

Keywords: esophageal squamous cell carcinoma; FSCN1; MMP14; invadopodia; anti-oncomir; prognosis

MicroRNA-133a regulates the mRNAs of two invadopodia-related proteins, FSCN1 and MMP14, in esophageal cancer

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Background: FSCN1 and matrix metalloproteinase 14 (MMP14) are both invadopodia-related proteins. We herein elucidate the tumourigenicity of these proteins and identify novel therapeutic agents in esophageal squamous cell carcinoma (ESCC).

Methods: FSCN1 and MMP14 were evaluated by immunohistochemistry and quantitative PCR, and microRNA (miR)-133a was also evaluated by PCR in surgical ESCC specimens. The roles of *FSCN1*, *MMP14* and miR-133a were established in ESCC cells.

Results: The expression of FSCN1 or MMP14 was an independent poor prognostic factor according to a multivariate analysis of immunohistochemistry, and their co-expression correlated with the poorest overall survival (OS) out of all the examined factors. Additionally, their mRNAs significantly correlated and both inversely correlated with miR-133a in surgical specimens. Transfection of a miR-133a mimic decreased the mRNA and protein levels of both FSCN1 and MMP14 in ESCC cells. The knockdown of *FSCN1* or *MMP14* and transfection of a miR-133a mimic inhibited the proliferation and invasion of ESCC cells. Patients with a lower miR-133a expression have a significantly poorer OS than those with a higher expression.

Conclusion: The combined expression of FSCN1 and MMP14 is associated with a poor prognosis, and miR-133a, which regulates their mRNAs, can serve as a strong tumour suppressor of ESCC.

Esophageal squamous cell carcinoma (ESCC) is one of the most malignant cancers of the gastrointestinal tract. Although multimodal therapy has improved the prognosis, the outcome of ESCC is still unsatisfactory. The poor outcomes of ESCC are related to its diagnosis at advanced stages and its propensity for metastasis, even when tumours are superficial (Pennathur *et al*, 2013).

FSCN1 is an evolutionarily conserved actin bundling protein that is localised in microspikes and filopodia underneath the plasma membrane. FSCN1 is widely expressed during embryogenesis in neural and mesenchymal tissues, but in adults, it is largely restricted to specific tissues, including the brain, endothelium and testes (Machesky and Li, 2010). In cancer cells, aberrantly expressed FSCN1 stabilises actin filaments in invasive foot structures termed invadopodia (Figure 1A). Invadopodia promote the degradation of the extracellular matrix (ECM) by coordinating

the presentation of matrix metalloprotease 14 (MMP14, also known as membrane type-1 MMP (MT1 MMP)) to the tips of the protruding structure and by increasing the secretion of MMP2 and MMP9 (Murphy and Courtneidge, 2011).

MMPs are zinc-dependent endopeptidases that not only degrade a variety of ECM molecules but also process a number of bioactive molecules. MMPs can degrade collagen, elastin, laminin, fibronectin and proteoglycan and are known to have important roles in cancer growth, migration, invasion, angiogenesis and metastasis (Chambers and Matrisian, 1997). There are two types of MMPs, secreted MMPs and membrane-anchored MMPs. MMP14 is one of the membrane-anchored MMPs, and MMP2 and MMP9 are secreted MMPs. MMP14 has a central role in tumour invasion and not only degrades the ECM itself (Zarrabi *et al*, 2011) but also promotes the secretion of proMMP2 and proMMP9 by

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Revised 13 September 2013; accepted 4 October 2013; published online 5 November 2013

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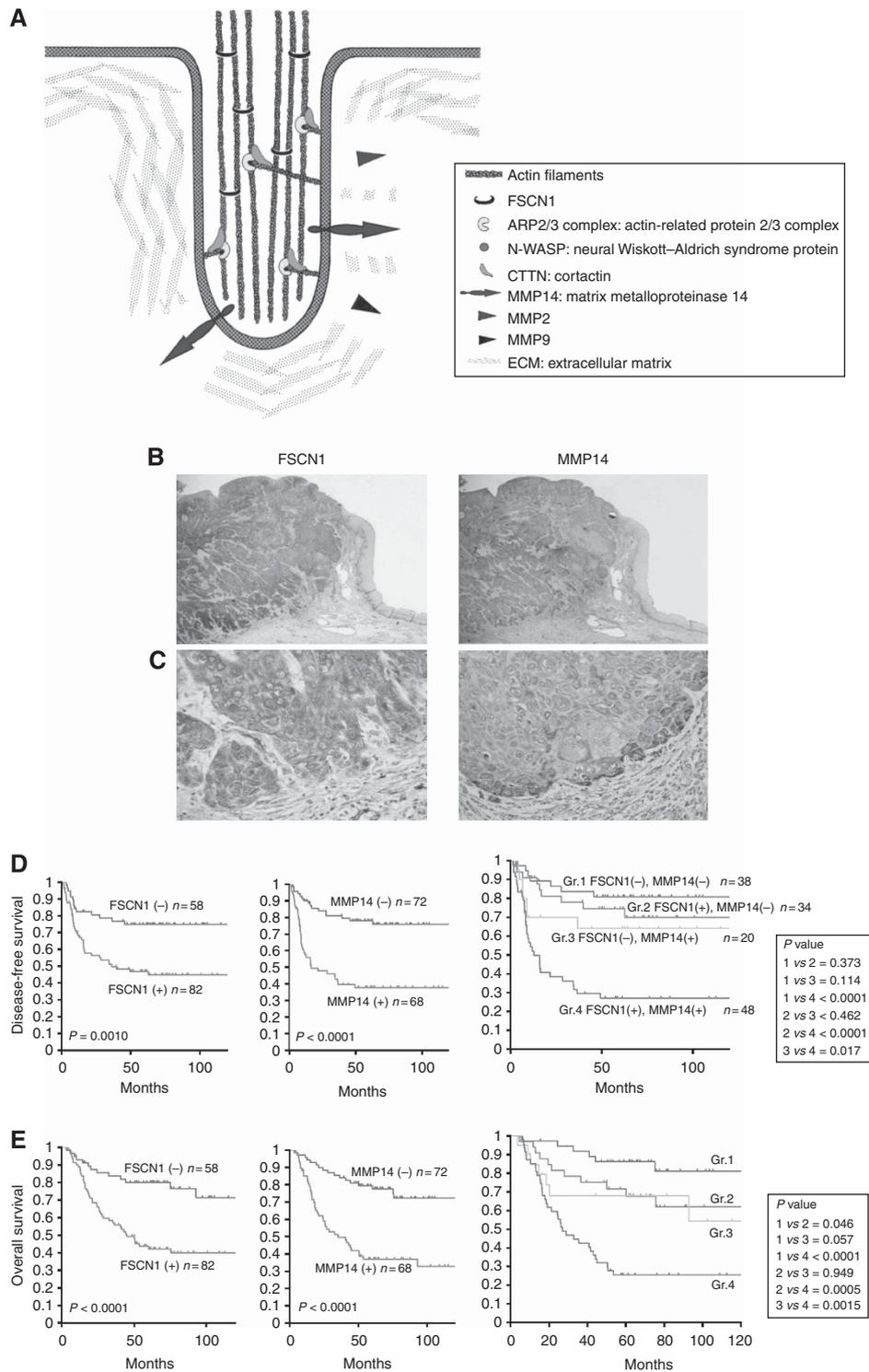


