CD47 expression regulated by the miR-133a tumor suppressor is a novel prognostic marker in esophageal squamous cell carcinoma

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Abstract. CD47 inhibits phagocytosis and its overexpression is correlated with poor prognosis in patients with several types of cancer. It has also been reported that *CD47* expression in multiple sclerosis is regulated by microRNAs. However, the regulatory mechanism of *CD47* in cancer tissues has not been yet clarified. Re-analysis of a public microarray database revealed that miR-133a is downregulated in esophageal squamous cell carcinoma (ESCC). Moreover, *in silico* algorithms predicted that miR-133a is a regulator of *CD47*. The purpose of this study was to clarify the clinical significance of *CD47* and its regulatory mechanism by miR-133a in ESCC. Quantitative real-time RT-PCR was used to evaluate *CD47* and miR-133a expression in 102 cases of curative resected ESCC and adjacent non-cancerous tissue. The regulation of *CD47* by miR-133a was examined with precursor miR-133a-transfected cells. A mouse xenograft model was used to investigate the ability of miR-133a to suppress tumor progression. High expression levels of *CD47* were associated with lymph node metastasis (P=0.049). Multivariate analysis showed that *CD47* expression was an independent prognostic factor (P=0.045). miR-133a expression was significantly lower in cancer tissues compared to adjacent non-cancerous tissues (P<0.001). *In vitro* assays showed that miR-133a is a direct regulator of *CD47*. miR-133a significantly inhibited tumorigenesis and growth *in vivo*. *CD47* expression is a novel prognostic marker in ESCC that is directly inhibited by the miR-133a tumor suppressor. This correlation could provide new insight into the mechanism of cancer progression and a promising candidate for target therapy in ESCC.

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Introduction

Esophageal squamous cell carcinoma (ESCC) has one of the highest mortality rates among patients with solid tumors due to the fact that it is a highly aggressive malignancy with early lymphatic and hematogenous dissemination (1,2). Recent advances in perioperative management, combined with chemotherapy or chemoradiotherapy, have led to improved survival rates. However, the prognosis for patients with advanced disease remains poor and unsatisfactory (3-5). Discovering suitable biomarkers of malignancy could enhance monitoring for cancer recurrence, and the development of new therapeutic approaches is essential for the improvement of survival rates.

CD47 is a widely expressed transmembrane protein that has been implicated in multiple cellular processes, including a function as a 'do not eat me' signal inhibiting macrophage activity by binding the signal regulatory protein α, which is expressed on phagocytes (6-8). Whereas this function is partly attributed to 'self recognition' in normal physiological conditions, several human cancers appear to upregulate CD47 to evade the tumor immunosurveillance system (9-11). Overexpression of *CD47* correlates with poor prognosis in acute myeloid leukemia (12), acute lymphoblastic leukemia (13) and breast cancer (14). However, the clinicopathological and prognostic significance of *CD47* expression in ESCC remains unclear.

It was reported that *CD47* expression in multiple sclerosis is regulated by microRNAs (miRNAs) including miR-34a, miR-155 and miR-326 (15). However, the mechanism of *CD47* regulation in cancer tissues has not yet been clarified. miRNAs are small non-coding RNAs of 18-25 nucleotides that partially bind the 3' untranslated region (3'-UTR) of their target mRNA, resulting in mRNA degradation and/or translational repression (16). By downregulating their target gene expression, miRNAs play an essential role in several cellular processes, such as proliferation, differentiation and apoptosis (17,18). Moreover, miRNAs also function as oncogenes or tumor suppressors depending on their targets. Therefore, miRNA has garnered attention as a new diagnostic and therapeutic tool for human malignancies (19,20).

In a search for *CD47* regulatory miRNAs downregulated in ESCC, we re-analyzed GSE6188, the Gene Expression Omnibus public microarray database from the National Center for Biotechnical Information (21). Five downregulated miRNAs were found in ESCC. Among them, miR-133a is downregulated in the miRNA expression signatures of several types of human malignancies relative to normal adjacent tissue: head and neck squamous cell carcinoma (22-24), bladder cancer (25,26), colorectal cancer (27), and rhabdomyosarcoma (28,29). Transfection of miR-133a induced reductions in cell proliferation, migration and invasion (25). These data suggested that miR-133a may function as a tumor suppressor. Furthermore, the TargetScan 5.0 *in silico* prediction algorithm (30) also indicated that miR-133a is a conserved regulatory miRNA of *CD47*. Based on these results, we focused on the miR-133a tumor suppressor as a direct regulator of *CD47*.

