as an oncomir.

More specifically, we demonstrated that the molecular mechanism by which miR-224 promotes CRC cell proliferation was due, at least in part, to acceleration of the G1-S phase transition, downregulation of p21Cip1 and p27Kip1, and upregulation of cyclin D1 in cells overexpressing miR-224. Previous studies revealed that the PI3K/AKT signal transduction cascade was required for cell cycle progression through the G1 phase (25). In addition,
activation of PI3K/AKT decreases the cellular levels of p21Cip1 and p27Kip1, and induces cyclin D1 expression, thereby promoting cell proliferation (14, 26). The modulation of these cell cycle regulators by PI3K/AKT is accomplished either directly or indirectly through inhibiting the phosphorylation and activation of FOXO3a (14, 27, 28). Our data demonstrated that miR-224 activates PI3K/Akt signaling and promotes cell survival through modulation of FOXO3a, p21Cip1, p27Kip1 and Cyclin D1 expression. This pathway represents a new mechanism which may possibly underlie the development of CRC.

We further explored the mechanism by which miR-224 could activate the PI3K/Akt signaling cascade. Bioinformatic algorithms predicted that both isoforms of PH domain leucine-rich-repeats protein phosphatase (PHLPP), PHLPP1 and PHLPP2, were bona fide target genes of miR-224. PHLPP1 and PHLPP2 belong to a novel family of Ser/Thr protein phosphatases and play central roles in maintaining cell survival suppression through negatively regulating the signaling pathways activated by AKT, PKC, MAPK and Mst1 (29-32). Evidence has recently emerged to suggest that PHLPP1 and PHLPP2 act as tumor suppressor genes, and their expression is frequently depleted in a variety of human cancers, including breast cancer, prostate cancer and CRC (26, 33). As Ser/Thr protein phosphatases, PHLPP1 and PHLPP2 can directly dephosphorylate AKT to inhibit AKT signaling activity, which promotes apoptosis (15, 30, 33). Overexpression of PHLPP1 and PHLPP2 in cancer cell lines decreases cell proliferation and tumorigenesis both in vitro and in xenograft models (26, 30, 33-37). In CRC cells, stable overexpression of PHLPP1 or PHLPP2 blocked the G2/M transition or induced G1 cell cycle arrest, thus decreasing the rate of cell proliferation (26). Collectively, these studies suggest that restoration of the expression or function of the PHLPPs may represent a potential novel therapeutic intervention strategy for CRC. However, precise details of the mechanisms which regulate PHLPP1 and
PHLPP2 remain elusive.

Our results also confirmed that both PHLPP1 and PHLPP2 were direct targets of miR-224. In addition, ectopic overexpression of PHLPP1 or PHLPP2 (without the 3′-UTR) significantly abrogated the miR-224-induced proliferation of CRC cells in vitro. Finally, expression analyses of miR-224, PHLPP1 and PHLPP2 in clinical CRC tissues revealed significant negative correlations between miR-224 and the expression of PHLPP1 and PHLPP2. Taken together, these results suggest that the effects of miR-224 on the proliferation of CRC cells may be mediated via downregulation of PHLPP1 and PHLPP2 via miR-224 directly targeting the 3′-UTRs of these genes. Thus, our current study uncovers what we believe to be a novel mechanism leading to downregulation of PHLPP1 and PHLPP2 in cancer cells.

In summary, the present study provides, for the first time, an essential link between miR-224-mediated tumor growth and downregulation of PHLPP1 and PHLPP2 in CRC. Our findings suggest an important role for miR-224 in the proliferation of CRC cells. Understanding the precise roles played by miR-224 in the initiation and progression of CRC will not only increase our understanding of the biology of this tumor type, but may also allow the development of a novel therapeutic strategy based on inhibition of miR-224.

ACKNOWLEDGMENTS

This work was supported by the National Natural Science Foundation of China (No. 30901791, 81172055, 81071735), Major projects of National Natural Science Foundation of China (No. 81090422), State Key Program of National Natural Science Foundation of China (U1201226), National Basic Research Program of China (973 Program, No. 2010CB529402 and 2010CB529403), Guangdong Provincial Natural Science Foundation of China (No. S2012010009643), and
Zhu Jiang Science & Technology New Star Foundation in Guangzhou city (2012046), Science and Technology Innovation Foundation of Guangdong Higher Education (CXZD1016) and Key Program of National Natural Science Foundation of Guangdong, China (2010B031500012).
References


FIGURE LEGENDS

Fig.1. MiR-224 is overexpressed in CRC. A, Real-time PCR analysis of miR-224 expression in 43 paired human CRC tissues and the matched adjacent normal tissues; miR-224 expression was normalized to U6 and expressed relative to the matched adjacent normal tissues. Each bar represents the mean of three independent experiments. B, Mean expression of miR-224 in the 43 paired human CRC tissues and the matched adjacent normal tissues. C, Mean expression of miR-224 by T classification. ** P<0.01. D, Kaplan-Meier overall survival analysis for CRC patients stratified by high miR-224 expression (> median; n = 50) or low miR-224 expression (< median; n = 57).

