miR-200b suppresses invasiveness and modulates the cytoskeletal and adhesive machinery in esophageal squamous cell carcinoma cells via targeting Kindlin-2

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To further our understanding of the pathobiology of esophageal squamous cell carcinoma (ESCC), we previously performed microRNA profiling that revealed downregulation of miR-200b in ESCC. Using quantitative real-time PCR applied to 88 patient samples, we confirmed that ESCC tumors expressed significantly lower levels of miR-200b compared with the respective adjacent benign tissues (P = 0.003). Importantly, downregulation of miR-200b significantly correlated with shortened survival (P = 0.025), lymph node metastasis (P = 0.002) and advanced clinical stage (P = 0.020) in ESCC patients. Quantitative mass spectrometry identified 57 putative miR-200b targets, including Kindlin-2, previously implicated in the regulation of tumor invasiveness and actin cytoskeleton in other cell types. Enforced expression of miR-200b mimic in ESCC cells led to a decrease of Kindlin-2 expression, whereas transfection of miR-200b inhibitor induced Kindlin-2 expression. Furthermore, transfection of miR-200b mimic or knockdown of Kindlin-2 in ESCC cells decreased cell protrusion and focal adhesion (FA) formation, reduced cell spreading and invasiveness/migration. Enforced expression of Kindlin-2 largely abrogated the inhibitory effects of miR-200b on ESCC cell invasiveness. Mechanistic studies revealed that Rho-family guanosine triphosphatases and FA kinase mediated the biological effects of the miR-200—Kindlin-2 axis in ESCC cells. To conclude, loss of miR-200b, a frequent biochemical defect in ESCC, correlates with aggressive clinical features. The tumor suppressor effects of miR-200b may be due to its suppression of Kindlin-2, a novel target of miR-200b that modulates actin cytoskeleton, FA formation and the migratory/invasiveness properties of ESCC.

Introduction

Esophageal cancer represents the sixth leading cause of cancer deaths and the eighth most common type of cancer worldwide (1,2). Despite the use of multimodal treatments such as radical surgery, chemotherapy and radiotherapy, the 5 year overall survival rate of patients with esophageal squamous cell carcinoma (ESCC) remains <14% (2–4). A poor survival rate in ESCC patients is highly associated with a frequent local invasion and distant metastasis, and >50% of patients have either unresectable cancer or radiographically visible metastases at diagnosis (2,5). The absence of the serosa and the presence of the extensive lymphatics in the esophagus are probably some of the contributing factors to the high frequency of local invasion and metastasis in ESCC (5). However, our understanding of the molecular mechanisms that regulate local invasiveness and metastatic potential of ESCC remain incomplete. To improve the overall outcome for patients with ESCC, it is important to understand the molecular mechanisms underlying these processes, thereby useful biomarkers and novel therapeutic targets can be discovered.

Over the past few years, microRNAs (miRNAs) have been recognized as critical regulators of cancer invasion and metastasis, either as promoters or as suppressors (6–8). Deregulation of miRNAs in cancer may result in aberrant expression of proteins that regulate cancer cell invasiveness, such as cytoskeletal regulatory proteins, cell adhesion molecules or proteins regulating epithelial-to-mesenchymal transition (9). Recently, the miR-200 family was identified as potent suppressors of epithelial-to-mesenchymal transition by directly targeting the E-cadherin transcriptional repressors, ZEB1 and ZEB2, thereby suppressing tumor invasion and/or metastasis (10–14). The miR-200 family is comprised of five members that are encoded within two clusters: the miR-200b-200a-429 cluster (miR-200b cluster) located on chr1p36 and the miR-200c-141 cluster located on chr12p13. Our previous investigation using miRNA profiling has revealed that the miR-200b cluster members are consistently downregulated in ESCC (15). Recently, a study showed that a high level of miR-200c correlated with chemoresistance and a short survival in ESCC patients (16). However, our knowledge about the exact roles played by the miR-200 family in ESCC and the underlying molecular mechanisms remain relatively unclear.