The purpose of this study was to clarify the clinical significance and regulatory mechanism of *CD47* in ESCC. Firstly, we examined the expression level of *CD47* in ESCC and adjacent non-cancerous tissue. Secondly, we evaluated the *CD47* regulation in ESCC by miR-133a *in silico* and *in vitro*, and investigated the functional analysis of the miR-133a using a mouse xenograft model.

Materials and methods

Clinical samples and RNA isolation. The pair of primary ESCC and corresponding normal esophageal epithelia were obtained from 102 ESCC patients (89 males and 13 females), who had undergone potentially curative surgery at the Department of General Surgical Science, Gunma University, between 1990 and 2007, after obtaining their written informed consent. The patients' ages ranged from 42 to 83 years, with a mean of 64.9. The median follow-up period for survivors was 40 months (range, 1-126 months). The pathological features of the specimens were classified based on the 6th edition of the TNM classification of the International Union against Cancer (UICC). The operations were classified as curative surgeries; there was no evidence of residual tumors, and the resected margins were microscopically free of tumors (R0). Normal tissues were obtained far from the center of the cancer in surgical specimens. All specimens were immediately frozen in liquid nitrogen and stored at -80˚C until RNA extraction. Total RNA was extracted using the miRNeasy Mini kit (Qiagen) according to the manufacturer's instructions. The quantity of isolated RNA was measured by the ND-1000 spectrophotometer (NanoDrop Technologies).

Evaluation of CD47 expression in clinical samples. cDNA for *CD47* mRNA quantitative real-time reverse transcriptase PCR (RT-PCR) was synthesized from 1 μg total RNA with the Omniscript Reverse Transcriptase kit (Qiagen) in a reaction volume of 20 μl (60 min at 37˚C and 5 min at 93˚C before being put on ice). The cDNA samples were stored at -30˚C until needed for analyses. The sequences of the *CD47* primers were as follows: sense primer, 5'-GGCAATGACGAAGGA GGTTA-3'; antisense primer, 5'-ATCCGGTGGTATGGAT GAGA-3'. The following β-actin (the internal control) primers were used: sense primer, 5'-CTCCTCCTGAGCGCAAGT ACTC-3'; antisense primer, 5'-TCCTGCTTGCTGATCCA CATC-3'. Real-time monitoring of PCR reactions was performed using the Light Cycler System and SYBR-Green I Master (Roche) according to the manufacturer's instructions. Quantitative real-time PCR was performed with the following cycling conditions; initial denaturation at 95˚C for 10 min, followed by 40 cycles of 95˚C for 10 sec, annealing at 60˚C for 10 sec, and extension at 72˚C for 10 sec. After amplification, the relative expression level of *CD47* was obtained by dividing the amount of *CD47* mRNA by the amount of β-actin mRNA in each sample.

Re-analysis for GSE6188. The Gene Expression Omnibus public microarray database GSE6188 from the National Center for Biotechnical Information (21) was re-analyzed by Subio Platform Inc., Tokyo, Japan. Significantly different (≥2-fold change) probes between cancerous tissues (153 samples) and normal tissues (104 samples) were extracted. The clinical status of the original tissue samples was determined in over half of the 48 donors with 2 cancerous tissues and 2 adjacent normal tissues.

Validation of miR-133a expression in clinical samples. cDNA for miR-133a quantitative real-time RT-PCR was synthesized from 10 ng of total RNA using the TaqMan microRNA Reverse Transcription Kit and specific stem-loop reverse transcription primers (Applied Biosystems) according to the manufacturer's protocol. The 15 μl reactions were incubated in a 96-well plate using the following temperature profile: 16˚C for 30 min followed by 40˚C for 30 min and 85˚C for 5 min. PCR was performed in a LightCycler™ 480 System (Roche). The 20 μl of PCR mix including the LightCycler 480 Probes Master kit (Roche) was incubated in a 96-well optical plate at 95˚C for 10 min and then followed by 45 cycles of 95˚C for 10 sec and 60˚C for 30 sec. The expression levels of miR-133a were normalized to that of the small nuclear RNA RNU6B and analyzed using the $2^{-\Delta\Delta Ct}$ method.

