MICRORNA-10B PROMOTES HUMAN ESOPHAGEAL CANCER CELL MIGRATION AND INVASION THROUGH KLF4

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Running head: miR-10b regulates KLF4 in esophageal cancer

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Recently, microRNAs (miRNAs) have emerged as regulators of cancer metastasis through acting on multiple signaling pathways involved in metastasis. In this study, we have analyzed the level of miR-10b and cell motility and invasiveness in several human esophageal squamous cell carcinoma (ESCC) cell lines. Our results reveal a significant correlation of miR-10b level with cell motility and invasiveness. Overexpression of miR-10b in KYSE140 cells increased cell motility and invasiveness, whereas inhibition of miR-10b in EC9706 cells reduced cell invasiveness albeit it did not alter cell motility. Additionally, we identified KLF4, a known tumor suppressor gene which has been reported to suppress esophageal cancer cell migration and invasion, as a direct target of miR-10b. Furthermore, overexpression of miR-10b in KYSE140 and KYSE450 cells led to a reduction of endogenous KLF4 protein, while silencing of miR-10b in EC9706 cells caused upregulation of KLF4 protein. Co-expression of miR-10b and KLF4 in KYSE140 cells and co-expression of siRNA for KLF4 mRNA and miR-10b-AS in EC9706 cells partially abrogated the effect of miR-10b on cell migration and invasion. Finally, analyses of miR-10b level in 40 human esophageal cancer samples and their paired normal adjacent tissues revealed an elevated expression of miR-10b in 95% (38/40) of cancer tissues, although no significant correlation of miR-10b level with clinical metastasis status was observed in these samples.

MicroRNAs, a class of small noncoding RNAs, have been identified as a new kind of gene expression regulators through targeting mRNAs for translational repression or cleavage (1-3). Lately, emerging evidence suggests important roles of miRNAs in apoptosis (4), hematopoietic development (5), cell proliferation (6), skin morphogenesis (7) and neural development (8). Deregulation of miRNAs has also been reported in a variety of tumors, including breast cancer, leukemia, lung cancer, and colon cancer (9-11), which indicated a significant correlation between miRNAs and human malignancy. miRNA expression profilings in esophageal cancers revealed a distinct miRNA signature (12, 13) and some miRNAs showed correlation with several clinicopathologic parameters (13). Furthermore, miR-21 is reported to regulate the proliferation and invasion in ESCC (14) and the miR-106b-25 polycistron is activated by genomic amplification and is potentially involved in esophageal neoplastic progression (15), providing evidences of a causal role of miRNAs in esophageal cancer development.

Recent studies show that miRNAs may act as activators or inhibitors of tumor metastasis by acting on multiple signaling pathways involved in metastasis (16-18). Ma et al (16) found that miR-10b initiates invasion and metastasis in breast cancer. MiR-10b, induced by the prometastatic transcription factor TWIST1, proceeds to inhibit translation of mRNA of HOXD10, a transcription factor already known...
for its roles in cell motility (19), resulting in increased expression of a pro-metastatic gene, RHOC. This study has provided the first evidence for a role of miRNA in tumor metastasis. Subsequently several additional miRNAs have been reported to act on various steps of metastasis (17, 18).

Krüppel-like factor 4 (KLF4), a zinc-finger protein of the Krüppel-like factor family, plays a role in cell-cycle regulation, differentiation and rises in response to DNA damage, serum starvation and contact inhibition (20, 21). In line with these studies, loss of KLF4 expression has been reported in several human tumors, including colorectal, stomach, esophageal and bladder cancers (22-25), which indicate its tumor suppressor role. However, KLF4 also exhibits oncogenic properties. Overexpression of KLF4 could be detected in oropharynx (26) and mammary carcinomas (27). Moreover, it has been reported to inhibit metastasis of several cancers, including esophageal (28), pancreatic (23) and colorectal cancer cells (29). In addition, KLF4 mRNA is targeted by miR-145 (30), implicating the post-transcriptional control of KLF4.