Fig.2. Upregulation of miR-224 promotes the proliferation and tumorigenicity of CRC cells in vitro and in vivo. A, Anchorage-independent colony formation assays. Representative micrographs are shown (left); the numbers of colonies containing more than 50 cells (right) were scored. Each bar represents the mean of three independent experiments. * P < 0.05. B and D, Tumor xenograft model. Cells were injected into the hindlimbs of nude mice (n = 6). Data points are presented as the mean ± SD tumor volume. C and E, histopathology of xenograft tumors. The tumor sections were under H&E staining and IHC staining using antibody against Ki-67.

Fig.3. Overexpression of miR-224 promotes the G1/S transition and cell cycle progression in CRC cells. A, Flow cytometric analysis of the percentage of cells in
various phases of the cell cycle. B, Western blotting analysis of indicated proteins. The protein expression levels were quantified by comparing the gray level of each band using Quantity One Software. C, Relative FOXO3a luciferase reporter gene activity and real-time PCR analysis of the expression of p21, p27, and cyclin D1 mRNA; GAPDH was used as a loading control. Error bars represent the mean ± SD of three independent experiments. * P < 0.05.

**Fig.4.** Inhibition of miR-224 inhibits cell cycle progression and suppresses the proliferation of colon cancer cells. A, Flow cytometric analysis of cell cycle distribution. B and C, Inhibition of miR-224 inhibits cell growth as determined by MTT assays (B) and colony formation assays (C). Errorbars represent mean ± SD from 3 independent experiments. *, P < 0.01.

**Fig.5.** MiR-224 directly targets the 3′-UTRs of *PHLPP1* and *PHLPP2*. A, Predicted miR-224 target sequences in the 3′-UTRs of *PHLPP1* and *PHLPP2*. The nucleotides mutated in the 3′-UTRs of *PHLPP1* and *PHLPP2* are highlighted in red. B, Western blotting analysis of PHLPP1 and PHLPP2 expression in the indicated cells. The protein expression levels were quantified by comparing the gray level of each band using Quantity One Software. C, RNA-immunoprecipitation (RIP) analysis, as assessed by immunoprecipitation of Ago1 in HCT116 cells. IgG immunoprecipitation was used as a negative control. D, Luciferase activity assays of the indicated cells. Error bars represent the mean ± SD of three independent experiments. * P < 0.05.
Fig.6.  PHLPP1 and PHLPP2 cooperatively play a crucial role in miR-224-induced colon cancer cell growth. A, Western blotting analysis of PHLPP1 and PHLPP2 expression in the indicated cells. B, Western blotting analysis of indicated proteins in miR-224-overexpressing cells transfected with PHLPP1, PHLPP2 (without the 3’-UTRs) or empty vector. The protein expression levels were quantified by comparing the gray level of each band using Quantity One Software. C, Flow cytometric analysis of cell cycle distribution in the indicated cells. D, MTT analyses of indicated cells. Each bar represents the mean ± SD of three independent experiments. * P < 0.05.
### Table 1: Correlation between Clinicopathologic Features and miR-224 expression in 110 CRC tissues

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<td>21</td>
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<td>30</td>
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<td>Distant Metastasis</td>
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<tr>
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<td>30</td>
</tr>
<tr>
<td>Yes</td>
<td>8</td>
<td>21</td>
</tr>
</tbody>
</table>
Figure 1

A

Increasing fold of miR-224 (T/N)

Patients

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43

B

Relative expression of miR-224

Normal (n=43) Tumor (n=43)

C

Relative expression of miR-224 by T classification

Normal (n=43) T (n=30) T2 (n=30) T3 (n=30) T4 (n=30)

D

Overall survival

Cumulative Survival (%)

Survival time (months)

Low n=59 (<median) High n=51 (<median)

P=0.004
Figure 2

A

Vector  miR-224

SW620

HCT116

B

Days

Tumor volume (mm$^3$)

SW620-Vector

SW620-miR-224

C

H&E

Ki-67

Vector

miR-224

D

Days

Tumor volume (mm$^3$)

HCT116-Vector

HCT116-miR-224

E

H&E

Ki-67

Vector

miR-224
Figure 3

A

![Graph A showing cell number distribution for SW620 and HCT116 cells with and without miR-224 expression.]