ESCC cell line. The human ESCC cell line TE-8 was kindly provided by Dr T. Nishihira (Institute of Development, Aging and Cancer, Tohoku University School of Medicine, Sendai, Japan). TE-8 cells were maintained in Roswell Park Memorial Institute (RPMI-1640 medium) (Wako Pure Chemical Industries) supplemented with 10% fetal bovine serum and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) and cultured in a humidified 5% CO_2 incubator at 37˚C.

Transfection of miR-133a precursor (Pre-miR-133a). Pre-miR miRNA Precursor Molecule mimicking miR-133a (Pre-miR-133a; Applied Biosystems) or non-specific control miRNA (Pre-miR Negative Control #1; Applied Biosystems) was transfected at 30 nmol/l into TE-8 cells using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instructions.

Plasmid construction. miR-133a target sequences in the 3'-UTR region of CD47 (Fig. 3A) were predicted using TargetScan (release 5.1: April 2009) and amplified from the genomic DNA of normal cells. The amplified fragment was inserted into the *Xho*I restriction sites of the dual-luciferase plasmid pmirGLO vector (Promega) using the In-Fusion®

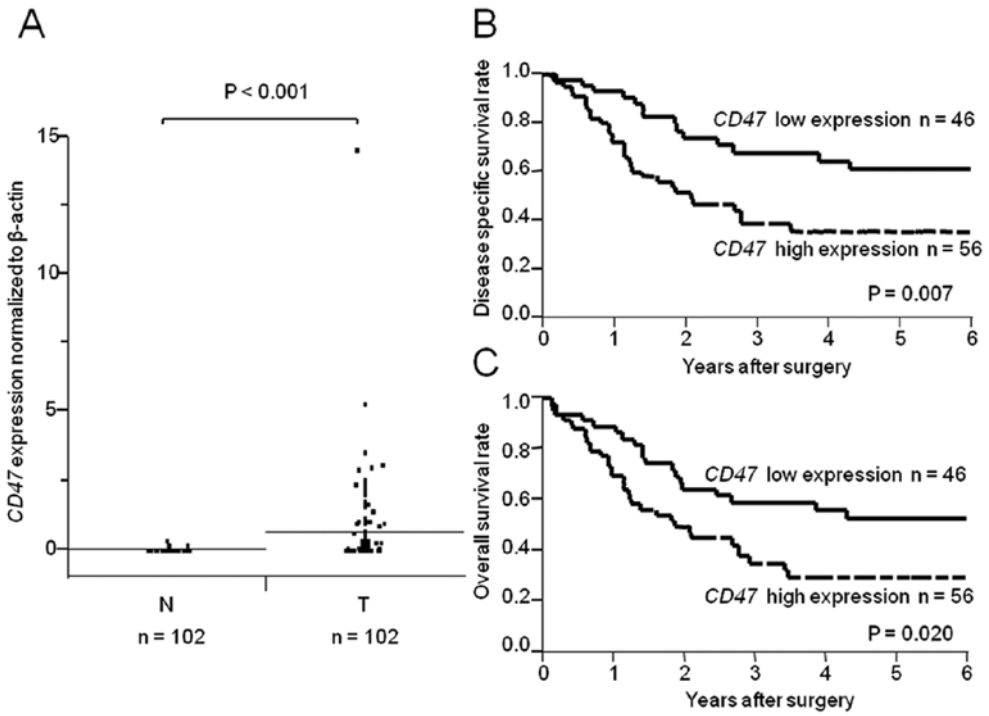

Figure 1. *CD47* expression and prognosis in 102 ESCC cases. (A) *CD47* levels assessed by real-time RT-PCR in cancerous (T) and non-cancerous (N) tissues from ESCC patients (n=102). *CD47* levels in T were significantly higher than those in N (P<0.001). Horizontal lines indicate each mean. (B and C) Kaplan-Meier curves based on *CD47* expression in ESCC. Disease-specific (5-year survival rates, high expression, 35.4%; low expression, 60.8%; P=0.007) and overall survival rates (5-year survival rates: high expression, 29.4%; low expression, 52.6%; P=0.020) of ESCC patients with high *CD47* expression were significantly lower than the rates among those with low *CD47* expression.

