MicroRNA-146a suppresses tumor malignancy via targeting vimentin in esophageal squamous cell carcinoma cells with lower fibronectin membrane assembly

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Abstract

**Background:** Esophageal squamous cell carcinoma (ESCC) is widely prevalent in Taiwan, and high metastatic spread of ESCC leads to poor survival rate. Fibronectin (FN) assembly on the cell membrane may induce ESCC mobility. Micro-RNAs (MiRNAs) are abundant in and participate in tumorigenesis in many cancers. However, the role of MiRNA in FN assembly-related ESCC mobility remains unexplored.

**Methods:** We divided ESCC CE81T cells into high-FN assembly (CE81FN+) and low-FN assembly (CE81FN−) groups by flow cytometry. MiRNA microarray analysis identified miR-146a expression as the most down-regulated miRNA in comparison of CE81FN+ and CE81FN− cells.

**Results:** Cell proliferation and migration were decreased when CE81FN+ cells overexpressed transgenic miR-146a compared to the parental cells, indicating an inverse correlation between low miR-146a expression and high proliferation as well as motility of FN assembly ESCC cells. Furthermore, vimentin is the target gene of miR-146a involved in ESCC tumorigenesis. MiR-146a suppressed cell proliferation, migration, and invasion of CE81FN+ cells through the inhibition of vimentin expression, as confirmed by real-time PCR, Western blotting and Transwell™ assay. Analysis of one hundred and thirty-six paired ESCC patient specimens revealed that low miR-146a and high vimentin levels were frequently detected in tumor, and that the former was associated with late tumor stages (III and IV). Notably, either low miR-146a expression or high vimentin level was significantly associated with poor overall survival rate among ESCC patients.

**Conclusions:** This is the first report to link FN assembly in the cell membrane with miR-146a, vimentin and ESCC tumorigenesis both in vitro and in ESCC patients.

**Keywords:** ESCC, miR-146a, Vimentin, Cell migration, Invasion

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In addition, the prognosis of ESCC is poor and 5-year survival rate is less than 15%, due to the high rates of tumor invasion and metastasis [3–5]. The progression of metastasis in ESCC is complex, involving extracellular matrix (ECM)-cell interaction, cell signaling network, gene regulation and epithelial mesenchymal transition (EMT). These processes turn benign tumor cells into malignant ones. However, the mechanism whereby the regulation of ECM-affected EMT beings about deterioration of cell behaviors during carcinogenesis is complex and poorly understood.

Fibronectin (FN) is an adhesive glycoprotein present in ECM and plasma, which also functions as a mesenchymal marker involved in EMT-related signaling pathways [6–8]. Overexpression of FN has been reported in the malignant development of various cancer types including breast, lung, melanoma, colorectal, and ovarian cancers [7, 9–11]. Up-regulation of FN is also associated with ESCC tumor progression and the degree of ESCC tumor invasion [12]. Emerging evidence indicates that early cancer metastasis is activated by the interaction of ECM with the tumor cell surface receptors, such as FN-integrin interaction [13]. Overexpression of FN together with vimentin is associated with advanced stage and poor prognosis of ESCC patients [14]. Vimentin and FN are the markers of epithelial to mesenchymal transition (EMT), which participate in tumor cell migration and invasion [6]. Recent study indicated that EMT may be regulated by miRNAs, such as miR-1199-5p [15]. However, the relationship among FN assembly, miRNA and target genes during ESCC tumorigenesis remains unclear. This study aimed to clarify the role of microRNAs and target genes in ESCC tumorigenesis under low and high FN assembly conditions.

MiRNAs are small, noncoding, single-stranded RNA molecules harboring 20–23 nucleotides, which post-transcriptionally regulate the target gene expression in diverse physiological or pathological processes through the degradation of mRNAs or blockage of translation by annealing to the complementary mRNA coding sequences [6, 16, 17]. Dysfunction of miRNA regulation affects cellular homeostasis and triggers various diseases including cancers. MiRNA may function either as an oncogene or a tumor suppressor depending on its target gene [12, 18]. Therefore, whether miRNAs participate in FN-related tumorigenesis warrants further exploration. MiR-146a functions either as a tumor suppressor or an oncogene depending on the types of cancer cells [19]. There has been reported that miR-146a as a tumor suppressor is significantly decreased in both cancerous tissue and serum of ESCC [20]. However, the role of miR-146a and its target genes have not been characterized in ESCC.

Here, we showed that polymeric fibronectin assembly on the cell membrane promotes cell motility through the regulation of miR-146a and the target gene vimentin. Vimentin functions as a mesenchymal marker and participates in EMT. We confirmed that overexpression of miR-146a significantly suppresses cell proliferation, colony and tumor formation, as well as migration and invasion through inhibition of vimentin. Finally, the results of our analysis of clinical ESCC specimens support the notion that suppression of miR146a and up-regulation of vimentin promotes ESCC tumorigenesis.