Figure 1. A schematic drawing of invadopodia and the expression profiles of FSCN1 and matrix metalloproteinase 14 (MMP14) in 140 ESCC samples. **(A)** FSCN1 bundles actin filaments and contributes to the stabilisation of invadopodia. CTTN (also known as cortactin), also one of the actin-binding proteins, promotes actin branching by the activation of the ARP 2/3 complex in synergy with N-WASP at the edge of the migrating cells. Invadopodia present MMP14 and secrete MMP2 and MMP9. **(B)** A microscopic view of the immunohistochemical analysis of ESCC adjacent to the normal epithelium. The data for FSCN1 (left panel) and MMP14 (right panel) are shown ($\times 20$); **(C)** invasive front ($\times 400$). **(D)** Kaplan–Meier survival analysis of the disease-free survival in ESCC cases with positive vs negative FSCN1 staining (left panel), MMP14 staining (middle panel) and different FSCN1/MMP14 expression patterns (right panel). **(E)** Kaplan–Meier survival analysis of the overall survival in ESCC cases with positive vs negative FSCN1 (left panel), MMP14 (middle panel) and FSCN1/MMP14 expression patterns (right panel).

cancer-associated fibroblasts and activates them (Egawa *et al*, 2006). MMP14 cleaves other functional proteins such as CD44, the integrin ν chain, low-density lipoprotein receptor-related protein, interleukin 8 and pro-tumour necrosis factor. Thus the proteolytic

activity of MMP14 contributes to the maintenance of the tumour microenvironment and promotes tumour growth and invasion (Egawa *et al*, 2006). In addition, recent studies have revealed that MMP14 regulates both cell growth and motility by a

non-proteolytic mechanism (D'Alessio *et al*, 2008; Sakamoto and Seiki, 2009).

The effect of *FSCN1* on cell invasiveness occurs as a result of its cooperation with MMPs, such as MMP2 and MMP9, in several cancers (Xie *et al*, 2005; Onodera *et al*, 2009; Ozerhan *et al*, 2010; Al-Alwan *et al*, 2011; Hayashi *et al*, 2011; Hanker *et al*, 2013). However, secreted MMPs are not crucial for basement membrane invasion, whereas MMP14 is (Sabeh *et al*, 2004; Hotary *et al*, 2006; Li *et al*, 2008; Poincloux *et al*, 2009). There are no previous reports investigating the correlation between *FSCN1* and MMP14. We herein show the significance of *FSCN1* and MMP14 expression in ESCC specimens and demonstrate that these molecules are regulated by one microRNA. Moreover, this microRNA, miR-133a, exerts its tumour-suppressive activities by regulating these molecules.

MATERIALS AND METHODS

Clinical ESCC specimens and ESCC cell lines. Formalin-fixed and paraffin-embedded tissue blocks were obtained from 140 patients, and frozen tissues were obtained from 84 patients. All patients underwent primary surgical treatment and were pathologically proven to have ESCC at the Chiba University Hospital, Chiba, Japan from 1997 to 2011. Written consent for tissue donation was obtained from each patient. The protocol was approved by the Institutional Review Board of Chiba University.

The TE2 human ESCC cell lines were provided by the Cell Resource Center for Biomedical Research Institute of Development, Aging and Cancer, Tohoku University, Sendai, Japan. T.Tn human ESCC cell lines were provided by the Japanese Cancer Research Resources Bank. All cells were cultured in Dulbecco's modified Eagle's medium nutrient mixture (DMEM; Life Technologies, Grand Island, NY, USA) supplemented with 10% fetal bovine serum at 37 °C in a 5% CO₂ atmosphere.

Western blot analysis. Anti-human MMP14 rabbit polyclonal IgG (1 : 250; Millipore, Temecula, CA, USA; Cat. No. AB6004) and anti-human *FSCN1* mouse monoclonal immunoglobulin (IG) G (1 : 100; Dako, Tokyo, Japan; code: M3567) were used as primary antibodies. The western blot analysis using MMP14-transfected A431 cells as a positive control, T.Tn control cell and two MMP14-silenced cells revealed that the MMP14 antibody recognised MMP14, and no non-specific bands were observed. In addition, a western blot analysis of T.Tn control cells and two *FSCN1*-silenced cells was performed and revealed that the *FSCN1* antibody recognised *FSCN1*, and no non-specific bands were observed.

The cells were harvested and lysed with RIPA (Radio-Immunoprecipitation Assay) buffer 72 h after transfection. The western blot analysis was performed on the XV PANTERA Gel 7.5–15% (DRC, Tokyo, Japan). The proteins in the gels were transferred electrophoretically onto nitrocellulose membranes. The membranes were incubated with primary antibodies against *FSCN1*, MMP14 and β -actin (1 : 1000; Abcam Limited, Cambridge, UK; clone No. mAbcam 8226). The expression of each protein was detected using secondary antibodies conjugated to peroxidase-labelled polymers. Bands were visualised using the Amersham ECL Prime Western blotting reagent (GE Healthcare Life Sciences, Uppsala, Sweden). Densitometry was done using the ImageJ software program (NIH, Bethesda, MD, USA).