Dry-Down PCR Cloning kit (Clontech). Plasmid sequences were confirmed by sequencing using the following primers: sense, 5'-GCAAGATCGCCGTGTAATTCTAG-3'; anti-sense, 5'-AGAGGCCTCAGCAGGTCATA-3'.

Luciferase assay. TE-8 cells were seeded in a 96-well plate and co-transfected with 0.2 μg of pmirGLO vecter, 100 nmol/l of Pre-miR-133a and 0.5 μl of Lipofectamine RNAiMAX (Invitrogen) in 50 μl of Opti-MEM Reduced-Serum Medium (Invitrogen). Pre-miR Negative Control #1 was used as a control. Twenty-four hours following transfection, the activities of the firefly and Renilla luciferases in cell lysates were measured using the Dual-Glo® Luciferase Assay System (Promega) and the Fluoroskan Ascent FL (Thermo Fischer Scientific). Firefly luciferase activity was normalized to Renilla luciferase activity. All transfection experiments were conducted in triplicate.

Protein expression analysis. Western blot analysis was used to confirm the expression of CD47 in Pre-miR-133a transfected cells. Total protein was extracted from TE-8 cells 48 h after transfection using PROPREP™ protein extraction solution (Intron Biotechnology, Inc.). Total protein (40 μg) was electrophoresed in an Any kD™ Mini-PROTEAN® TGX™ Precast Gel (Bio-Rad Laboratories, Hercules, CA, USA) and then electrotransferred to a Hybond-enhanced nitrocellulose membrane (Amersham Pharmacia Biotech) at 200 mA for 180 min at 4˚C. The membrane was blocked with 5% skim milk and CD47 protein was detected using CD47 mouse monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:100. β-actin mouse monoclonal antibody (clone AC-74; Sigma) diluted 1:1000 served as a control. Bands on the membrane were detected using Retiga-4000R and QCapture Pro 6.0, an enhanced chemiluminescence detection system, according to the manufacturer's instructions (QImaging).

In vivo assay. Forty-eight hours following transfection with Pre-miR-133a or Pre-miR Negative Control #1, 6-week-old female BALB/c nude mice received subcutaneous injections of $4x10^6$ of TE-8 cells. The tumor volume was determined by caliper measurements at day 14 and calculated using the formula: Volume = S x S x L/2, where S is the short length of the tumor in mm and L is the long length of the tumor in mm.

Statistical analysis. Differences between two groups were estimated using the Student's t-test, the χ^2 test, and the repeated-measures ANOVA. Kaplan-Meier curves were generated for disease-specific and overall survival, and statistical significance was determined using the log-rank test. Univariate and multivariate survival analyses were carried out using the Cox proportional hazards regression model. A probability value of <0.05 was considered significant. All statistical analyses were performed using the JMP5.0 software (SAS Institute Inc.).

Results

Association between CD47 expression and clinicopathological findings in ESCC patients. *CD47* expression was higher in a subset of ESCC samples compared with corresponding non-cancerous tissues (Fig. 1A; P<0.001). We divided the 102

Table I. Relationship between *CD47* expression and clinicopathological features.

Factors	*CD47* expression, n		P-value
	Low (n=46)	High (n=56)	
Gender			
Male	40	49	0.935
Female	6	7	
Age (years) (mean ± SD)	65.0±8.0	64.8±7.9	0.864
Tumor depth			
T1	12	7	0.248
T2	7	6	
T3	23	37	
T4	4	6	
Lymph node metastasis			
N0	20	14	0.049[a]
N1	26	42	
Distant metastasis			
M0	41	46	0.316
M1	5	10	
Lymphatic invasion			
Negative	40	54	0.074
Positive	6	2	
Venous invasion			
Negative	34	47	0.214
Positive	12	9	
Tumor stage			
I	8	4	0.148
II	17	16	
III	16	23	
IV	5	13	

[a]P<0.05.