In this study, we firstly confirmed that the miR-200b cluster members are frequently downregulated in ESCC, and this abnormality significantly correlates with a poor prognosis and unfavorable clinicopathological features in ESCC patients. Our in vitro studies suggest that miR-200b strongly represses the invasiveness and modulates the cytoskeletal and adhesive machinery in ESCC cells. Lastly, mass spectrometry (MS) studies allowed us to discover Kindlin-2 as an important mediator of the tumor suppressor functions of miR-200b.

Materials and methods

Clinical samples

Human ESCC samples and adjacent non-tumorous esophageal epithelial tissues were collected between October 2007 and December 2008 directly after surgical resection at the Department of Tumor Surgery of Shantou Central Hospital, China. The cases were selected based on a clear pathological diagnosis, follow-up data, and had not received previous local or systemic treatment. The histological characterization and clinicopathological staging of the samples were performed in accordance with the seventh edition of American Joint Committee on Cancer Tumor-Node-Metastasis staging system. Detailed clinical information of the ESCC patients is described in Supplementary Table 1, available at Carcinogenesis Online. The study was approved by the ethical committee of the Central Hospital of Shantou City and the ethical committee of Shantou University Medical College, and written informed consent was obtained from all surgical patients to use resected samples for research.

Human ESCC cell lines

KYSE510, KYSE180, KYSE150, KYSE70 and TE3 human ESCC cell lines were kindly provided by Dr Ming-Zhou Guo, Department of Gastroenterology.

Abbreviations: 3′-UTR, 3′-untranslated region; ESCC, esophageal squamous cell carcinoma; FA, focal adhesion; FAK, focal adhesion kinase; GTP, guanosine triphosphate; HPLC, high-performance liquid chromatography; IHC, immunohistochemistry; miRNA, microRNA; MS, mass spectrometry; qRT-PCR, quantitative real-time PCR; SILAC, stable isotope labeling with amino acids in cell culture; siRNA, small interfering RNA.
and Hepatology, Chinese PLA General Hospital, Beijing, China. EC109 and EC9706 were obtained from Chinese Academy of Medical Sciences, Beijing, China. The ESCC cell lines used in this study were cultured according to the methods described below: KYSE510, KYSE180, KYSE150, KYSE70 and TE3 cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum. EC109 and EC9706 cells were maintained in Dulbecco’s modified Eagle’s medium plus 10% newborn calf serum.

**In vitro invasion and migration assays**

Cell invasion, migration assays were performed as described below: 48 h after transfection with 10 nM miR-200b mimic or 200 nM anti-200b or their corresponding negative control RNA, cells were starved for 24 h and 1 × 10⁷ starved cells were seeded into transwells (BD Biosciences) with 8 μm pore size membranes coated with or without matrigel (for invasion and migration assays, respectively). After 48 h, cells within the transwells were removed and migrated/invaded cells on the bottom of the transwells were stained with crystal violet. Photos of migrated/invaded cells on the transwell membrane were taken under ×200 magnification, and the numbers of migratory/invaded cells were counted from at least five different fields.

**RNA isolation and qRT–PCR**

Total RNA was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA). For the quantitative real-time PCR (qRT–PCR) of miR-200a, miR-200b, miR-429 and RN156B (U6, endogenous control), TaqMan MicroRNA Assay kits (Applied Biosystems) were used and real-time PCR reaction was carried out using ABI 7500 fast real-time PCR system (Applied Biosystems) as described before (15).

**Vector construction and site-directed mutagenesis**

The coding region of Kindlin-2 was amplified and cloned (BamHI and Xhol) into the eukaryotic expression vector pcDNA3.1 (Invitrogen) to generate pcDNA3.1-Kindlin-2 expression vector. To construct the luciferase reporter vector, the 3′-untranslated region (3′-UTR) of FERM2, CDK2, CFL2, HMOX1, PAF1, PAK2, RALB and RDX containing the putative miR-200b binding site(s) were amplified and cloned (XbaI and FseI) into pcDNA3.5-Control vector (Promega). All the primers used for gene cloning are described in Supplementary Table 2, available at Carcinogenesis Online. For site-directed mutagenesis, Fast Mutagenesis System (TransGen Biotech, Beijing, China) was used, and the experiment was performed according to the manufacturer’s instructions. Primers for mutagenesis are listed in Supplementary Table 2, available at Carcinogenesis Online.