Immunohistochemistry. The sections were mechanically deparaffinised and incubated in Target Retrieval Solution (Dako, Carpinteria, CA, USA) at 95 °C for 40 min. After the endogenous peroxidase activity was blocked with methanol containing 3% hydrogen peroxide (Dako) for 30 min, the tissue sections were incubated with *FSCN1* antibody or MMP14 antibody at 4 °C overnight. The sections were then incubated in secondary antibody

at 37 °C for 60 min. Subsequently, the sections were counterstained with hematoxylin for 1 min. The evaluation of immunohistochemical (IHC) staining was done as described previously (Usui *et al*, 2012).

Fluorescence microscopy. Cells cultured on coverslips were fixed in 4% paraformaldehyde for 10 min and permeabilised with 0.1% Triton X-100 for 5 min. After blocking with 10% FBS/PBS, the cells were incubated with anti-human *FSCN1* antibody at 37 °C for 60 min. Then, cells were incubated with Alexa Fluor 532 goat anti-mouse IgG (Invitrogen, Carlsbad, CA, USA) as secondary antibodies and Acti-stain 488 Fluorescent Phalloidin (Cytoskeleton, Denver, CO, USA) for F-actin at 37 °C for 60 min. ProLong Gold antifade reagent with DAPI (Invitrogen) was used for the counterstaining of DNA and for the antifade reagent.

microRNA target prediction. miRNA targets were predicted by the algorithms of the miRWalk online prediction software program (<http://www.umm.uni-heidelberg.de/apps/zmf/mirwalk/>). The miRwalk miRNA target prediction was performed using 3'UTRs incorporating eight other established miRNA-target prediction programs (Diana-microT, miRanda, miRDB, PICTAR, PITAm, RNA22, RNAhybrid, Targetscan) (Dweep *et al*, 2011).

Target site inhibition assays. The miScript Target Protector for the miR-133a-binding site in the 3'UTR of *MMP14* mRNA (target-binding site sequence provided: 5'-CCAGAGGGGCAGGAGGGACCAAAAAGGAAAATGAGGAC-3') and Negative control miScript Target Protector with no homology to any known mammalian gene (Qiagen, Hilden, Germany) was used. The miScript Target Protector is a single-stranded, modified RNA sequence that specially interferes with the interaction of a miRNA with a single target, while leaving the regulation of other targets of the same miRNA unaffected. Mature miRNA molecules (*mirVana* miRNA hsa-miR-133a mimic; assay ID: MH10413, Applied Biosystems, Foster City, CA, USA) and miScript Target Protector or Negative control miScript Target Protector were co-transfected into ESCC cell lines. After 72 h of transfection, total RNA was isolated.

Transfection of the small interfering RNAs, microRNA mimic and microRNA inhibitor. The small interfering RNA (siRNA) sequences (Stealth RNAi siRNA, Invitrogen) targeting *FSCN1* (si*FSCN1*-1: Cat. No. HSS186009, si*FSCN1*-2: Cat. No. HSS110044), *MMP14* (si*MMP14*-1: Cat. No. HSS106639, si*MMP14*-2: Cat. No. HSS106640), the negative control (Negative Control Duplex medium GC Duplex No. 2: Cat. No. 12935-112), the miR-133a mimic (*mirVana* miRNA Mimic, assay ID MC10413) and the negative control (*mirVana* miRNA Mimic Negative Control No. 1, Applied Biosystems), the miR-133a inhibitor (*mirVana* miRNA Inhibitor, assay ID MH10413) and the negative control (*mirVana* miRNA Inhibitor Negative Control No. 1, Applied Biosystems) were transfected into cell lines by electroporation using the Neon Transfection System (Invitrogen).

Quantitative reverse transcription PCR (qRT-PCR). Total RNA was extracted from frozen surgical specimens and cell lines using Trizol reagent (Invitrogen) and reverse transcribed into cDNA using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems). Real-time PCR was done using the SsoFast EvaGreen Supermix (Bio-Rad, Hercules, CA, USA) with the following primers (PCReady PCR and Sequencing Primers, Operon, Biotechnology, Tokyo, Japan): *FSCN1*, 5'-CAACGATGGCGCCTCAA-3' and 5'-CCTTGTTATAGTCGCAGAAGTTCGAA-3'; and *MMP14*, 5'-AAGAGGAGAAGAGCAAACAG-3' and 5'-CGGTA GGCAGTGAAGTTCG-3'. *ACTA1* was used as an internal control, and the sequences used were 5'-CCTTCATCGGTATGGAGTC-3' and 5'-GTTGGCATAACAGTCCCTT-3'.

The miR-133a expression levels (assay ID: 002246) were analysed by TaqMan quantitative real-time PCR (TaqMan MicroRNA Assay, Applied Biosystems) and normalised to the RNU6B level (assay ID: 001093). All reactions were performed in triplicate.

Cell proliferation, migration and invasion assays in ESCC cell lines. After transfection of si*FSCN1*, si*MMP14* or the miR-133a mimic, cells were plated in 96-well plates at 5×10^3 cells per well in 100 μ l of medium containing 10% FBS. After 120 h, the cell proliferation was assessed with the Cell Counting Kit-8 (Dojindo, Kumamoto, Japan). Triplicate wells were measured for cell viability in each treatment group. Cell motility was determined using a micropore chamber assay, and the cell invasion was determined using a Matrigel-coated (BD Biosciences, Bedford, MA, USA) and collagen I-coated micropore membrane filter with 8 μ m pores. The collagen I-coated chambers were prepared as described previously (Wang *et al.*, 2010).

In brief, 5×10^4 cells were seeded onto the top chamber of a 24-well micropore polycarbonate membrane filter with 8 μ m pores (BD Biosciences), and the bottom chamber was filled with DMEM containing 10% FBS as a chemoattractant. After 24 h of incubation, the membranes were fixed and stained by the Diff-Quik reagent (International Reagents, Kobe, Japan), and the cells on the upper surface were carefully removed with a cotton swab. Cell migration/invasion was quantified by counting the average number of migrated/invaded cells in four random high-powered fields per filter.

Statistical analyses. The categorical variables of the 140 clinical samples examined by IHC were compared by the chi-square test. The survival analysis using the Kaplan–Meier method was performed by the log-rank test. The relationship between two variables and numerical values obtained by real-time RT–PCR were analysed using *t*-tests. The relationships among miR-133a expression, *FSCN1* expression and *MMP14* expression were analysed using the Spearman's rank correlation. Statistical significance was defined as $P < 0.05$. All statistical analyses were performed using the JMP statistical package (SAS Institute, Cary, NC, USA).