**Methods**

**Stable cell lines, cell culture and construction of vimentin 3′UTR luciferase reporter plasmid**

CE81FN+ and CE81FN− cells were sorted from a human ESCC cell line CE81T (ATCC® HTB56™) by flow cytometry. CE81T cells incubated with anti-FN antibody-conjugated magnetic beads and Magnetic bead-bound CE81T cells were designated as CE81FN+ cells. The FN unbound cells were named as CE81FN− cells. For the quantification purpose, the sorted cells stained with anti-FN polyclonal antibodies and analyzed by flow cytometry. CE81FN+ + miR-146a and CE81FN+ + CON stable cell lines were established using lentiviral infection from the parental CE81FN+ cells. The above cell lines and human embryonic kidney 293T cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Gibco, Maryland, USA) containing 10% fetal bovine serum (FBS; Biological Industries, Kibbutz Beit haemek, Israel), penicillin (200 U/ml; Sigma, Missouri, USA) and streptomycin (100 μg/ml; Sigma) at 37 °C in a 5% CO2 incubator. ESCC cell lines KYSE150 (RRID: CVCL_1348) and KYSE70 (RRID: CVCL_1356) were cultured in RPMI1640 medium (Gibco). For construction of vimentin 3′-UTR luciferase reporter plasmid, the PMIR-REPORT™ (Thermo Fisher Scientific, Illinois, USA) was used following the manufacturer’s instructions. The target sequences of 3′-UTR region of wild- and mutant-type vimentin are provided in the Additional file 1: Table S1.

**Transfection**

Stable CE81FN+ cell lines overexpressing miR-146a or miR-control were established by transfection with premiR-146a (Pre-miR™ miRNA precursor; Applied Biosystems, Massachusetts, USA) or pre-miR-control (5 μmole/L) using lentiviral infection. Transient transfection of anti-miR-146a (100 pmol/L) (Anti-miR™ miRNA inhibitor; Applied Biosystems), siRNA-vimentin (Invitrogen, California, USA), plasmid pcDNA3-vimentin (a gift from Dr. Ming-Der Perng, National Tsing-Hua University, Hsinchu, Taiwan) were transfected by Lipofectamine 2000™ following the manufacturer’s instructions.
The negative small interfering RNA controls used were pre-miRNA (Applied Biosystems), anti-miRNA (Applied Biosystems), and si-RNA (Invitrogen). The plasmid pcDNA3-Luc was used as a vector control. The cells (5 × 10^5/well) were transfected with the above materials in a six-well plate for 48 h.

**Western blotting**
Cells were lysed and protein samples were collected by centrifugation at 13,600 rpm for 20 min at 4 °C. The concentration of protein samples was determined by the Coomassie protein assay kit (Thermo Fisher Scientific). An equal amount of protein was loaded and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The separated protein bands were electrically transferred to a PVDF membrane (MILLIPORE, Massachusetts, USA). The membrane was blocked with 5% non-fat milk in Tris-buffered saline and Tween 20 (TBST) at RT for 1 h and then incubated with the specific primary antibodies. The following antibodies were used: vimentin (1:1000, MILLIPORE) and β-actin (1:5000, Sigma). The blots were incubated with ECL (Millipore) and captured by BioSpectrum AC (UVP). The results of Western blotting were quantified by density analysis using Vision Works TM LS image acquisition and analysis software.

**miRNA detection and real-time polymerase chain reaction (PCR)**
Total RNA was extracted from tissues or cultured cells using the Trizol™ reagent (Invitrogen) according to manufacturer’s instructions. The RNA pellet dissolved in DEPC water, and Ncode™ VILO™ cDNA synthesis miRNA kit (Invitrogen) was used for cDNA synthesis. The cDNA synthesized using MultiScribe™ Reverse Transcriptase in the presence of a universal Reverse primer (Invitrogen). The conditions of the thermal cycler were programmed as follows: 25 °C 10 min, 37 °C 120 min, 85 °C 5 min and finally at 4 °C. Real-time PCR was conducted to amplify the cDNA with SYBR Green SuperMix™ reagent (Invitrogen), the universal primer and miR-146a-specific primer (Additional file 1: Table S1). The mixture (20 μl) was loaded into a 96-well plate and analyzed by using a real-time PCR machine (Applied Biosystems). Data were normalized with endogenous U54, and the relative expression was calculated with the formula 2 − ΔCt values. For identifying the potential target gene of miR-146a, two groups were designed for this experiment. First group CE81FN+ cells transfected with N.C. and mimic-miR-146a and another group is CE81FN+ + CON and CE81FN+ + 146a stable cell lines. The comparison of these two gene expression profiles by Illumina™ oligonucleotide microarray analysis.