**Oligonucleotide transfection**

Syn-hsa-miR-200b miScript miRNA Mimic, Anti-hsa-miR-200b miScript miRNA Inhibitor, Kindlin-2 small interfering RNA (siRNA) and their corresponding negative control RNAs were all purchased from Qiagen. The miRNA Inhibitor, Kindlin-2 small interfering RNA (siRNA) and their corresponding negative control RNAs were all purchased from Qiagen. Syn-hsa-miR-200b miScript miRNA Mimic, Anti-hsa-miR-200b miScript miRNA Inhibitor, Kindlin-2 small interfering RNA (siRNA) and their corresponding negative control RNAs were all purchased from Qiagen. Syn-hsa-miR-200b miScript miRNA Mimic, Anti-hsa-miR-200b miScript miRNA Inhibitor, Kindlin-2 small interfering RNA (siRNA) and their corresponding negative control RNAs were all purchased from Qiagen. Syn-hsa-miR-200b miScript miRNA Mimic, Anti-hsa-miR-200b miScript miRNA Inhibitor, Kindlin-2 small interfering RNA (siRNA) and their corresponding negative control RNAs were all purchased from Qiagen. Syn-hsa-miR-200b miScript miRNA Mimic, Anti-hsa-miR-200b miScript miRNA Inhibitor, Kindlin-2 small interfering RNA (siRNA) and their corresponding negative control RNAs were all purchased from Qiagen. Syn-hsa-miR-200b miScript miRNA Mimic, Anti-hsa-miR-200b miScript miRNA Inhibitor, Kindlin-2 small interfering RNA (siRNA) and their corresponding negative control RNAs were all purchased from Qiagen. Syn-hsa-miR-200b miScript miRNA Mimic, Anti-hsa-miR-200b miScript miRNA Inhibitor, Kindlin-2 small interfering RNA (siRNA) and their corresponding negative control RNAs were all purchased from Qiagen.

**Vector construction and site-directed mutagenesis**

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**Luciferase reporter assay**

The firefly luciferase construct was cotransfected with a control Renilla luciferase vector into EC109 cells in the presence of either miR-200b mimic or anti-200b or corresponding negative control RNA. After 48 h, luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega). Data are presented as ratios between firefly and Renilla fluorescence activities. The experiments were performed independently in triplicate.

**Western blot and Rho GTase activation assay**

Proteins were separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membrane (Millipore). The membranes were blocked with 5% non-fat milk and incubated with antibodies against Kindlin-2 (1:2000; Millipore), pFAK(Tyr597) (1:1000; Invitrogen), FAK (1:500; BD Transduction Laboratories) and β-actin (1:10 000; Sigma). The proteins were detected with Western Blot Luminol Reagent (Santa Cruz).

The pull down of the guanosine triphosphate (GTP)-bound form of Rho-family GTPases was performed using Rhoα and Cdc42/Rac1 activation assay kit (Cytoskeleton) in accordance with the manufacturer’s instructions. Protein quantitative analysis was performed by western blot analysis using antibodies against Rhoα, Cdc42 and Rac1 provided within the kits.

**Immunohistochemistry**

Paraffin-embedded tissue blocks were cut into 4 μm sections and processed for immunohistochemistry (IHC) with a protocol described previously (17). Antibodies against Kindlin-2 (1:500; Millipore) were used. Scoring was classified into four grades: no reactivity scored 0, faint reactivity scored 1, moderate reactivity scored 2 and strong reactivity scored 3.
groups of samples, Student’s t-test or Mann–Whitney U-test were used. Survival curves were plotted using the Kaplan–Meier method and compared using the log-rank test, and X-tile software was used for the selection of optimal cutpoints before analysis (18). The chi-square test or Fisher’s exact test was used to analyze the association of miR-200b-200a-429 expression and clinical-pathological parameters, and the same cutpoints were used as in the survival analysis. Differences were considered significant when the P value was <0.05.