RESULTS

Expression of two invadopodia-related proteins, *FSCN1* and *MMP14*, in clinical ESCC specimens. Neither *FSCN1* nor *MMP14* was detected in the normal esophageal epithelium by IHC staining. Positive staining for both *FSCN1* and *MMP14* was apparent in cancer cells, especially in the cytoplasm, and strong staining was observed at the invasive front of tumours (Figure 1B and C).

Relationship between *FSCN1* or *MMP14* expression and the clinicopathological features. Among the 140 ESCC samples, 82 (58.6%) were positive for *FSCN1* and 68 (48.6%) were positive for *MMP14*. Patients with positive *FSCN1* showed significantly higher T stages ($P < 0.0005$), higher clinical stages ($P < 0.0005$) and a higher rate of recurrence ($P < 0.005$ by the chi-square test), and patients with positive *MMP14* tumours showed significantly higher T stages ($P < 0.05$), higher N stages ($P < 0.005$), higher clinical stages ($P < 0.001$), a higher level of lymphatic invasion ($P < 0.05$), a higher level of vascular invasion ($P < 0.05$) and higher rates of recurrence ($P < 0.0001$) (Table 1). Both the *FSCN1*-positive and *MMP14*-positive patients had a significantly shorter disease-free survival rate (DFS) ($P = 0.0010$ and $P < 0.0001$, respectively) and OS ($P < 0.0001$ and $P < 0.0001$, respectively) (Figure 1D and E). The 5-year survival rates were 79.9% vs 43.8% for those with no *FSCN1* staining and positive

FSCN1 staining, respectively. The 5-year survival rates were 79.4% and 36.9% for those without and with *MMP14* expression, respectively.

Relationship between *FSCN1* and *MMP14* expression and the survival time. We classified patients into four groups: group 1 included those who were both *FSCN1* and *MMP14* negative ($n = 38$, 27.1%), group 2 included those who were *FSCN1* positive and *MMP14* negative ($n = 34$, 24.3%), group 3 included those who were *MMP14* positive and *FSCN1* negative ($n = 20$, 14.3%) and group 4 included patients who were positive for both *FSCN1* and *MMP14* ($n = 48$, 34.3%). Out of the 68 cases with positive *MMP14* expression, 48 cases (70.6%) also showed positive *FSCN1* expression. However, 34 out of the 72 cases with negative *MMP14* staining (47.2%) showed a positive *FSCN1* expression. *MMP14* expression was significantly correlated with the *FSCN1* expression ($P = 0.0050$). A survival analysis showed significant differences in the DFS and OS among the four groups of patients, with the fourth group having a significantly lower survival rate compared with the other groups (Figure 1D and E). The 5-year survival rates of each group were 86.2, 71.7, 69.1 and 25.4% for groups 1, 2, 3 and 4, respectively.

A multivariable Cox proportional hazards analysis was performed to identify the independent factors associated with the OS. Positive *FSCN1* staining, positive *MMP14* staining, the T stage and the N stage were identified as significant independent prognostic factors (Table 2).

Effects of transfection of siRNAs targeting *FSCN1* mRNA and *MMP14* mRNA on ESCC cell lines. The introduction of each of the four siRNA sequences (si*FSCN1*-1, si*FSCN1*-2, si*MMP14*-1 and si*MMP14*-2) into T.Tn and TE2 cells led to a significant decrease of the target mRNA and a remarkable decrease in the amount of the target protein. However, the knockdown of *FSCN1* did not lead to a decrease in *MMP14* mRNA or protein in the two cell lines. Similarly, the knockdown of *MMP14* did not affect the expression of *FSCN1* mRNA or protein (Figure 2A–C).

Immunofluorescent staining of T.Tn and TE2 cells demonstrated that si*FSCN1* cells lost their normal spindle shape as seen in negative controls and also exhibited a more rounded morphology (Figure 2D, the data for TE2 cells not shown). However, the si*MMP14*-transfected cells did not show morphological changes (data not shown).

In the proliferation assays, transfection of each of the four siRNAs led to a significant decrease in cell growth 120 h after transfection (Figure 2E).

In the migration assay, the Matrigel invasion assay and the collagen I invasion assay, the rates of penetration of cells through the membrane were significantly decreased by the transfection of the each of the siRNAs. There was no significant difference between the number of si*FSCN1*-transfected cells that penetrated the Matrigel-coated membrane and that of the cells that penetrated the collagen I-coated membrane. However, the rate of si*MMP14*-transfected cells that penetrated the collagen I membrane was significantly lower than that of the cells that penetrated the Matrigel-coated membrane (33.3% vs 54.6% of the negative control (si*MMP14*-1, $P = 0.0395$) and 12.3% vs 25.2% (si*MMP14*-2, $P = 0.0362$) in T.Tn cells and 33.6% vs 54.4% (si*MMP14*-1, $P = 0.0027$) and 14.3% vs 23.9% (si*MMP14*-2, $P = 0.0026$) in TE2 cells (Figure 2F)).

Effects of miR-133a mimic, miR-133a inhibitor and Target Protector on ESCC cell lines. We previously reported that miR-133a regulates *FSCN1* (Kano *et al.*, 2010). A bioinformatic analysis performed using the 'miRwalk' method identified one conserved sequence site for miR-133a in the 3' UTR of *MMP14*.

The introduction of a miR-133a mimic into T.Tn and TE2 cells led to significant decreases in both the *FSCN1* and *MMP14* mRNA

Table 1. Relationship between the *FSCN1* and *MMP14* expression and clinicopathological features

	FSCN1 expression			MMP14 expression		
	- (n = 58)	+ (n = 82)	P-value	- (n = 72)	+ (n = 68)	P-value
Age, years						
< 65 (n = 81)	37	44	0.23	42	39	0.91
≥ 65 (n = 59)	21	38		30	29	
Gender						
Male (n = 121)	50	7	0.89	63	57	0.70
Female (n = 20)	8	12		9	11	
Tumour depth						
Tis, T1 (n = 60)	36	24	0.00011	38	22	0.015
T2, T3, T4 (n = 80)	22	58		34	46	
Regional lymph nodes						
N0 (n = 66)	33	33	0.052	43	23	0.0022
N1 (n = 74)	25	49		29	45	
Stage						
stage0, I (n = 35)	24	11	0.00017	27	8	0.00090
stageII, III, IV (n = 105)	34	71		45	60	
Lymphatic invasion						
ly0 (n = 51)	25	26	0.17	33	18	0.017
ly1, 2, 3 (n = 89)	33	56		39	50	
Vascular invasion						
v0 (n = 57)	28	29	0.13	36	21	0.021
v1, 2, 3 (n = 83)	30	53		36	47	
Histology						
Well, mod (n = 111)	45	66	0.68	59	52	0.42
Poor (n = 29)	13	16		13	16	
Recurrence						
Recurrence (n = 56)	14	42	0.0013	16	40	0.000010
No recurrence (n = 84)	44	40		56	28	

Abbreviation: MMP14 = matrix metalloproteinase 14.