In this study, we have identified a significant correlation between the level of miR-10b and human ESCC cell motility and invasiveness. Furthermore, we have verified a functional role of miR-10b in ESCC cell migration and invasion. Additionally we have identified KLF4 mRNA as a direct target of miR-10b and shown that KLF4 can partly inhibit ESCC cell migration and invasion initiated by miR-10b. Finally, we found an elevated expression of miR-10b in 95% (38/40) of human esophageal cancer tissues than the normal counterparts, although no significant correlation of miR-10b expression with clinical metastasis status was observed.

**Experimental Procedures**

*Cell line, cell culture and transfection- Human ESCC cell lines KYSE30, KYSE70, KYSE140, KYSE150, KYSE410, KYSE450, KYSE510 and EC9706 were all established from human ESCC patients (31-33). Among them, KYSE series were generous gifts from Dr. Y. Shimada at Hyogo College of Medicine (Hyogo, Japan), and EC9706 was established and maintained in our lab.*

Lipofection2000 (Invitrogen, Carlsbad, CA) was used for DNA plasmid transfection, and Hiperfect (Qiagen GmbH, D-40724 Hiden, Germany) was used for oligonucleotide transfection according to the manufacturers’ protocols. miRNA inhibitor (miR-10b-AS) was chemically enhanced by 2'O-4'C-methylene modification. siRNA sequence for KLF4 knock-down was GGACGGCTGTGGATGGAAA (34). Single strand 2'-OME-antisense-EGFP was used as a negative control for miR-10b-AS and double strand nonsense oligonucleotide for siRNA.

*Tissue specimens- Forty paired tissue specimens (tumor and adjacent normal mucosa) of primary human ESCC were provided by the First Affiliated Hospital of Anhui Medical University (Anhui province, China). All of the tissues were obtained at the time of surgery and immediately stored in liquid nitrogen until use. None of the patients had received radiotherapy or chemotherapy before surgery. Patients diagnosed with metastasis had lymph node metastasis verified by pathological analysis. For all the samples, clinicopathologic characteristics (age, gender, differentiation, tumor depth and tumor-node metastasis) are shown in Table 1. This study was approved by the ethical committees of the Chinese Academy of Medical Sciences Cancer Institute and the First Affiliated Hospital of Anhui Medical University, and informed contents were obtained from the patients.*

*Real-time quantitative PCR- For real-time RT-PCR of miR-10b and KLF4 mRNA, total RNA was isolated using Trizol solution (Invitrogen). Reverse transcription was*
performed using MMLV Reverse Transcriptase (Epicentre, Paris, France). MiR-10b was reverse transcribed by the looped primer which binds to six nucleotides at the 3’ portion of miR-10 molecules. Reverse transcription of KLF4 mRNA was performed according to manufacturer’s protocol. Real-time PCR was performed using RT² Real-Time™ SYBR Green (SuperArray, Frederick, MD) according to the manufacturer’s protocol. The U6 small nuclear RNA and GAPDH mRNA were used as internal controls for miR-10b and KLF4 mRNA, respectively. All reactions were run in triplicate. The ΔΔCt method for relative quantification of gene expression was used to determine miRNAs and KLF4 mRNA expression levels. Looped RT-primer for miR-10: GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGGATACGACCACAAA. PCR primers for miR-10b: GGATACCCTGTAGAACCGAA/CAGTGCGTGTCGTGGAGT. PCR primers for miR-10a: GGATACCCTGTAGATCCGAA/CAGTGCGTGTCGTGGAGT. Primers for U6: TGGGGTTATACATTGTGAGAGGA/GTGTGCATTACGGAGTTCAGAGGTT. Primers for KLF4 mRNA: CGAACCCACACAGGTGAGAA/TACGGTAGTGCCTGGTCAGTTC. Primers for GAPDH mRNA: GGTCGGAGTCAACGGATTTG/ATGAGCCACCTTCTCCAT.