ESCC patients into two groups according to the level of *CD47* expression in their cancerous tissue. The cut-off point was the median level of *CD47* expression in non-cancerous tissues. The correlation between *CD47* expression and the clinicopathologic characteristics of patients is shown in Table I. The *CD47* high expression group (n=56) showed more extensive lymph node metastasis than the low expression group (n=46; P=0.049). However, no significant differences were observed regarding gender, age, tumor depth, distant metastasis, lymphatic invasion, venous invasion, or TNM stage.

Prognostic significance of CD47 expression in ESCC patients. The 5-year disease-specific survival rates of ESCC patients in the *CD47* high expression group were significantly lower than those in the low *CD47* expression group (high expression, 35.4%; low expression, 60.8%; P=0.007; Fig. 1B). The 5-year overall survival rates of ESCC patients in the high expression group were also significantly lower than those in the low expression group (high expression, 29.4%; low expression, 52.6%; P=0.020; Fig. 1C). Univariate analysis of disease-specific survival revealed that the relative level of tumor depth, lymph node metastasis, distant metastasis, lymphatic invasion, and *CD47* expression were prognostic factors (Table II). Multivariate analysis of the five factors found to be significant in univariate analysis showed that high expression of *CD47* (P=0.045), tumor depth (P=0.042) and lymph node metastasis (P=0.019; Table II) are independent prognostic factors of poor survival.

Re-analysis of GSE6188 and miR-133a expression in ESCC. In search for *CD47* regulatory miRNAs downregulated in ESCC, we found five downregulated miRNAs during re-analysis of a public microarray database (Fig. 2A). Among them, miR-133a expression was validated in 102 clinical samples of ESCC and adjacent non-cancerous tissues. The expression of miR-133a was significantly lower in cancerous tissues compared to non-cancerous tissues (P<0.001; Fig. 2B).

miR-133a directly binds the 3'-UTR of CD47 and regulates its protein expression in ESCC. miR-133a was predicted to be an *in silico* regulator of *CD47* by TargetScan 5.0 (30) (Fig. 3A). We found an inverse correlation between miR-133a and CD47 expression in 102 clinical samples of ESCC. High levels of miR-133a were associated with low *CD47* expression (P<0.001; Fig. 3B). The luciferase reporter assay demonstrated that the luminescence intensity was significantly decreased in Pre-miR-133a transfectants (Fig. 3C; P<0.001), suggesting that miR-133a has actual target sites in the 3'-UTR of *CD47* mRNA in ESCC. The transient transfection of Pre-miR-133a also repressed CD47 protein levels in ESCC (Fig. 3D).

Tumor suppressive activity of miR-133a in vivo. A mouse xenograft model was used to evaluate the tumor suppressive activity of miR-133a (Fig. 4A). Pre-miR-133a strongly inhibited the tumorigenic potential of TE-8 cells *in vivo* (5 of 10; 50%) compared with the negative control (8 of 8; 100%) (P=0.036, Fig. 4B). Moreover, Pre-miR-133a significantly inhibited tumor volume growth on Day 14 after injection (P=0.007; Fig. 4B).

Discussion

The expression level of miR-133a in primary ESCC was lower than in corresponding normal esophageal tissues, a finding which is consistent with the results of a previous microarray expression study (21). Because tumor suppressive miRNAs are usually underexpressed in tumors, it has been suggested that miR-133a functions as a tumor suppressor. Indeed, this study revealed that miR-133a inhibited tumor growth in an ESCC xenograft mouse model. Kano *et al* demonstrated that transfection of miR-133a inhibits cell proliferation and cell invasion in ESCC through the regulation of *FSCN1*, which is usually increased in tumors compared to normal epithelium (31). *FSCN1* overexpression is significantly associated with poor prognosis in ESCC (32). These *in vivo* and *in vitro* findings suggest that miR-133a contributes to decreases in cell proliferation. Furthermore, our data revealed that miR-133a inhibits tumor xenograftment, suggesting that miR-133a could play a key role in tumorigenesis in ESCC.

Table II. Results of univariate and multivariate analysis of clinicopathological factors affecting disease-specific survival rates following surgery.