Table 2. Cox multivariate analysis results

Terms	Risk ratio (95% CI)	P-value
Age > 65 years only	0.682 (0.391–1.160)	0.16
Male sex	1.564 (0.733–3.836)	0.28
T2,T3,T4	2.315 (1.065–5.626)	0.045
N1	2.088 (1.094–4.230)	0.032
Stage II,III,IV	0.535 (0.151–1.882)	0.33
Lymphatic invasion	1.230 (0.673–2.351)	0.51
Vascular invasion	1.451 (0.808–2.720)	0.23
Poor tumour differentiation	1.104 (0.567–2.002)	0.76
FSCN1-positive staining	2.348 (1.200–4.862)	0.016
MMP14-positive staining	2.458 (1.364–4.595)	0.0035

Abbreviations: CI = confidence interval; MMP14 = matrix metalloproteinase 14.

levels (56.8% and 42.6% of the negative control in T.Tn cells and 57.0% and 42.5% in the TE2 cells; Figure 3A). On the other hand, the introduction of a miR-133a inhibitor into the two cell lines led to significant increases in both the *FSCN1* mRNA and *MMP14* mRNA levels (140.4% and 157.6% of the negative control in T.Tn cells and 131.0% and 135.0% in TE2 cells; Figure 3B).

Then, target site inhibition assays were performed to investigate the interaction between miR-133a and *MMP14* mRNA. The two cell lines were co-transfected with the miR-133a mimic and miScript Target Protector designed to interfere with the interaction of miR-133a with *MMP14* mRNA by protecting the miR-133a-binding site of the *MMP14* mRNA. The results showed a significantly higher expression of *MMP14* mRNA than that in the cells transfected with the miR-133a and negative control miScript Target Protector (143.4% of the negative control in T.Tn cells and 135.0% in TE2 cells; Figure 3C). These results suggested that miR-133a directly regulates *MMP14* mRNA. The introduction of a miR-133a mimic into T.Tn and TE2 cells led to a decrease in the *FSCN1* and *MMP14* protein levels (Figure 3D and E).