Transwell™ migration and invasion assay
Cell migration and invasion were investigated using Transwells with pore size of 8 μm (Falcon, BD Labware, Massachusetts, USA). For cell migration, the cells (10^5 cells/plate) were seeded into the upper chamber with 200 μl of serum free DMEM, and 600 μl of DMEM supplemented with 10% PBS was seeded into the lower chamber. For cell invasion, the upper chamber was coated with a 1 mg/ml matrigel (BD Biosciences, California, USA) in advance and incubated at 37 °C for 1 h. The cells were seeded into the upper chamber and incubated at 37 °C in a 5% CO2 incubator for 48 and 108 h, respectively. After incubation, the upper chamber was carefully removed using cotton swabs, and the cells that migrated to the bottom chamber were washed twice, fixed with 1% formaldehyde for 15 min and stained with 0.1% crystal violet (Sigma) for 10 min at RT. The migrated cells were counted in 10 randomly selected fields under a light microscope.

**Immunohistochemistry (IHC) and immunofluorescence staining (IFA)**
CE81FN+, CE81FN−, CE81FN+ + CON and CE81FN+ + 146a cells were seeded (4 × 10^5 cells/well) onto glass coverslips in a 6-well plate. The cells were fixed in 3.7% paraformaldehyde for 30 min, and washed by 1X PBS for 5 min followed by treatment with 0.1% Triton-X-100 for 30 min. Cells were blocked in 1X blocking buffer for 1 h at RT, and then treated with the monoclonal anti-vimentin antibody (Abcam, Cambridge, UK) for 16 h at 4 °C. After PBS washing for 10 min, cells were treated with secondary mouse monoclonal antibody and kept in the dark for 1 h. After PBS washing, cells were counterstained with Hoechst 33342 staining solution (Abcam) for 30 min and then mounted with glycerol gelatin (Sigma). The protein level of vimentin was evaluated by IHC staining of tumor and non-tumor tissues of the ESCC patient specimens. The anti-vimentin monoclonal antibody (Abcam) was used to detect vimentin protein. The secondary antibody was used followed by Streptavidin labeling (Dako, Cytomation, Carpinteria, USA). The slides were then treated with AEC solution for 20 min at RT, and counterstained with 10% hematoxylin (Muto Pure Chemicals, Tokyo, Japan) and mounted with glycerol gelatin (Sigma).
ESCC patient specimens
One hundred and thirty six ESCC specimens were analyzed in this study. Informed consent was obtained from all patients and the study was approved by the Institutional Review Board, National Cheng Kung University Hospital, Tainan, Taiwan. All clinical samples were used in accordance with the guidelines of the Declaration of Helsinki. A total of 68-paired ESCC specimens were used for the detection of miRNA expression by real-time PCR, and a different set of 68 paired ESCC specimens were used for staining of miR-146a expression by in situ hybridization and vimentin expression by IHC and ISH staining (Table 3).

Statistical analysis
Two-tailed Student’s t test was used. Data are shown as mean ± SD. Overall survival curves were calculated according to the Kaplan–Meier method (log-rank test). p < 0.05 was considered to be statistically significant.

Results
FN assembly on ESCC cell membrane correlated with miR-146a expression and cell migration
Vascular arrest and metastasis of the circulating tumor cells in the lungs could be mediated by the binding between polymeric FN assembled on the surface of tumor cells and endothelial dipeptidyl peptidase IV (DPP IV) [10]. Therefore, we sorted out the ESCC CE81T cells into two groups by flow cytometry according to the expression level of FN that was observed, i.e., high FN assembly on the cell surfaces (CE81FN+ cells) and the other with low FN assembly (CE81FN− cells) (Fig. 1a, b). Figure 1a demonstrated that the abundant FN assembly on the membrane of CE81FN+ cells compared to that on CE81FN− cells was shown by immunofluorescence staining (Fig. 1a, arrow). Figure 1b indicated that the fluorescence signals from CE81FN− and CE81FN+ cells merged and quantified by flow cytometry. Next, we investigated the possibility that miRNAs participate in FN assembly-related cell migration. Therefore, we conducted the miRNA microarray screening. The miRNA expression profile showed that miR-146a was the most down-regulated miRNA in CE81FN+ cells compared to CE81FN− cells (Table 1, 5.02 fold). Similarly, the expression level of miR-146a was significantly lower in CE81FN+ cells than in CE81FN− cells as confirmed by real-time PCR (Fig. 1c). We then measured the migration ability of these two cell lines by Transwell™ migration assay at 48 h post-seeding. CE81FN+ cells showed a significantly higher migration rate compared to CE81FN− cells (Fig. 1d). In summary,
highly expressed in CE81FN cells. P values were less than 0.05 analyzed by Biomedical Engineering Center, Industrial Technology Research miRNA-microarray, which contains 932 probes. The microarray data was PCR (Fig. 2a). CE81FN cells were compared to the gene expression profiles of either miR-146a or control miRNA from the paren- cell lines CE81FN cells. We compared the gene expression profiles of miR-146a gene in ESCC cells. Prediction and validation of vimentin as a target migration. abundant FN assembly on cell membranes was associ- ated with low miR-146a expression and high ESCC cell migration.