Plasmid construction- Genomic sequence of the human miR-10b gene was cloned from mixed DNA of normal esophageal tissues, inserted into pcDNA3.0 (Invitrogen) and named as pcDNA3.0-miR-10b. The full-length 3’UTR of KLF4 was subcloned into the pIS0 luciferase plasmid in our lab (36). Primers for miR-10b: GCCGGAATTCAGCTAGCAGCTCTGG/CCGGAATTCTGGAGAGGCTCATCAAG. Primers for KLF4 3’UTR: CCGGAGCTCTTTAAATCCCAAGACAGTGGATATG/CGGTCTAGAGGTTCAGTTAATTTAAAAACTTATTCTCACCTTG. Primers for mutation: TTTATGGTGATATCCCAACACTTCCA/GATATCACCCAATAAAATTATATCCGGTGA.

Luciferase reporter assay- Cells of 90% confluence in 96-well plates were transiently transfected with firefly luciferase reporter gene constructs and miR-10b expressing plasmid. After 48h, luciferase activity was measured using the Dual-Luciferase Reporter Assay System according to the manufacturer’s protocol (Promega, Madison, WI), normalized for transfection efficiency by cotransfecting with 0.5 ng of pRL-SV40 Renilla (Luciferase Assay System; Promega). For each plasmid construct, 3 independent transfection experiments were performed in triplicate.

Western blot- Western blot were performed as previously described (37). Protein bands were detected by chemiluminescence using the ECL system (Vigorous Biotech, Beijing, China) according to the manufacture’s protocol. Intensity of the bands was quantified by
densitometry.

Half-life analysis- KYSE140 cells of 90% confluence were transfected with pcDNA3.0-miR-10b and control plasmid. 48 hours after, cells were treated with 100 μg/ml cyclohexamide. At the indicated time points, cell extracts were prepared and analyzed by Western Blot with KLF4 and control β-actin antibodies. Band intensities were quantified by densitometry, and the resulting KLF4/β-actin ratios plotted in a nonlinear regression plot.

Statistical analysis- Statistical analysis was performed on SPSS13.0. Pearson’s correlation analysis was used to examine the correlation between miR-10b and migration and invasion potential and the correlation between miR-10b and KLF4 protein levels of human ESCC cell lines. Differences between two groups were explored by the student’s t-test. For comparison of paired tissues, paired student’s t-test was used to determine statistical significance. Values were presented as mean ± SD. A p value < 0.01 was considered to be statistically significant, which is labelled as * in figures.

RESULTS

miR-10b expression correlates with cell motility and invasiveness in human ESCC cell lines. Real-time RT-PCR (q-PCR) analysis of eight human ESCC cell lines revealed that miR-10b levels varied considerably among the cell lines (Fig. 1A). The highest expression level of miR-10b was observed in EC9706 cells, which was ~70-fold higher than that in KYSE70, KYSE140 and KYSE150 cells.

To test the correlation between miR-10b level and migration and invasion potential in human ESCC cell lines, we performed transwell assays on the ESCC cell lines (Fig. 1B). In the KYSE series, statistically significant correlations of miR-10b level with both cell migration and invasion potential were observed ($r = 0.74$ and $r = 0.76$, respectively, with $p$ value < 0.05). Taking into account EC9706 cell line, highly significant correlations were found between miR-10b and cell migration and invasion potential ($r = 0.98$ and $r = 0.97$, respectively, with $p$ value < 0.001). These results suggested a causal role of miR-10b in migration and invasion of human ESCC cell lines.

miR-10b is closely relative to miR-10a, which differs from miR-10b by a single nucleotide (Fig. 2A). To rule out potential cross reactivity of miR-10a, we overexpressed miR-10b in KYSE140 cells. Total RNA was converted to cDNA using a looped RT primer (Fig. 2A). Mature miR-10b and miR-10a were measured by real-time PCR using miRNA specific primers (Fig. 2A). The results showed that the miR-10b level was dramatically elevated (>1000 folds), whereas the increase of miR-10a was much less (Fig. 2B). Transfection of antisense inhibitor miR-10b-AS into EC9706 significantly reduced the level of miR-10b but not that of miR-10a (Fig. 2C), indicating that miR-10b PCR primers were relatively specific, exhibiting weak cross reactivity with cDNA reverted from mature miR-10a.