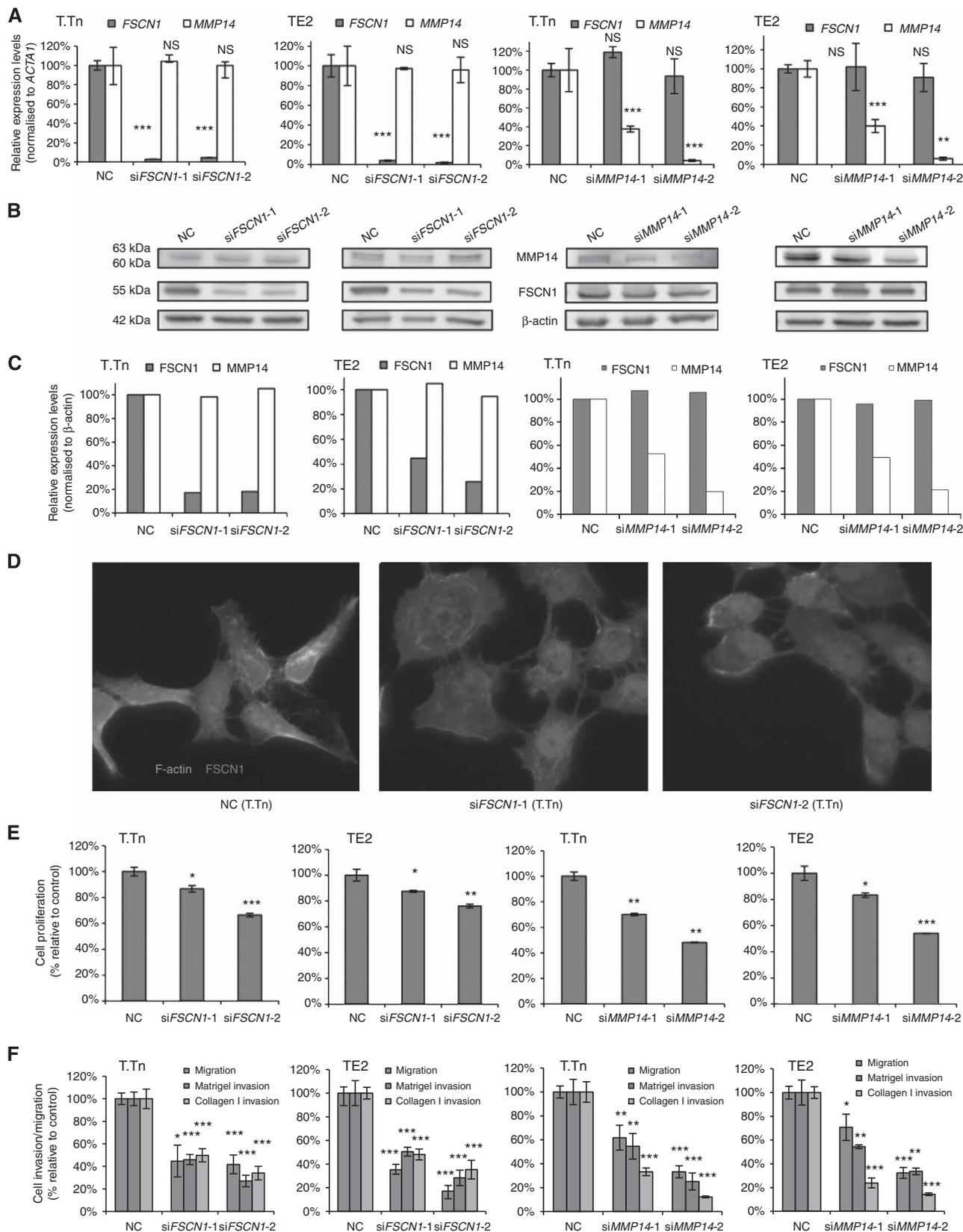


Figure 2. The effects of *FSCN1* and *MMP14* knockdown on the mRNA levels, protein levels, morphological changes, proliferation and migration/invasion activities in ESCC cell lines 72 h after the transfection. Two siFSCN1, siFSCN1-1 and siFSCN1-2, and two siMMP14, siMMP14-1 and siMMP14-2, were transfected into ESCC cell lines (T.Tn and TE2 cells). (A) Results of the evaluation of the mRNA expression levels by real-time RT-PCR. (B and C) The results of an evaluation of the protein expression levels determined by a western blot analysis. The MMP14 protein was seen as two bands, 63 kDa for the latent form and 60 kDa for the active form. (D) Morphological changes of T.Tn cells. The cells were stained with an anti-FSCN1 antibody (red), phalloidin (green) and DAPI (blue). (E) Results of the proliferation assay. (F) Results of the migration/Matrigel invasion/collagen I invasion assay. Abbreviations: NC, negative control, NS, not significant. **P* < 0.05, ***P* < 0.01, ****P* < 0.005. The full colour version of this figure is available at *British Journal of Cancer* online.

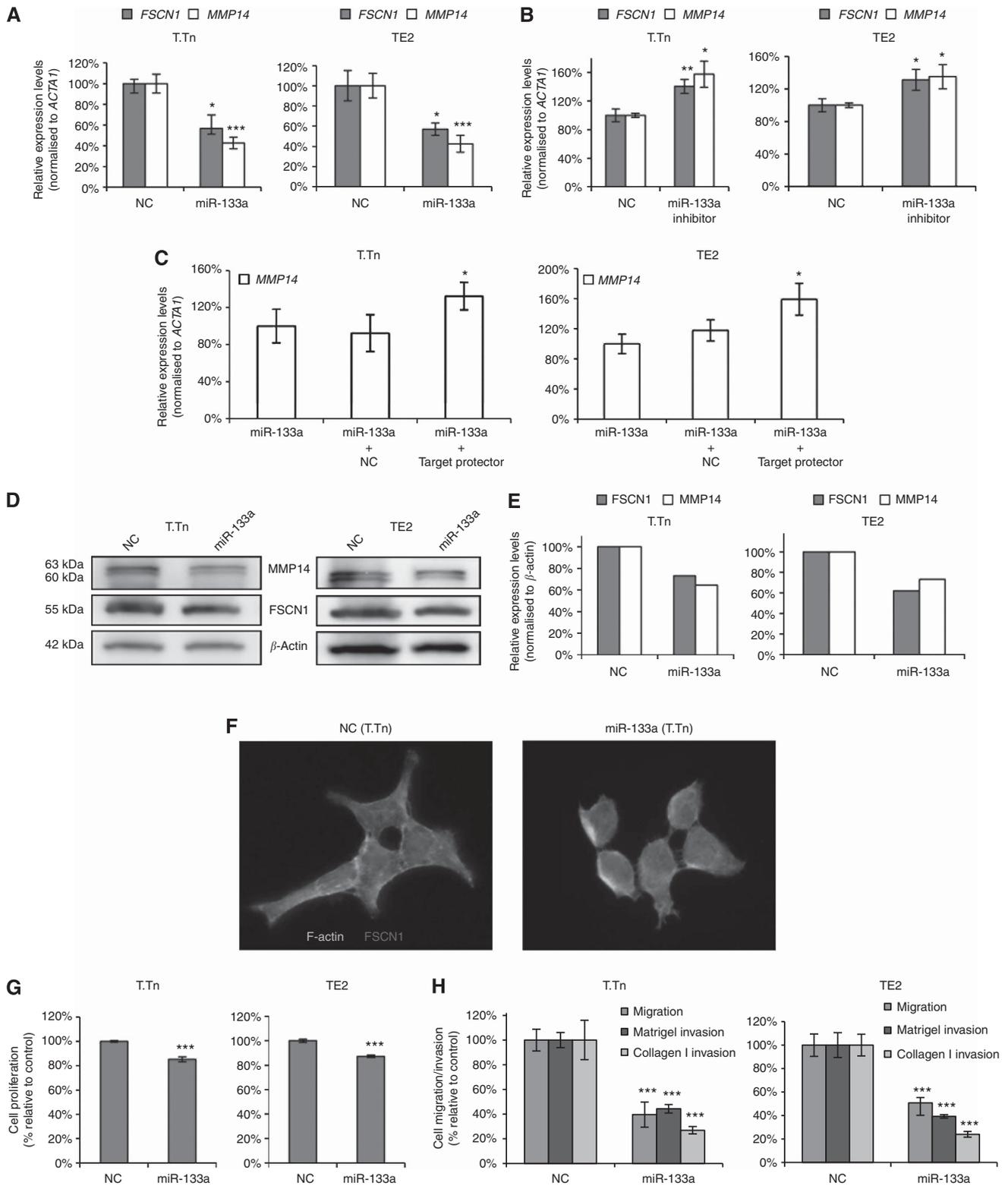


Figure 3. The effects of the miR-133a mimic, miR-133a inhibitor and Target Protector transfection on the mRNA and protein levels, and the effects of miR-133a mimic transfection on the proliferation and migration/invasion activities in ESCC cell lines 72 h after transfection. **(A)** The effects of miR-133a mimic transfection on the mRNA levels as evaluated by real-time RT-PCR. **(B)** The effects on the mRNA levels following miR-133a inhibitor transfection, as evaluated by real-time RT-PCR. **(C)** The effects of the co-transfection of the miR-133a mimic and Target Protector on the mRNA levels as evaluated by real-time RT-PCR. **(D and E)** The effects of the transfection of the miR-133a mimic on the protein levels as evaluated by a western blot analysis. **(F)** Morphological changes of T.Tn cells. **(G)** Results of the proliferation assay. **(H)** Results of the migration/Matrigel invasion/collagen I invasion assay. Abbreviation: NC = negative control. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$.

Immunofluorescent staining of the cells demonstrated that miR-133a-transfected cells lost their normal spindle shape as seen in negative controls and exhibited a more rounded morphology (Figure 3F, the data for TE2 cells not shown).