### Table 1 Differentially expressed miRNAs in CE81FN+ cells compared to CE81FN− cells

| Hsa-miR-ID      | P-value | Fold changea | Regulation (FN-P/FN-N)b |
|-----------------|---------|--------------|-------------------------|
| Hsa-miR-99a     | 0.01    | 2.16         | Up                      |
| Hsa-miR-921     | 0.04    | 1.88         | Up                      |
| Hsa-miR-320b    | 0.00    | 1.60         | Up                      |
| Hsa-miR-320c    | 0.00    | 1.60         | Up                      |
| Hsa-miR-1268    | 0.02    | 1.59         | Up                      |
| Hsa-miR-146a    | 0.02    | 5.02         | Down                    |
| Hsa-miR-301a    | 0.04    | 1.83         | Down                    |
| Hsa-miR-141     | 0.03    | 1.82         | Down                    |
| Hsa-miR-345     | 0.01    | 1.58         | Down                    |
| Hsa-miR-660     | 0.02    | 1.55         | Down                    |

Total RNA extracted from CE81FN+ and CE81FN− cells was screened by the miRNA-microarray, which contains 932 probes. The microarray data was analyzed by Biomedical Engineering Center, Industrial Technology Research Institute, Hsinchu, Taiwan. All the P values were less than 0.05

- a The Fold change of CE81FN+ vs. CE81FN− is the log ratio value of real-time PCR analysis
- b FN-P/FN-N represent as FN-Positive and FN-Negative expression CE81T cells

The protein level of vimentin was suppressed in CE81FN+ + 146a cells

We compared the protein level of vimentin in the two stable cell lines CE81FN+ + 146a and CE81FN+ + CON. Accordingly, the vimentin protein level was low at CE81FN+ + 146a cells in compared to CE81FN+CON cells by Western blotting (Fig. 3a) as well as by immuno- fluorescence staining (Fig. 3b, green). We constructed the pMIR-luciferase reporter plasmid harboring either the wild-type or mutant vimentin 3’UTR. The lucif- erase activity was assessed to confirm miR-146a targeting vimentin 3’UTR. HEK293T cells were co-transfected with either wild-type or mutant-type vimentin 3’UTR plasmid and pre-miR-146a or control microRNA (N.C.) for 48 h (Additional file 1: Figure S2). Our data showed that overexpression of miR-146a resulted in significant suppression of wild-type vimentin luciferase reporter activity compared to the control microRNA (N.C.). How- ever, miR-146a showed no significant suppression of the mutant vimentin luciferase reporter activity (Additional file 1: Figure S2). Furthermore, human breast cancer cells: MDA-MB-231 FN+/MCF7 FN+ [21] as well as lung cancer cells: CL1-5 FN+/CL1-0 FN+ [11] showing higher migration of FN+ cells. In addition, we transiently transfected two ESCC cell lines KYSE 150 (high miR-146a expression) and KYSE 70 (low miR-146a expression) (Additional file 1: Figure S3A) with anti-miR-146a or miR-146a, respectively, and evaluated the level of vimen- tin protein (Additional file 1: Figure S3B and S3C) and cell invasion capability (Additional file 1: Figure S3D). Altogether, these data together with the results in Fig. 2 imply that fibronectin assembly mediated miR-146a nega- tive regulation of vimentin expression is a general event in various cancer cells.

**MiR-146a inhibited ESCC cell invasion by targeting vimentin**

To confirmed the role of miR-146a together with its neg- ative regulated vimentin in the tumorigenesis of ESCC cells, the CE81FN+ + CON stable cell line shown in Fig. 3 was transiently transfected with scramble microRNA or mimic-miR-146a, and miR-146a overexpression was
confirmed by real-time PCR (Fig. 4a). Accordingly, we detected decreased protein level of vimentin (Fig. 4b, left panel), accompanied with decreased invasion capability of the CE81\textsuperscript{FN+} + CON cells overexpressing mimic-miR-146a (Fig. 4c, miR-146a vs. N.C.). To verify the relationship between vimentin and miR-146a as well as their effects on cell motility, we showed that the protein level of vimentin increased in CE81\textsuperscript{FN+} + CON cells co-transfected with mimic-miR-146a and pcDNA3-vimentin (Fig. 4b, right panel), accompanied by increased cell invasion compared to the vector control (Fig. 4c, miR-146a + pcDNA3.1-Vim vs. miR-146a + Vector).