miR-10b promotes human ESCC cell migration and invasion. To determine whether miR-10b regulates human ESCC cell migration and invasion, we selected EC9706 cells which show strong migration and invasion potential, and KYSE140 cells which show weak migration and invasion potential, for further study. We first performed loss-of-function analysis by silencing miR-10b with miR-10b-AS in EC9706 cells. Inhibition of miR-10b in EC9706 cells led to a 73% reduction in invasion assay compared with control cells, whereas no change was observed in migration potential (Fig. 2D, E). We then transfected pcDNA-3.0-miR-10b into KYSE140 cells, and a two fold increase in cell migration as well as invasion potential was observed (Fig. 2F, G). In addition, overexpression of miR-10b had no effect on cell proliferations in KYSE140 cells (data not shown). These observations indicated a positive role of miR-10b in migration and invasion of human ESCC cell lines.
**KLF4 mRNA is a direct target of miR-10b.** To explore the molecular mechanism of miR-10b function, we used TargetScan4.0 to predict targets of miR-10b in human. Several genes, which have been reported to suppress cell migration or invasion, were selected for further analysis. Among them, *KLF4* was of particular interest, since it was found to be down-regulated in human esophageal cancer (22, 25, 38, 39) and correlated with cancer metastasis or migration of cancer cells including human esophageal cancer (28).

As shown in Fig. 3A, there was a miR-10b binding site at nt295-301 of the *KLF4* 3′UTR. Comparing the human sequence for interspecies homology, we found that the miR-10b target sequence at nt295-301 of the *KLF4* 3′UTR is highly conserved among different species (Fig. 3A). To determine whether *KLF4* is a direct target of miR-10b, we constructed pIS0-KLF4-3′UTR and pIS0-KLF4-3′UTR-mut (Fig. 3B). Cotransfection of KYSE140 cells with pIS0-KLF4-3′UTR and pcDNA3.0-miR-10b caused a 30% decrease in the luciferase activity compared to the negative control. This suppression was rescued by the four-nucleotide substitution in the core binding sites (Fig. 3C). The similar effect was also found in KYSE450 cells (Fig. 3C).

**miR-10b regulates endogenous KLF4 protein expression.** To test whether miR-10b expression affected endogenous KLF4 expression, we transfected pcDNA3.0-miR-10b and control plasmid into KYSE140 and KYSE450 cells, a decrease of KLF4 protein level in KYSE140 and KYSE450 cells was observed. Consistent with these results, silencing of miR-10b in EC9706 cells showed an increase of KLF4 protein level (Fig. 4A). Meanwhile we found that HOXD10 was also regulated by miR-10b in these three cell lines, with a corresponding change of RHOC level (Fig. 4A). Real-time q-PCR of *KLF4* mRNA showed that miR-10b overexpression or inhibition had no effect on *KLF4* mRNA level (Fig. 4B), which indicated the post-transcriptional regulation of miR-10b on *KLF4* mRNA. We then examined KLF4 protein levels in eight ESCC cell lines (Fig. 4C) and compared them with miR-10b levels, but no significant correlation was found between KLF4 protein levels and miR-10b levels.

KLF4 is an unstable protein and ubiquitin-mediated proteasome is involved in its degradation. When new protein synthesis was blocked, KLF4 underwent rapid turnover and exhibited a relatively short half-life of 2 h (40). To rule out any effect of miR-10b on KLF4 protein stability, the half-life of KLF4 was measured. KYSE140 cells transfected with miR-10b expressing plasmid were treated with cyclohexamide to halt new protein synthesis. At the indicated time points, cell extracts were prepared and analyzed by Western Blot (Fig. 4D). Analysis of KLF4/β-actin ratios by regression plot showed that the half-life of KLF4 in miR-10b overexpressing cells was comparable to control cells (Fig. 4E). Therefore, miR-10b does not change the rate of KLF4 protein degradation. The decreased KLF4 expression in miR-10b overexpressing cells was due to the posttranscriptional regulation of miR-10b.

**KLF4 partly suppresses migration and invasion initiated by miR-10b.** Next, we investigated whether KLF4 suppressed cell migration and invasion in KYSE140 and EC9706 cells. KLF4 has been reported to suppress metastasis of several cancers including esophageal cancer cells (28). As shown in Fig. 5A and 5B, silencing of KLF4 by siRNA in KYSE140 cells led to increased cell migration and invasiveness, while overexpression of KLF4 in EC9706 cells resulted in a decrease in cell migration and invasion, demonstrating a negative role of KLF4 protein in migration and invasion in human ESCC cells.