In the proliferation assay, there was a small but significant decrease in cell growth (85.3% ($P=0.0027$) of negative control in T.Tn and 87.2% ($P<0.001$) in TE2 cells) (Figure 3G). There were also significant decreases in the migration, the Matrigel invasion and the collagen I invasion. As was observed in the siMMP14-transfected cells, the rate of miR-133a-transfected cells that penetrated the collagen I-coated membrane was significantly lower than that of the cells that penetrated the Matrigel-coated membrane (27.9% vs 44.2% of the negative control ($P=0.00390$) in T.Tn cells and 24.0% vs 39.2% ($P=0.0342$) in TE2 cells (Figure 3H)).

Relationships between miR-133a, *FSCN1* and *MMP14* mRNA expression in 84 ESCC clinical specimens. Total RNA was isolated from 84 matched non-cancerous esophageal epithelia and ESCC tissues from which the miR-133a, *FSCN1* and *MMP14* levels were determined by real-time PCR. In all the 84 matched normal and tumour specimens, the expression levels of miR-133a were

significantly lower in the tumour tissues than in the normal tissues ($P=0.0125$; Figure 4A). The Spearman's rank correlation test revealed a significant correlation between the expression of *FSCN1* and *MMP14* ($r=0.524$, $P<0.0001$; Figure 4B) and an inverse correlation between miR-133a and *FSCN1* ($r=-0.314$, $P=0.0040$, Figure 4C) and between miR-133a and *MMP14* ($r=-0.249$, $P=0.0235$, Figure 4D).

We divided the patients into two groups, high and low miR-133a groups, based on the relative miR-133a expression levels normalised to that of RNU6B. The cutoff value was set at 0.012. A survival analysis using the Kaplan–Meier method and the log-rank test showed a significant decrease in the OS in the low miR-133a group ($P=0.0407$, Figure 4E), and the 5-year survival rate was 21.2% (vs 49.4% in the high miR-133a group).

DISCUSSION

These findings showed that *FSCN1* and *MMP14*, which are both located in the invadopodia of cancer cells, contribute to tumour

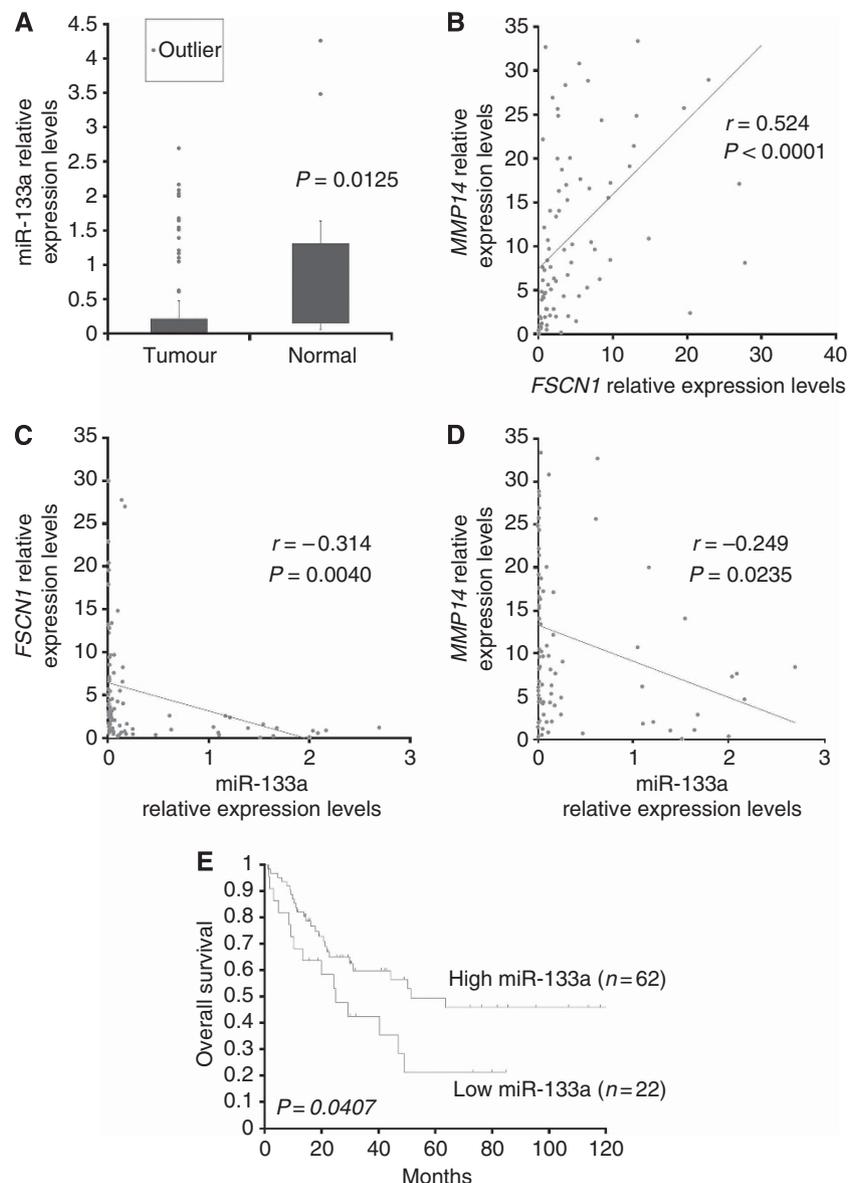


Figure 4. The expression profiles of *FSCN1* and *MMP14* in 84 ESCC samples. (A) The expression levels of miR-133a in tumour tissues and normal tissues. (B) Correlation between *FSCN1* and *MMP14*. (C) Correlation between miR-133a and *FSCN1*. (D) Correlation between miR-133a and *MMP14*. (E) Results of the Kaplan–Meier survival analysis of the OS in the high miR-133a group vs the low miR-133a group.

growth, migration and invasion, and the overexpression of each of them is an independent poor prognostic factor for ESCC. The co-expression of these two molecules correlated with the poorest 5-year survival out of all of the factors examined. miR-133a also regulates both of these invadopodia-related genes and exerts a tumour-suppressive activity in ESCC.