Similarly, we transiently introduced anti-scramble microRNA (anti-N.C.), and anti-miR-146a into CE81\textsuperscript{FN+} + 146a stable cells as shown in Fig. 4d. We found that the endogenous miR-146a level was significantly decreased (>35%) in CE81\textsuperscript{FN+} + 146a cells with anti-miR-146a compared to the CE81\textsuperscript{FN+} + 146a cells with
Table 2 The most up and down-regulated mRNA expression profile of ESCC cell lines by oligonucleotide microarray analysis

| Gene symbol | Regulation | log FC* |
|-------------|------------|---------|
| NDRG1       | Up         | 4.07    |
| SLC2A3      | Up         | 3.70    |
| SMG1        | Up         | 2.54    |
| IL1A        | Up         | 2.51    |
| LIMCH1      | Up         | 2.51    |
| TMEM123     | Up         | 2.33    |
| DDX21       | Up         | 2.27    |
| HERC5       | Up         | 2.25    |
| NUDT21      | Up         | 2.25    |
| PLOD2       | Up         | 2.25    |
| BIRC3       | Up         | 2.23    |
| IFIT2       | Up         | 2.21    |
| SMC3        | Up         | 2.13    |
| FAM60A      | Up         | 2.08    |
| UBE2V2      | Up         | 2.07    |
| ITGA9       | Up         | 2.05    |
| FAM18B      | Up         | 2.04    |
| CETN3       | Up         | 2.04    |
| ITGB1       | Up         | 2.01    |
| NMD3        | Up         | 1.99    |
| HSPA1A      | Down       | –1.94   |
| ATFS        | Down       | –1.77   |
| PTHLH       | Down       | –1.66   |
| HSPA1B      | Down       | –1.62   |
| C16orf53    | Down       | –1.61   |
| GRWD1       | Down       | –1.59   |
| MPDU1       | Down       | –1.56   |
| TRPM2       | Down       | –1.56   |
| DDX54       | Down       | –1.55   |
| PTHLH       | Down       | –1.51   |
| S100A2      | Down       | –1.47   |
| LFNG        | Down       | –1.47   |
| MEPCE       | Down       | –1.44   |
| DDX39       | Down       | –1.43   |
| AVEN        | Down       | –1.41   |
| MPDU1       | Down       | –1.40   |
| POLR2L      | Down       | –1.39   |
| LYPD1       | Down       | –1.38   |
| LANCL2      | Down       | –1.38   |
| VIMb        | Down       | –0.41   |

1—CE81 FN+ cells were transiently transfected with N.C. or mimic-miR-146a.
2—CE81 FN+ + CON and CE81 FN+ + 146a stable cell lines. The cells from 1 and 2 were under the microarray analyzed by illumina gene expression system. The illumina chip contains 47,231 probes and over 31,000 genes. The data shows the differential gene expression profile of 1 and 2.

* Represents as log of fold change of 2.

b Vimentin (VIM) expression was down-regulated in this microarray.

anti-miR-146a compared to the scramble anti-N.C. group (Fig. 4e, left panel). Accordingly, the invasion of CE81 FN+ + 146a cells transiently expressing anti-miR-146a was significantly higher compared to the anti-N.C. group (Fig. 4f, anti-miR-146a vs. anti-N.C.). We further silenced the expression of vimentin in the CE81 FN+ + 146a + anti-miR-146a cells with a small interfering RNA of vimentin (si-Vim) (Fig. 4e, right panel). Consequently, the invasion of the CE81 FN+ + 146a + anti-miR-146a + si-vimentin cells was significantly decreased compared to the CE81 FN+ + 146a + anti-miR-146a + si–N.C. cells (Fig. 4f, anti-miR-146a + si-vimentin vs. anti-miR-146a + si–N.C.). The negative regulation between miR-146a and vimentin was further confirmed using HEK293T cells due to its high transfection efficiency (Additional file 1: Figure S4). Similar results were observed in another two ESCC cell lines as shown in Additional file 1: Figure S2.

Taken together, above data clearly imply that miR-146a suppresses cell invasion through downregulation of vimentin expression in various ESCC cells.