Results

Decreased expression of the miR-200b cluster in ESCC correlates with shorter patient survival, lymph node metastasis and advanced clinical stage

Our previous studies using miRNA profiling applied to three cases of ESCC revealed a downregulation of the miR-200b-200a-429 cluster in ESCC, as compared with adjacent benign esophageal tissues (15). In the present study, we aimed to confirm these findings using qRT–PCR applied to 88 pairs of ESCC and the adjacent benign esophageal tissues. As illustrated in Figure 1A, the three miR-200b cluster members (namely, miR-200b, miR-200a and miR-429) are clustered within a short segment of chromosome 1. Using Wilcoxon signed-rank test, we confirmed that the expression levels of the miR-200b cluster members were significantly lower in ESCC when compared with the adjacent benign esophageal tissues (Figure 1B, P < 0.05). We also found that the expression levels of these three miRNAs significantly correlated with each other in ESCC (Figure 1C, P < 0.001).

Kaplan–Meier analysis was performed to compare the prognosis of patients carrying tumors with different expression levels of the three miR-200b cluster members. As shown in Figure 1D, ESCC patients carrying tumors expressing low levels of the miR-200b cluster had a significantly worse prognosis compared to those with high expression levels (Figure 1D, P < 0.05). The optimal cutpoint values for the survival analysis were generated using X-tile software (18).

Figure 1. Expression status of the miR-200b cluster in ESCC specimens and their prognostic values in patients with ESCC. (A) A schematic representation of the localization of the miR-200b cluster members within the genome. (B) Expression levels of the miR-200b cluster members were examined by TaqMan real-time PCR in 88 cases of ESCC specimens (tumor) and adjacent non-tumor esophageal epithelial tissues (non-Tumor). U6 was used as an endogenous control. Data are presented as median ± interquartile range, and Wilcoxon signed-rank test was used in statistical analysis. (C) The expression levels of the miR-200b cluster members in ESCC were compared with each other. Pearson’s correlation analysis was performed. (D) Kaplan–Meier survival curves for ESCC patients were plotted on miRNA expression levels, and survival difference was analyzed by log-rank test. The optimal cutpoint values for the survival analysis were generated using X-tile software (18). (E) The expression levels of the miR-200b cluster members were compared between tumors with and without lymph node metastasis (indicated as LN+ and LN−, respectively). Data are plotted as mean ± standard error of the mean. (F) Expression levels of miR-200b in a panel of seven ESCC cell lines were detected by TaqMan real-time PCR. The mean expression level of miR-200b in non-tumorous esophageal epithelial tissues (NT) from the 88 patient cohort was used as the normal control, extreme values outside the normal distribution were excluded.
miR-200b targets Kindlin-2 in ESCC

Among the three newly identified miR-200b targets, Kindlin-2 was shown previously to promote cancer cell migration and invasiveness (23,24). Our following observations support the concept that Kindlin-2 is indeed a target of miR-200b in ESCC. First, in silico prediction revealed two potential binding sites of miR-200b on Kindlin-2 3′-UTR, and simultaneous mutation of both potential binding sites abolished the suppression of miR-200b mimic on the luciferase activity (Figure 2D). Second, an inverse correlation between the expression of Kindlin-2 and miR-200b can be observed in a panel of six ESCC cell lines (Figure 1F and Figure 2E). Third, to test whether miR-200b indeed regulates the protein expression of Kindlin-2, three cell lines (i.e. EC9706, EC109 and KYSE510) that expressed the lowest level of miR-200b and the highest level of Kindlin-2 were chosen to transfet with miR-200b mimic, whereas KYSE150 cells that had the highest expression of miR-200b and the lowest expression of Kindlin-2 were transfected with miR-200b inhibitor. As shown in Figure 2E, transfection of miR-200b mimic reduced Kindlin-2 expression in all three ESCC cell lines tested, and inhibition of endogenous miR-200b in KYSE150 cells increased Kindlin-2 expression. Lastly, we found a significant inverse correlation between Kindlin-2 protein expression (by IHC) and miR-200b levels (by qRT–PCR) in ESCC samples (Figure 2F, P = 0.033).