To further establish a functional connection between miR-10b and KLF4, we tested whether KLF4 deregulation was required for regulation of miR-10b on cell migration and invasion. pcDNA3.1-KLF4, which carried the whole KLF4 coding sequence without 3′UTR, and
pcDNA3.0-miR-10b were cotransfected into KYSE140 cells. Interestingly, we found that expression of KLF4 partly abrogated migration and invasion initiated by miR-10b in KYSE140 cells (Fig. 5C). To further test this, we co-transfected EC9706 cells with siRNA for KLF4 mRNA and miR-10b-AS and found that the effect of miR-10b-AS was partially attenuated by siRNA for KLF4 mRNA (Fig. 5D). These results indicate that KLF4 serves as a target of miR-10b, contributing to the effect of miR-10b on cell migration and invasion. In parallel, KLF4 protein level of both experiments was analyzed by Western Blot.

miR-10b is significantly elevated in human ESCC tissues. Finally, we studied whether the expression level of miR-10b in vivo was associated with the metastasis outcome in human ESCC patients. We measured miR-10b level in 40 tumor specimens and their paired normal adjacent tissues. Among these tissues, 20 pairs were node-positive and the others were node-negative, which were verified by pathological analysis. As shown in Fig. 6, an elevated miR-10b level was detected in 95% (38/40) of the tumor tissues compared with their corresponding normal tissues, with a mean fold of 3.39. The elevation of miR-10b levels in cancer tissues was highly significant with \( p < 0.001 \), indicating an important role of miR-10b in human ESCC. However, student’s \( t \)-test, used to examine the difference of miR-10b level between node-positive and node-negative tissues, yielded no significant correlation of miR-10b levels with clinical metastasis status, with \( p \) value = 0.242.

DISCUSSION

MiRNAs, a class of small noncoding RNAs, have emerged as important regulators of protein posttranscriptional regulation. Increased numbers of reports reveal the aberrant expression of several miRNAs in tumors, correlation with certain oncogenes or tumor suppressors, or in response to chemotherapy or radiation (9, 41, 42). In the past two years, accumulating data have pointed to a regulatory role for miRNAs in cancer metastasis, which was first discovered by Ma et al who found that miR-10b initiates breast cancer invasion and metastasis. In this study, we investigated the role and the functional target of miR-10b in human ESCC.

We first analyzed miR-10b levels and migration and invasion potential in eight ESCC cell lines. The results showed that miR-10b level and migration and invasion potential varied significantly among different cell lines. The migration potential of the cell lines that we observed was consistent with a previous study in which they evaluated migration potential of 12 ESCC cell lines (43). Statistically significant correlations were observed between miR-10b expression and the migration and invasion potential in ESCC cells, which suggested a causal role of miR-10b in human ESCC cell migration and invasion in culture. We subsequently confirmed that miR-10b promoted migration and invasion in human ESCC cells, which was similar with that in breast cancer cells (16).

Next we explored the molecular mechanism underlying its function and found that KLF4 was a direct target of miR-10b. KLF4 is a transcription factor which is involved in cell-cycle regulation, apoptosis and differentiation, its expression can be increased by DNA damage, serum deprivation and contact inhibition (20, 21). Recently, KLF4 has been shown to inhibit cell migration and invasion in TE2 of human ESCC cell line (28). In addition, KLF4 can inhibit pancreatic cancer metastasis in vivo (23). Consistent with these results, KLF4 overexpression reduces cell migration and invasion in RKO colon cancer cells (29). These observations, taken together, indicate that KLF4 has a negative role in migration and invasion of cancer cells, which is opposite to the positive role of miR-10b. To explore the role of KLF4 in the promotion of migration and invasion by
miR-10b, we first confirmed the suppression role of KLF4 protein on cell migration and invasion in KYSE140 and EC9706 cells. To further establish the functional connection between miR-10b and KLF4, we overexpressed KLF4 and miR-10b in KYSE140 cells and silenced KLF4 and miR-10b in EC9706 cells, and found that KLF4 protein could partly abrogated the function of miR-10b. Therefore, KLF4 protein might have a role in miR-10b regulation of cell migration and invasion. KLF4 overexpression suppressed both cell migration and invasion in EC9706 cells, whereas silencing of miR-10b did not lead to changes of EC9706 cell migration. Additionally, KLF4 overexpression did not completely counteract the effect of miR-10b, which indicated the existence of other targets of miR-10b. In fact, it is already known that HOXD10 is a direct and functional target of miR-10b, which resulted in an increased expression of a well-characterized pro-metastatic gene RHOC (16). We confirmed that HOXD10 is regulated by miR-10b with a corresponding change of RHOC in the three ESCC cell lines.