**MiR-146a and vimentin expression levels correlated with ESCC tumor stage and overall survival rate of ESCC patients**

To clarify the significance of miR-146a and the target gene vimentin in clinical ESCC patients, two groups of ESCC patient specimens were analyzed (Table 3). The first set of 68 ESCC patient specimens (Group I, Table 3) was used for measuring miR-146a level by real-time PCR. The miR-146a level was significantly higher in the early ESCC tumor stages (I + II) compared to the late tumor stages (III + IV) (Additional file 1: Figure S5). The expression levels of miR-146a and vimentin in the second set of 68 paired ESCC patient specimens (non-tumor vs. tumor) in the tissue array (Group II, Table 3) were measured by ISH and IHC staining. The level of miR-146a was significantly lower in tumorous ESCC compared to non-tumorous ESCC by ISH staining (Fig. 5a). In contrast, vimentin protein level was significantly higher in tumorous cells compared to non-tumorous cells by IHC staining (Fig. 5b). The expressions of miR-146a and vimentin in tissue sections of two representative ESCC patients were evaluated by ISH and IHC staining, respectively. We detected low miR-146a and high vimentin expression in the tumorous cells of these two representative patients (Fig. 5c), which were consistent with the results presented above. Furthermore, the associations of miR-146a and vimentin with overall survival rate among these ESCC patients were analyzed by Kaplan–Meier analysis and log rank test. Our data revealed that the poor overall survival rate of ESCC patients 2 years after surgery were
significantly correlated with either low miR-146a expression (cut off value: < 0.8) or high vimentin expression (cut off value: > 1.8) but not with miR-146a + vimentin (Fig. 5d, e and Additional file 1: Table S2). We conducted the clinicopathologic parameter analysis of miR-146a, vimentin and miR-146a + vimentin using sixty-eight ESCC patients (Table 3, Group II). No further correlation of miR-146a, vimentin and miR-146a + vimentin with age and gender was detected (Additional file 1: Table S2). Above data imply that the negative correlation between miR-146a and vimentin affects ESCC tumorigenesis and tumor progression, both in vitro and in ESCC patient specimens.

**Discussion**

In this study, we demonstrated that the level of membrane-bound FN was negatively correlated with the expression of miR-146a which suppressed ESCC cell motility by targeting vimentin, a marker of EMT during normal development or metastatic progression. We found that the miR-146a-vimentin axis is involved in tumor formation, tumor stage and overall survival rate in
of tumor cells, including stimulation of cell proliferation, differentiation, and metastatic cascade in melanoma. Zhang et al. J Biomed Sci (2020) 27:102

| Characteristics | Group I (ESCC patient specimens) | Group II (non-tumor vs. tumor) |
|-----------------|----------------------------------|-------------------------------|
| Patients no     | 68                               | 68                            |
| Age (years)     | 57                               | 57                            |
| Median age      | 36–87                            | 34–82                         |
| Sex             | Male/female                       |                                |
| Male            | 64/4                             |                                |
| Female          | 4/6                              |                                |
| Tumor stages    |                                  |                                |
| I               | 8                                | 6                             |
| II              | 19                               | 14                            |
| III             | 31                               | 43                            |
| IV              | 10                               | 5                             |
| Two-year survival |                |                                |
| ≥ 24 months    | 32                               | 18                            |
| < 24 months    | 36                               | 50                            |

Group I is the characteristics of ESCC patients for measuring the miR-146a RNA expression levels by real-time RT-PCR

Group II is the characteristics of ESCC patient specimens (non-tumor vs. tumor) for direct detection of miR-146a and vimentin expression levels by ISH and IHC staining

Table 3 Characteristics of Group I and Group II of ESCC clinical specimens

ESCC patients. This is the first report to reveal that miR-146a suppresses migration and invasion through the negative regulation of vimentin in ESCC cells.

Expression of FN that is assembled on the cell membrane as a polymeric form plays a pivotal role in promoting tumor progression, including cell migration, invasion, and cancer metastasis [7, 9, 11]. Indeed, aggregation of FN on the cell membrane is highly-correlated with metastasis in various cancer cell types [10, 22, 23]. FN, a metastatic related gene, is also highly expressed in diverse tumors [23], and modulates multiple functions of tumor cells, including stimulation of cell proliferation, differentiation, and metastatic cascade in melanoma and ovarian cancers via activation of various cell surface receptors such as integrin [22, 24]. Zhang et al. reported that FN expression is up-regulated during lung cancer metastasis [25]. Here, we found that FN assembled on the ESCC tumor cell membrane was highly associated with low expression level of miR-146a, which led to increased ESCC cell invasion through increased vimentin expression.

Increased of ESCC tumorigenesis through the targeting of PTEN or REPS2 by miR-21 and miR-373 have been reported [26, 27]. In contrast, the expression levels of miR-205 and miR-375 decreased in tumorous parts of ESCC patient specimens by targeting Zeb and PDK1 genes [28, 29]. The above findings indicate the complexity of miRNAs, their target genes, as well as their functions in ESCC tumorigenesis.