miR-200b and Kindlin-2 regulate invasiveness and migratory function in ESCC

Transfection of miR-200b mimic into three ESCC cell lines (EC9706, EC109 and KYSE510, Figure 3A), all of which had a low level of miR-200b expression, induced a significant decrease in their cell migration and invasiveness (Figure 3B and C). Accordingly, when we inhibited miR-200b using an inhibitor in KYSE150 (Figure 3A), an ESCC cell line that expressed miR-200b at the highest level among all ESCC cell lines examined, we found a significant increase in cell migration and invasiveness (Figure 3B and C). To determine whether Kindlin-2 is a mediator of the biological function of miR-200b, two representative cell lines with low miR-200b expression and high Kindlin-2 expression (i.e. EC109 and KYSE510) were used as cell models in our subsequent biological analyses. As shown in Figure 3D and E, siRNA knockdown of Kindlin-2 reduced ESCC cell invasiveness in both EC109 and KYSE510 cells. Furthermore, enforced expression of Kindlin-2 in both EC109 and KYSE510 cells largely restored the invasiveness that was suppressed by miR-200b (Figure 3F and G).

Both miR-200b and Kindlin-2 regulate actin cytoskeleton and focal adhesion formation in ESCC cells

Previous studies have shown that Kindlin-2 regulates cell–extracellular matrix interaction, cytoskeletal structure, focal adhesion (FA) formation and cell spreading (31–33). Based on these observations, we hypothesized that miR-200b may regulate these biological functions via its downregulation of Kindlin-2. This hypothesis was supported by multiple experimental observations. First, after transfection of miR-200b mimic into two ESCC cell lines that express a low level of miR-200b (i.e. EC109 and KYSE510), cell spreading and protrusion formation, two key features dictated by actin cytoskeleton (34–36), were substantially inhibited (Figure 4A). Second, confocal microscopy analysis showed that enforced expression of miR-200b mimic markedly suppressed actin cytoskeleton reorganization, as evidenced by decreased formation of stress fibers and filopodia protrusions in EC109 cells, and diminished podosomes in KYSE510 cells (Figure 4B and C). Third, miR-200b mimic repressed the formation of large mature FAs in both cell lines, as revealed by confocal microscopy (Figure 4B and C). Finally, quantitative analysis showed that the cell spreading areas were dramatically reduced by miR-200b mimic transfection in both cell lines (Figure 4D). As shown in Figure 5A–C, siRNA knockdown of Kindlin-2 in EC109 and KYSE510 largely mimicked the biological effects of transfection of miR-200b mimic, i.e. reduced cell spreading area, decreased stress fibers and FA formation.

Both miR-200b and Kindlin-2 regulate the activity of the Rho-family GTPases

Since members of the Rho-family GTPases have been demonstrated as master regulators of actin cytoskeleton reorganization (37), we speculated that miR-200b might have influenced the cytoskeletal activities of Rho-family GTPases. To validate this hypothesis, we performed a series of experiments using EC109 cells, which were used as the positive control in our subsequent validation analysis. The luciferase reporters containing the 3′-UTR of HMOX1, CDK2, FERM2, PAF (KIAA0101), PAK2, RALB and RDX (23–30), CFL2, which was shown previously to be a miR-200b target gene (7), served as the positive control in our subsequent validation analysis. Our experimental approach was to clone the 3′-UTRs of these eight candidate genes into a luciferase reporter vector, as detailed in Materials and methods. By performing bidirectional screening using miR-200b mimic and miR-200b inhibitor, our studies allowed us to validate three novel miR-200b targets, including CDK2, PAF and Kindlin-2 (Figure 2C). The luciferase reporters containing the 3′-UTR of the other four putative miR-200b targets were shown not to respond accordingly to the manipulation of miR-200b expression levels, probably due to the fact that their protein expression was regulated by miR-200b through indirect mechanisms other than direct binding of miR-200b to their 3′-UTR.
structure by regulating Rho-family GTPases. In support of this concept, transfection with miR-200b mimic significantly repressed the expression levels of GTP-bound Cdc42 and RhoA in both EC109 and KYSE510 cells, suggesting a decreased activity of these Rho-family GTPases (Figure 6A). However, the active form of another member of Rho-family GTPases, Rac1, was undetectable in both EC109 and KYSE510 cells (Supplementary Figure 2, available at Carcinogenesis Online). As shown in Figure 6B, Kindlin-2 knockdown produced similar biological effects as miR-200b mimic transfection in both EC109 and KYSE510 cells.
miR-200b targets Kindlin-2 in ESCC