We further investigated KLF4 protein levels in eight ESCC cell lines and compared them with miR-10b levels, but no significant correlation was found between KLF4 protein levels and miR-10b levels. Since KLF4 has an important role in many biological processes, its expression may be regulated by many different regulators (21, 44). Therefore, miR-10b, as one of its posttranscriptional regulators, could partly influence its protein level, but may not have a correlation with its expression level.

MiR-10b was initially found to be downregulated in breast cancer specimens compared with normal breast tissues (45), the downregulation in breast cancer was further confirmed by others (16, 46). Conversely, miR-10b was subsequently found to be significantly upregulated in human glioblastoma multiforme (47) and pancreatic adenocarcinomas (48). In the present study, we detected miR-10b level in 40 primary human ESCC tissues and their corresponding normal counterparts, and found that 95% (38/40) of the cancer tissues had increased miR-10b expression levels than the paired normal tissues, with \( p \) value < 0.001, which was in line with the discoveries in human glioblastoma multiforme (47) and pancreatic adenocarcinomas (48). However, no significant correlation was found between the miR-10b level and the metastasis outcome of the patients, which gave rise to the controversy of two groups (16, 46). Ma et al showed that miR-10b was overexpressed in about 50% (9/18) of metastasis-positive tissues compared with metastasis-free tissues or normal breast tissues (16). However, Gee et al studied 219 patients and found that miR-10b did not have any significant correlation with factors such as the presence or number of tumor-involved lymph nodes (46). Our data demonstrated no significant correlation of miR-10b levels with clinical metastasis status. However this is not necessarily in conflict with the finding that miR-10b promoted migration and invasion of human ESCC cells. First, the primary cancer was a heterogeneous mass of cells, such as mesenchymal cells, blood cells and cancer cells. Metastatic or invasive cells might be only a small fraction in the whole cancer samples. Thus it is unlikely to correlate miR-10b with the clinical metastasis status in whole tissue samples. Second, the few metastatic or invasive cells may be at the growing edge of cancer, which may not be contained in the dissected samples. In addition, although miR-10b has been shown to initiate cancer metastasis, considering the multi-steps in cancer metastasis, we are not sure which steps miR-10b may be involved in. Moreover, 40 pairs of cancer samples may not be enough to test the correlation of miR-10b with the clinical metastasis status. Therefore, it is not surprising that no correlations were found between miR-10b level and clinical metastasis status in whole cancer samples.

In animal genomes, multiple precursors are found to produce similar mature products (49, 50), which form miRNA families. MiR-10a, a
close relative of miR-10b, is located at chromosome 17q21 within the cluster of the HOXB genes. The two miRNAs differ only in one nucleotide in their central nucleotides. They are identical at nucleotides 2-8, which seems to be the most important segment for target recognition (51). miR-10a has been studied in several tumors (52-54). According to TargetScan4.0 analysis, KLF4 mRNA target site for miR-10b overlaps with miR-10a. Moreover, comparing with miR-10b, miR-10a could form a perfectly complementary miRNA–target pairs with KLF4 mRNA. Thus miR-10a might have a similar role in human esophageal cancer which needs to be further investigated.

In conclusion, we have shown for the first time that the miR-10b level was significantly correlated with the migration and invasion potential of human ESCC cells. Furthermore, miR-10b overexpression promoted cell migration and invasion in human ESCC cell lines. Additionally, we identified KLF4 mRNA as a direct and functional target of miR-10b. Finally, we revealed an elevated expression of miR-10b in ESCC tissue specimens, albeit there was no significant correlation of miR-10b levels with clinical metastasis status. A future challenge will be to identify additional targets of miR-10b to elucidate its role in esophageal cancer.