MiRNA plays critical roles in the regulation of tumorigenesis and various signaling pathways including, apoptosis, inflammation and immune responses [30, 31]. MiR-146 is further classified as miR-146a and miR-146b, which are located on chromosome 5 and 10, respectively. Only two nucleotides differ between the mature form of miR-146a and miR-146b at the 3’-end. However, the underlying mechanism involved in the regulation of miR-146a and miR-146b remains unclear [32]. Katakowski et al. reported that overexpression of miR-146b decreases glioma cell migration and invasion [33]. Moreover, it has also been shown that miR-146b is overexpressed in thyroid tumor, colorectal cancer, and melanoma [34]. In lung and pancreatic cancers, miR-146a is defined as an anti-tumor RNA, inhibits cell migration and invasion through suppression of EGFR signaling [35, 36]. It also plays a suppressive role in breast, gastric, and prostate cancers. In contrast, miR-146a functions as an oncogene in melanoma, which promotes tumor initiation and progression by activating Notch signaling [37, 38]. Therefore, miR-146a may function either as an oncogene or a tumor suppressor in tumorigenesis depending on the types of tumors. In addition, miR-146a negatively regulates the pro-inflammatory chemokine IL-8 and the innate immune response by interfering with the NF-κB pathway [31, 39]. In this study, we demonstrated that miR-146a plays a suppressive role in ESCC tumorigenesis and tumor progression, including cell proliferation, colony and tumor formation (data not shown), migration and invasion.

Since downregulation of miR-146a in ESCC cells is associated with high FN assembly on cell membranes, it is conceptually possible that both events are causally related. It has been reported that the transcription of miR-146a can be activated by NF-κB [40, 41] and activation of AKT is capable of triggering nuclear transportation of NF-κB to become an active transcription factor [42, 43]. Interestingly, we demonstrated that AKT is inactivated in suspended tumor cells with high FN assembly on the cell membrane [8]. These findings imply that FN assembly on tumor cell membrane may cause downregulation of miR-146a by suppressing the PI3K/AKT/NF-κB signaling pathway [44]. Furthermore, it has been shown that the metastatic suppressor, BRMS1, increases the level of miR-146a in cancer cells to reduce their metastatic potential [45]. Since FN assembly on tumor cell membranes is highly associated with the metastatic potential of tumor cells [9, 11, 46], it is likely that FN assembly decreases BRMS1 to circumvent metastatic suppression by reducing BRMS1-promoted miR-146a levels [45]. Furthermore, WWOX,
Fig. 5  Low miR-146a or high vimentin expression levels in the tumorous ESCC tissue array correlated with poor overall survival rate.  

**a** The miR-146a levels in the tumorous and adjacent non-tumorous cells of 68 paired ESCC patient specimens in the tissue array after ISH treatment were quantified by defining regions of interest (ROI) using automated cell acquisition and quantification software (Histoquest™) (P < 0.001).  

**b** The protein levels of vimentin in the same ESCC specimens used in (a) were stained by IHC followed by Histoquest™ quantification (P < 0.0001).  

**c** Two representative patient specimens containing tumorous and adjacent non-tumorous cells from the 68 ESCC patients were analyzed to determine miR-146a and vimentin expression levels after ISH and IHC treatment. Hematoxylin was used to stain nucleus and AEC (3-amino-9-ethylcarbazole) for miR-146a and vimentin expression. Scale bar 20 μm.  

The correlation of **d** miR-146a level (P < 0.0001) and **e** vimentin expression (P < 0.05) with ESCC patient overall survival rate were assessed by Kaplan–meier analysis and Log rank test.
a negative regulator of c-Myc, has been demonstrated to inhibit the FN expression level and metastatic ability of triple-negative breast cancer (TNBC) by upregulating miR-146a [47]. In line with these findings, upregulation of SOX5, a target gene of miR-146a-5p, and downregulation of miR-146a-5p have been reported in TNBC [48]. Overexpressing SOX5 significantly eliminates the effects of miR-146a-5p mimics in TNBC cells and increases the expression of mesenchymal markers including FN and vimentin [48]. Furthermore, miR-146a mimics decrease the TGF-β-induced fibronectin in orbital fibroblasts by lowering Smad4 and TRAF6 protein levels [49], indicating that miR-146a may lead to the inhibition of FN expression in ESCC cells. Altogether, these contradictory findings warrant further investigation of the causal relationship between miR-146a and FN assembly on ESCC cell membranes.

Guo et al. reported that functional SNPs of pre-miR-146a contribute to ESCC susceptibility and clinical outcome [50]. Here, we reveal that FN assembly on ESCC cell membrane was associated with decreased expression of miR-146a, leading to upregulation of vimentin and promotion of ESCC cell motility. More importantly, emerging evidence indicated that FN expression is highly associated with poor overall survival rates in variety of cancer types such as colorectal and gastric cancer [51, 52]. On the other hand, high expression of miR-146a were correlated with prolonged overall survival [53]. Based on our finding, the FN assembly is negative correlation with miR-146a regarding to cancer patients overall survival rates. Notably, we demonstrated that either low miR-146a or high vimentin expression was significantly correlated with tumor formation, tumor stages and poor overall survival rate of ESCC patients (Fig. 5). These findings may be valuable for developing a novel drug targeting strategy toward miR-146a and vimentin for treatment of late stages ESCC.