Since FA kinase (FAK) has been shown to play a crucial role in the regulation of the formation and turnover of FAs (38,39) as well as cell spreading (40,41), we examined the biological impact of miR-200b on FAK phosphorylation. As shown in Figure 6C, transfection of miR-200b mimic inhibited the phosphorylation of FAK on tyrosine 397 in both EC109 and KYSE510 cells. To determine whether Kindlin-2 plays a role in mediating the biological effects of miR-200b in the modulation of FAK activation, we inhibited Kindlin-2 expression using siRNA. As shown in Figure 6D, knockdown of Kindlin-2 by siRNA also decreased the expression of pFAK(Y397). In contrast, enforced expression of Kindlin-2 markedly restored pFAK(Y397) expression that was suppressed by miR-200b mimic transfection (Figure 6E).

Collectively, these findings suggest that Kindlin-2 is an important mediator of the biological functions of miR-200b in ESCC cells.

Discussion

During the past few years, mounting evidence has demonstrated that loss of the miR-200 family promotes tumor initiation by modulating the stemness of cancer stem cells (42–44), and enhances tumor malignant progression by regulating epithelial-to-mesenchymal transition and modulating the tumor microenvironment that favors metastasis (10–14,45). However, our understanding about the clinical significance and the biological role of the miR-200 family in ESCC is rather limited. In keeping with the previous concepts, we found that low expression of the members of the miR-200b-200a-429 cluster...
significantly correlate with short patient survival and aggressive phenotypes, the first study that confirms the clinical significance of the miR-200b cluster in ESCC.

Although we have presented compelling evidence that the miR-200b cluster is decreased in ESCC and has associated features suggesting tumor suppressor functions, it has been reported that miR-200c is overexpressed in ESCC, and high expression of miR-200c confers chemoresistance in ESCC cells (16). Correlating with these, the expression of the miR-200c and the miR-200b cluster is oppositely altered in ESCC, probably due to the fact that chr1p36 containing the miR-200b cluster is often deleted in ESCC (46), whereas chr12p13 containing the miR-200c-141 cluster is frequently amplified in ESCC (46,47).

Kindlin-2 is a FERM domain containing protein, which has been shown to modulate cell shape by linking the cytoskeleton with the cell–extracellular matrix adhesions (31–33). Importantly, Kindlin-2 belongs to Kindlin protein family, which is one of the only two known families of proteins that mediate the inside–out activation of integrin signaling pathways (48). Currently, the importance of Kindlin-2 in tumor malignant progression has been recognized; Kindlin-2 was

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Fig. 4. miR-200b represses ESCC cell spreading and FA formation. (A) The influence of miR-200b mimic transfection on ESCC cell morphology. Cells were seeded on glass slides (for 24h) or fibronectin-coated glass slides (for 1h). Arrowheads indicate cells with protrusions, and arrows indicate rounded cells with no visible protrusions. Scale bars: 30 μm. (B and C) The impact of miR-200b mimic transfection on actin cytoskeleton structure and FA formation was analyzed by confocal microscopy. Actin cytoskeleton and FAs were visualized by phallolidin staining (red) and paxillin labeling (green), respectively. For EC109 cells, stress fibers and filopodia are respectively noted by arrowheads and arrows in the F-actin fields; arrows in the paxillin field indicate FAs. For KYSE510 cells, arrowheads in the F-actin field indicate large podosomes, and arrows in the paxillin field indicate FAs. Scale bars: 20 μm. (D) miR-200b diminished cell spreading area in both EC109 and KYSE510 cells. Cell spreading area was measured with the software ImageJ, cells from at least three different fields were analyzed (**P < 0.01, Student’s t-test).
miR-200b targets Kindlin-2 in ESCC