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**FOOTNOTES**

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**FIGURE LEGENDS**

**Fig. 1.** Correlation of miR-10b with migration and invasion potential in human ESCC cell lines. *A.* MiR-10b expression in eight ESCC cell lines was measured by real-time qRT-PCR and the *U6* small nuclear RNA was used as internal control. *B.* Migration and invasion potential was assessed by transwell assays and quantified by relative cell numbers. Pearson’s correlation analysis was used to examine the correlation between miR-10b and migration and invasion potential of human ESCC cell lines.

**Fig. 2.** miR-10b promotes migration and invasion of human ESCC cell lines. *A.* Sequences of miR-10a and miR-10b, looped primer used for reverse transcription (RT) and miRNA specific primers for real-time PCR. *B* & *C.* In (B) KYSE140 cells transfected by pcDNA3.0-miR-10b and control vector and (C) EC9706 cells transfected by miR-10b-AS, miR-10a and miR-10b expression were measured by real-time qRT-PCR. Migration and invasion potential in both cell lines (D and F) treated as above were
measured by transwell assays, and were expressed as relative cell numbers (E and G). The results were means of three independent experiments ± SD.

**Fig. 3.** The KLF4 3’-untranslated region (3’UTR) is a target of miR-10b. A. A miR-10b target site resides at nt295-301 of the KLF4 3’UTR, and is highly conserved in different species. Upper panel: sequence alignment of miR-10b with binding sites on the KLF4 3’UTR. Lower panel: sequence of the miR-10b binding site within the KLF4 3’UTR of eight species. B. Diagram of the luciferase reporter plasmids: plasmid with the full length KLF4-3’UTR insert (pIS0-KLF4-3’UTR) and plasmid with a mutant KLF4-3’UTR (pIS0-KLF4-3’UTR-mut) which carried a substitution of four nucleotides within the miR-10b binding site. C. Luciferase activity assay demonstrates a direct targeting of the 3’UTR of KLF4 by miR-10b. KYSE140 and KYSE450 cells were transfected with pcDNA3.0-miR-10b and pIS0-KLF4-3’UTR/pIS0-KLF4-3’UTR-mut. pRL-SV40 Renilla was used for normalization of transfection efficiency. After 48 hours, luciferase activities were measured.

**Fig. 4.** miR-10b regulates KLF4 expression at the post-transcriptional level. A. KYSE140 and KYSE450 cells were transfected with pcDNA3.0-miR-10b and control vector. EC9706 were transfected with miR-10b-AS and nonsense sequence. After 48 hours, KLF4, HOXD10 and RHOC protein levels were analyzed by Western blot. B. In parallel, KLF4 mRNA in the three cell lines treated as above was measured by real-time RT-PCR. GAPDH was used as internal control. C. KLF4 protein level was tested by Western blot and band intensities of KLF4 protein were quantified by densitometry and normalized against β-actin. Pearson’s correlation analysis was used to examine the correlation between miR-10b and KLF4 protein levels of human ESCC cell lines. D & E. KYSE140 cells, at 48h after transfection of pcDNA3.0 and pcDNA-3.0-miR-10b, were treated with 100μg/ml cyclohexamide and harvested at the indicated time points. Whole cell extracts were analyzed by Western blot in (D). Band intensities of KLF4 protein were quantified by densitometry and analyzed in a nonlinear regression plot in (E). The results were means of three independent experiments.

**Fig. 5.** KLF4 partly suppresses migration and invasion initiated by miR-10b. A. KYSE140 cells were transfected with siRNA targeting KLF4 mRNA and nonsense sequence, and EC9706 cells were transfected with pcDNA3.1-KLF4 and pcDNA3.1. After 48 hours, transwell assays were performed to evaluate migration and invasion potential of these cells. B. KLF4 protein levels in these cells were analyzed by Western blot. C & D pcDNA3.1-KLF4, which carried the whole KLF4 coding sequence without 3’UTR, and pcDNA3.0-miR-10b were cotransfected into KYSE140 cells (C). EC9706 cells were transfected with siRNA for KLF4 mRNA and miR-10b-AS (D). Then migration and invasion assays were performed to evaluate the effect of KLF4 on the function of miR-10b. In parallel, KLF4 protein levels were analyzed by Western blot.