Vimentin is the major component of the intermediate filaments and, along with FN, contributes to epithelial-mesenchymal transition (EMT) as well as cancer cell metastasis. Similarly, overexpression of vimentin in ESCC cells is correlated with increased tumor growth, invasion, poor prognosis, and lymph node metastasis [54, 55]. Sudo et al. reported that the high expression of both FN and vimentin was associated with advanced tumor stage and poor prognosis in ESCC patients [14]. Actually, current research suggests that FN promotes EMT in variety of malignant cancer types and is one of well-known biomarkers of EMT for cancer metastasis [7]. Indeed, we found that downregulation of miR-146a led to upregulation of vimentin thereby promoting cell proliferation, colony and tumor formation as well as cell motility of ESCC cells that highly assemble FN on the cell membrane.

The discrepancy of the correlation between the expression level of vimentin and the level of cell invasion (Fig. 4) indicates the possibly that miR-146a may target other molecules such as IGSF1 (Fig. 2b), which are also required for ESCC cell motility. Nevertheless, this experiment disclosed the trend of a negative correlation between miR-146a and vimentin plus cell invasion. More importantly, the underlying mechanism for downregulation of miR-146a through epigenetic alteration during ESCC progression might be elucidated in the future. Taken together, from our findings we hypothesize that fibronectin assembly-related low miR-146a expression induces ESCC cell mobility through regulation of its targeting gene vimentin (Additional file 1: Figure S6).

MiRNA-96 decreases cancer cell invasion and migration by inhibition of K-ras gene expression and triggering of apoptosis [13]. MiR-200c suppresses proliferation by down-regulating mutant K-ras expression in breast and lung cancer cells [56]. Moreover in colorectal cancer, let-7 miRNA suppresses K-ras activity and p53 expression [57]. These studies imply that an association exists between various miRNAs and the ras gene. It has been reported that increased miR-146a suppresses the expression of EGFR, ERK1/2 and K-ras genes, resulting in the inhibition of cell migration, invasion and proliferation of pancreatic cancer and non-small cell lung cancer cells [35, 58]. The above findings imply that miR-146a affects the activity of K-ras. However, the regulatory relationship between K-ras and miR-146a in ESCC warrants further study.

In summary, we identified a new regulatory mechanism whereby miR-146a suppresses the cell motility of ESCC cells that highly assemble FN on cell membranes by inhibiting vimentin expression. Our findings warrant further exploration to determine the roles of these two genes in ESCC cell metastasis in vivo.

**Conclusions**

ESCC patients in Taiwan are at high risk of developing tumor cell metastasis. However, the underlying mechanism remains unclear. High fibronectin membrane assembly ESCC cells (CE81\textsuperscript{FN+}) showed increased migration and invasion compared to low fibronectin membrane assembly cells (CE81\textsuperscript{FN-}). MiR-146a expression was the most down-regulated miRNA in CE81\textsuperscript{FN+} cells compared to CE81\textsuperscript{FN-} cells. MiR-146a expression level was inversely correlated with the mobility of ESCC cells. We identified vimentin as the target of miR-146a in ESCC tumorigenesis and mobility, and further analysis confirmed this finding. Analysis of one hundred and thirty-six ESCC patient specimens disclosed that low miR-146a expression or high vimentin level was significantly associated with tumor formation and poor overall
survival rate. This is the first report to link FN cell membrane assembly with miR-146a, vimentin and ESCC tumorigenesis both in vitro and in ESCC patients.

Supplementary information

Abbreviations

ESCC: Esophageal squamous cell carcinoma; FN: Fibronectin; miRNA: Micro-RNA; CE81FN: CE81T cells with high-FN assembly; CE81TFN: CE81T cells with low-FN assembly; EMT: Epithelial mesenchymal transition; ECM: Extracellular matrix; RT-PCR: Reverse transcription-polymerase chain reaction.

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Authors’ contributions

Literature search: CHL, HYC, YSL, YFW, HSL, HCC. Figures and tables: CHL, HYC, YSL, SHL. Study design: CHL, HYC, YSL, YFW, HSL, HCC. Data collection: CHL, YSL, JTH, HSL, HCC. Data analysis: CHL, HYC, YSL, YFW, HSL, HCC. Data interpretation: CHL, SHL, YSL, YFW, HSL, HCC. Clinical specimens: W-WL. Writing: CHL, HYC, HSL, HCC. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analyzed in the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

An informed consent form was signed by all patients in accordance with the Declaration of Helsinki and the study was approved by the Institutional Review Board, National Cheng Kung University Hospital, Tainan, Taiwan R.O.C. (No. B-08-102-099).

Consent for publication

Not applicable.

Competing interests

All authors declare that they have no competing interests.

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