B

<table>
<thead>
<tr>
<th>SW620</th>
<th>HCT116</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vector</td>
<td>miR-224</td>
</tr>
<tr>
<td>p21</td>
<td><img src="image" alt="p21 expression" /></td>
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<tr>
<td>p27</td>
<td><img src="image" alt="p27 expression" /></td>
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<tr>
<td>cyclin D1</td>
<td><img src="image" alt="cyclin D1 expression" /></td>
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<tr>
<td>p-AKT ser473</td>
<td><img src="image" alt="p-AKT expression" /></td>
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<tr>
<td>AKT</td>
<td><img src="image" alt="AKT expression" /></td>
</tr>
<tr>
<td>p-FOXO3a ser253</td>
<td><img src="image" alt="p-FOXO3a expression" /></td>
</tr>
<tr>
<td>FOXO3a</td>
<td><img src="image" alt="FOXO3a expression" /></td>
</tr>
<tr>
<td>α-Tubulin</td>
<td><img src="image" alt="α-Tubulin expression" /></td>
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</table>

![Graph B showing relative protein expression for SW620 and HCT116 cells with and without miR-224 expression.]

C

![Graph C showing related FOXO3a luciferase activity and related p21, p27, and cyclin D1 mRNA expression for SW620 and HCT116 cells with and without miR-224 expression.]

* indicates statistical significance.
Figure 4

A

- SW620
  - G1=58.73%, S=29.25%, G2/M=12.02
  - miR-224-in
  - G1=65.48%, S=19.72%, G2/M=14.80

- HCT116
  - G1=53.32%, S=28.46%, G2/M=18.22
  - miR-224-in
  - G1=65.48%, S=18.63%, G2/M=15.89

B

- SW620-NC
- SW620-miR-224-in
- HCT116-NC
- HCT116-miR-224-in

C

- SW620 NC miR-224-in
- HCT116 NC miR-224-in

Cell growth curve

Days 1 2 3 4 5

Increasing fold

SW620 HCT116

*
Figure 6

A

<table>
<thead>
<tr>
<th>SW620-miR-224</th>
<th>HCT116-miR-224</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vector</td>
<td>PHLPP1</td>
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<tr>
<td>PHLPP1</td>
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<tr>
<td>α-Tubulin</td>
<td>PHLPP1</td>
</tr>
<tr>
<td>SW620-miR-224</td>
<td>Vector</td>
</tr>
<tr>
<td>PHLPP2</td>
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<tr>
<td>PHLPP2</td>
<td>HCT116-miR-224</td>
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<tr>
<td>α-Tubulin</td>
<td>PHLPP2</td>
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</tbody>
</table>

Bar graphs showing relative protein expression:
- miR-224 in SW620 and HCT116 cells with and without PHLPP1 or PHLPP2

B

<table>
<thead>
<tr>
<th>SW620-miR-224</th>
<th>HCT116-miR-224</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vector</td>
<td>PHLPP1</td>
</tr>
<tr>
<td>PHLPP1</td>
<td>PHLPP2</td>
</tr>
<tr>
<td>AKT (ser473)</td>
<td></td>
</tr>
<tr>
<td>p-AKT</td>
<td>PHLPP1</td>
</tr>
<tr>
<td>p-AKT</td>
<td>PHLPP2</td>
</tr>
<tr>
<td>FOXO3a (ser253)</td>
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</tr>
<tr>
<td>FOXO3a</td>
<td>PHLPP1</td>
</tr>
<tr>
<td>FOXO3a</td>
<td>PHLPP2</td>
</tr>
<tr>
<td>α-Tubulin</td>
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</tr>
</tbody>
</table>

Bar graphs showing relative protein expression:
- p-AKT, AKT, p-FOXO3a, FOXO3a in SW620 and HCT116 cells with and without PHLPP1 or PHLPP2

C

<table>
<thead>
<tr>
<th>SW620-miR-224</th>
<th>HCT116-miR-224</th>
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<tbody>
<tr>
<td>G1</td>
<td>S</td>
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<td>G2/M</td>
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</tr>
<tr>
<td>G1</td>
<td>S</td>
</tr>
<tr>
<td>G2/M</td>
<td></td>
</tr>
</tbody>
</table>

Bar graphs showing percentage of cells in different phases:
- SW620 and HCT116 cells with and without PHLPP1 or PHLPP2

D

Graph showing cell growth curve:
- SW620-miR-224 and HCT116-miR-224 with and without PHLPP1 or PHLPP2

* denotes statistically significant differences.
Clinical Cancer Research

MicroRNA-224 promotes cell proliferation and tumor growth in human colorectal cancer by repressing PHLPP1 and PHLPP2

Wen-Ting Liao, Ting-Ting Li, Zheng-Gen Wang, et al.

Clin Cancer Res Published OnlineFirst July 11, 2013.

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