The formation and turnover of FAs, cell–substratum contact sites that link integrins to the actin cytoskeleton, not only transmit extracellular signals into cells but also facilitate cells to migrate (23, 24). Indeed, miR-200b has been shown to target genes associated with actin cytoskeleton remodeling, such as WAVE3 and MARCKS (20, 51). Moreover, the list of proteins downregulated by miR-200b may contain proteins that could also mediate the biological function of miR-200b similar to that of Kindlin-2 but do not possess miR-200b binding site within the 3′-UTR of their encoding messenger RNA, in those cases, their expression is probably regulated by miR-200b through indirect mechanisms (e.g., modulated by direct miR-200b targets or by signaling pathways that could be modulated by other mechanisms targeted by miR-200b).

To conclude, our data reveal a novel mechanism that miR-200b suppresses ESCC aggressiveness and modulates the cytoskeletal and adhesive machinery via targeting Kindlin-2. Our findings also suggest

**Fig. 5.** Kindlin-2 knockdown suppresses cell spreading and FA formation in ESCC cells. (A) (Left panel) After siNC or siKindlin-2 transfection, cells were seeded on fibronectin-coated slides and allowed to adhere for 1 h before the detection of Kindlin-2 and F-actin cytoskeleton by immunofluorescence. Arrows indicate the colocalization of Kindlin-2 and cell spreading edges (marked with F-actin staining) in siNC-transfected cells. Scale bars: 10 μm. (Right panel) After siNC or siKindlin-2 transfection, cells were allowed to adhere for 12 h on fibronectin-coated slides. FAs and F-actin cytoskeleton were examined by immunofluorescence. Arrowheads indicate stress fibers and arrows indicate FAs as detected by paxillin staining. Scale bars: 20 μm. (B) In parallel with the experiments described in A, cell spreading area was measured with the software ImageJ after adhesion for 1 or 12 h on fibronectin-coated slides, cells from at least three different fields were analyzed (*P < 0.05, **P < 0.01, Student’s t-test). (C) In parallel with the experiments described in A, after adhesion for 12 h on fibronectin-coated slides, the average FA number was compared between siNC- and siKind2-transfected cells, cells from at least three different fields were analyzed (*P < 0.05, ***P < 0.001, Student’s t-test).
that miR-200b and its targets may serve as promising prognostic markers for ESCC and therapeutic targets for ESCC invasion intervention.

**Supplementary material**

Supplementary Figures 1 and 2 and Tables 1–5 can be found at [http://carcin.oxfordjournals.org/](http://carcin.oxfordjournals.org/)

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**Fig. 6.** Kindlin-2 is an important mediator of miR-200b in modulating the activity of the Rho-family GTPases and FAK. (A and B) Forty-eight hours after miR-200b mimic transfection (A) or Kindlin-2 knockdown (B), GTP-bound (active) forms of Cdc42 and RhoA were pulled down and detected by western blot. Total Cdc42 and RhoA expression from equal amounts of input lysates was detected, and actin was used as an internal control. (C and D) Forty-eight hours after miR-200b mimic transfection (C) or Kindlin-2 knockdown (D), phosphorylated FAK on tyrosine 397, i.e. pFAK(Y397), and total FAK was detected by western blot. Actin was used as an internal control. The results shown are representatives of three repeated experiments. (E) Forty-eight hours after the transfection of the indicated reagents, the indicated proteins were examined by western blot, and actin was used as an internal control. Vector: pcDNA3.1, Kind2: pcDNA3.1-Kindlin-2. The results shown are representatives of two repeated experiments.

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**Conflict of Interest Statement:** None declared.

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