**Fig. 6.** MiR-10b is significantly upregulated in human ESCC tissue samples. MiR-10b expression in forty paired tissue specimens (cancer and adjacent normal mucosa) of primary human ESCC was measured by relative qRT-PCR. The U6 small nuclear RNA was used as internal control. Student’s t-test was used to examine the difference of miR-10b level between node-positive and node-negative tissues.
Table 1 The clinicopathologic characteristics of 40 ESCC patients

<table>
<thead>
<tr>
<th>Clinicopathologic characteristics</th>
<th>Case distribution</th>
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<tbody>
<tr>
<td>Age (median)</td>
<td>38-72 (57)</td>
</tr>
<tr>
<td>Sex (male/female)</td>
<td>30/10</td>
</tr>
<tr>
<td>Differentiation (well/moderate/poor)</td>
<td>14/17/9</td>
</tr>
<tr>
<td>Tumor depth (sm/mp/ad)</td>
<td>4/9/27</td>
</tr>
<tr>
<td>Lymph node metastasis (negative/positive)</td>
<td>20/20</td>
</tr>
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Abbreviations: sm, submucosa; mp, muscularis propria; ad, adventitia.
Figure 2

A

miR-10a
UACCCUGUAAGAUCGAAAUUGUG
looped RT primer
primer for miR-10a
GGATACCTGTAGACCCGAA
miR-10b
UACCCUGUAAGAUCGAAAUUGUG
primer for miR-10b
GGATACCTGTAGACCCGAA
GTCGTATCCAGTGCTGCTGGG
AAACACCAGCATAGTCAGACGTTAACCGCTG

B

EC9706

C

Relative expression of
miR-10b by real-time RT-PCR

D

migration

E

Relative cell number

control
miR-10b-AS

F

cDNA3.0
pcDNA3.0-miR-10b

G

Relative cell number

cDNA3.0
cDNA3.0-miR-10b

migration
invasion
Figure 3

A

KLF4 3'UTR

miR-10b

H. sapiens
M. mulatta
P. troglodytes
C. porcellus
O. cuniculus
B. taurus
E. caballus
L. africana

5' GGAAGUUGUGGAUACAGGGUAU 3'
3' UGUUUAAGCCAAGAUGUCCUAU 5'

B

5' Firefly luciferase KLF4-3'UTR 3'

pIS0-KLF4-3'UTR
pIS0-KLF4-3'UTR-mut

GGAAGUUGUGGAUACAGGGUAU
GGAAGUUGUGGAUACTCUCAU

C

Luciferase Firefly:Renilla

pcDNA3.0 + - + - + - + -
pcDNA3.0-miR-10b - + - + - + -
plS0-KLF4-3'UTR + + - + + +
plS0-KLF4-3'UTR-mut - - + + - + +

KYSE140
KYSE450

15
Figure 5

A

![Graph showing relative cell number for control, KLF4+ siRNA, pcDNA3.1, and pcDNA3.1-KLF4 in KYSE140 and EC9706 cells.](image)

B

![Western blots showing KLF4 and β-actin expression levels for control, KLF4+ siRNA, pcDNA3.1, and pcDNA3.1-KLF4 in KYSE140 and EC9706 cells.](image)

C

![Graph showing relative cell number for pcDNA3.0, pcDNA3.0-miR-10b, pcDNA3.1, and pcDNA3.1-KLF4 in KYSE140 and EC9706 cells.](image)

D

![Graph showing relative cell number for control, KLF4+ siRNA, EGFP-AS, and miR-10b-AS in KYSE140 and EC9706 cells.](image)
Figure 6

![Graph showing relative expression of miR-10b in normal and cancer conditions for node-positive and node-negative samples.]

- Normal
- Cancer
MICRORNA-10B promotes human esophageal cancer cell migration and invasion through KLF4

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