Recently, Chen *et al* (2010) reported that migrastatin analogues exert their tumour-suppressive activity by inhibiting *FSCN1*. Migrastatin is a natural product secreted by *Streptomyces* (Nakae *et al*, 2000), and synthetic migrastatin analogues are potent inhibitors of tumour cell migration, invasion and tumour metastasis (Takemoto *et al*, 2001; Lecomte *et al*, 2011).

Xie *et al* (2005) reported that the effect of *FSCN1* on tumour cell invasion depended on the activation of MMP2 and MMP9. Onodera *et al* (2009) showed that tumour necrosis factor- α (TNF- α) induced the overproduction of *FSCN1*, which in turn upregulated MMP9 expression in two cholangiocarcinoma cell lines. Al-Alwan *et al* (2011) reported that *FSCN1* enhanced the urokinase-type plasminogen activator secretion after TNF- α activation via the enhancement of NF-kappa B activity, which in turn can activate MMP2 and MMP9. However, Sabeh and other researchers reported that secreted MMPs are not crucial for basement membrane invasion, whereas MMP14 is (Sabeh *et al*, 2004; Hotary *et al*, 2006; Li *et al*, 2008; Poincloux *et al*, 2009). Additionally, Onodera *et al* (2009) also reported that *FSCN1* siRNA did not influence the expression level of MMP9 under the normal culture conditions in cholangiocarcinoma cell lines, and similarly, our present study shows that *FSCN1* knockdown does not affect the expression level of MMP14.

In the si*FSCN1*-transfected cells, the inhibition of Matrigel invasion and collagen I invasion paralleled that of the migration. In contrast, in the si*MMP14*- or miR-133a-transfected cells the inhibition of collagen I invasion was stronger than that of Matrigel invasion, which still paralleled that of migration. These results are consistent with a previous report that collagen I, but not Matrigel matrices, provide an MMP-dependent barrier function for the basement membrane (Sodek *et al*, 2008) and reflect our finding that *FSCN1* knockdown does not affect the expression level of MMP14. In addition, although some studies have reported that inhibition of MMP14 did not affect the 2D proliferation, D'Alessio *et al* (2008) reported that MMP14 contributes to cell migration and proliferation *in vitro* (in the absence of ECM) through a proteolysis-independent mechanism. The finding of our study that the introduction of si*MMP14* led to a decrease of cell growth and migration is consistent with this previous report.

Accordingly, these results suggest that tumour-suppressive effect of *FSCN1* inhibitors, such as migrastatin analogues, may be limited.

Although inhibitors of MMPs, especially of MMP14, seem to be a strong candidate tumour suppressor, there are currently no synthetic or biological MMP inhibitors in clinical trials for cancer (Devy and Dransfield, 2011). MMP-based intervention strategies using compounds containing zinc-chelating groups, such as hydroxamates, have met with limited clinical success and had severe side effects. These are assumed to be due to the poor selectivity of these broad-spectrum compounds (Devy *et al*, 2009). The catalytic domains of all MMPs share high amino-acid similarity, and their active sites are extensively conserved, thus differentiating between MMPs with small molecule substrates or inhibitors is extremely difficult (Zarrabi *et al*, 2011).

Devy *et al* (2009) reported that antibody-based biotherapeutic agents may fulfill the selectivity, and they identified a highly selective antibody-based inhibitor of MMP14 (DX-2400). However, MMP14 is necessary for normal physiological processes, such as wound healing, the maintenance of bone (Holmbeck *et al*, 2005) and normal angiogenesis, so even selective inhibition of MMP14 might cause severe side effects.

We demonstrated that the prognosis of patients with both *FSCN1*- and *MMP14*-positive IHC staining was significantly worse than that of patients whose tumours were positive for only one of the proteins.

Because a *FSCN1* inhibitor might not exert sufficient tumour-suppressive activity, and a *MMP14* inhibitor might cause adverse effects, an upstream regulator of both *FSCN1* and *MMP14*, not an inhibitor of either of them, could serve as an ideal tumour-suppressive agent. We have previously reported that miR-133a regulates *FSCN1* mRNA (Kano *et al*, 2010). We found that miR-133a also regulates *MMP14* mRNA, and demonstrated an inverse correlation between miR-133a and *FSCN1* and between miR-133a and *MMP14*, and also demonstrated miR-133a to have tumour-suppressive effects. MicroRNAs are endogenous small (≤ 22 nucleotide) 'non-messenger' RNAs that target mRNAs for cleavage or translational repression (Ambros, 2004; Bartel, 2004). In animals, miRNAs are imprecisely complementary to their mRNA targets. The impreciseness makes it possible for one miRNA to target several mRNAs. A single mRNA can also be targeted by several miRNAs (Gomes *et al*, 2013). Many recent experimental and clinical studies have shown that the aberrant expression of miRNAs is associated with tumour aggressiveness. For example, it has been shown that some miRNAs can function as tumour promoters (oncomirs) and other miRNAs function as tumour suppressors (anti-oncomirs) (Zhang *et al*, 2007; Ghelani *et al*, 2012).

Promoting the activity of anti-oncomirs using the delivery of miRNA mimics could be a novel therapeutic option for cancer (Ghelani *et al*, 2012). As most normal cells already express the anti-oncomirs, the delivery of miRNA mimics to the cells is unlikely to induce adverse effects, as the cellular pathways affected by the mimic have already been activated or inactivated by the endogenous miRNA. However, it is not easy to deliver these agents successfully, because it is difficult for them to penetrate the cellular lipid bilayer membrane as a result of their negative charge and because they are easily degraded by RNase *in vivo*. Recently, numerous trials targeting miRNA have been conducted by researchers and biopharmaceutical companies using cationic lipids, polymers, nanoparticles and so on as delivery reagents for more stable, safer and more effective drug delivery systems (Uchino *et al*, 2013), and some have yielded good results without major adverse effects.

In conclusion, the combination of *FSCN1* and *MMP14* is a poor prognostic factor, and miR-133a, which directly regulates both *FSCN1* and *MMP14*, can serve as a strong tumour suppressor of ESCC.

ACKNOWLEDGEMENTS

We thank Dr Motoharu Seiki and Dr Naohiko Koshikawa (The Institute of Medical Science, the University of Tokyo, Tokyo, Japan) for the generous gift of the *MMP14*-transfected A431 cells. This study was financially supported by a Grant-Aid for Scientific Research (JSPS KAKENHI Grant no. 25861175) from the Japan Society for the Promotion of Science and the Ministry of Education, Culture, Sports, Science and Technology of Japan.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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