	Univariate analysis			Multivariate analysis		
Clinicopathological variable	RR	95% CI	P-value	RR	95% CI	P-value
Gender (male/female)	1.092	0.673-1.615	0.671	-	-	-
Age (<65/≥65)	1.065	0.7965-1.427	0.671	-	-	-
Depth of tumor invasion (T1/T2,3,4)	2.019	1.278-3.698	0.001[a]	1.665	1.018-3.143	0.042[a]
Lymph node metastasis (N0/N1)	1.941	1.356-2.958	<0.001[a]	1.584	1.073-2.488	0.019[a]
Distant metastasis (M0/M1)	1.662	1.153-2.304	0.008[a]	1.284	0.881-1.810	0.185
Lymphatic invasion (negative/positive)	2.354	1.098-9.913	0.023[a]	1.092	0.444-4.810	0.870
Venous invasion (negative/positive)	1.459	0.985-2.369	0.060	-	-	-
miR-133a expression (high/low)	1.117	0.835-1.499	0.455	-	-	-
CD47 expression (low/high)	1.515	1.118-2.097	0.007[a]	1.371	1.007-1.908	0.045[a]

RR, relative risk; CI, confidence interval; [a]P<0.05.

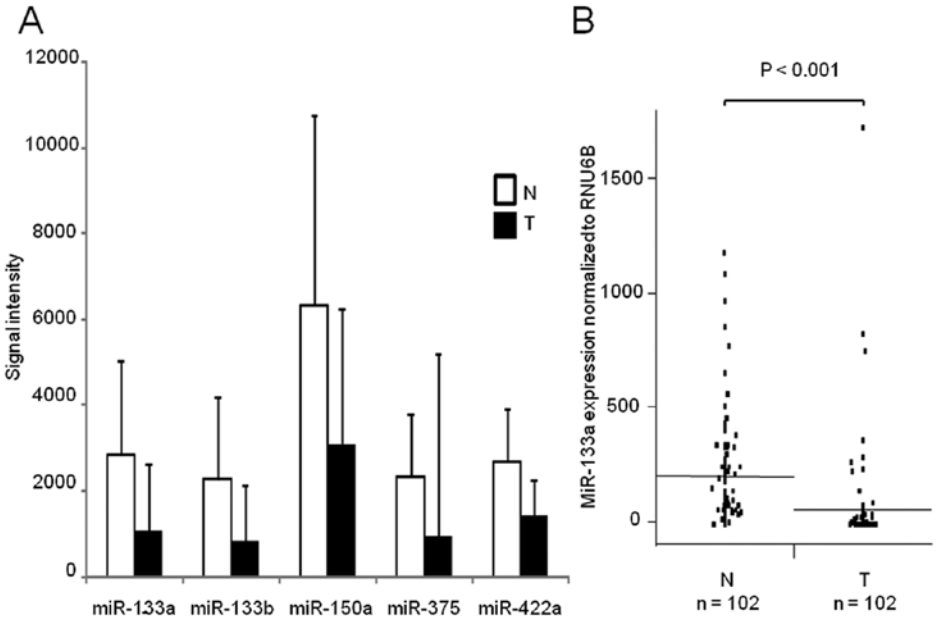

Figure 2. Re-analysis of a microarray database and miR-133a expression in 102 ESCC cases. (A) Five miRNAs downregulated in ESCC were found after re-analysis of the public microarray database GSE6188. The fold changes [cancerous tissues (T)/non-cancerous tissues (N)] were miR-133a, 0.366; miR-133b, 0.350; miR-150, 0.482; miR-375, 0.409; and miR-422a, 0.529. The error bar represents the mean ± SD. (B) miR-133a expression in T and N from ESCC patients (n=102) by real-time RT-PCR. miR-133a levels in T were significantly lower than those in N (P<0.001). Horizontal lines indicate each mean.

Luciferase assays supported the premise of a specific interaction between miR-133a and the 3'-UTR of *CD47*, a protein that inhibits phagocytosis. The negative modulatory effect of miR-133a was substantiated further by a strong inverse relationship between the expression levels of *CD47* mRNA and miR-133a. The significant reduction of *CD47* protein in an ESCC cell line following Pre-miR-133a transfection suggests that miR-133a directly regulates *CD47* expression. In contrast, it has been demonstrated that hematopoietic cells from *CD47* knockout mice were rapidly cleared from the bloodstream by macrophages in syngeneic wild-type mice (7,33). Chao *et al* also reported that monoclonal antibody against *CD47* enabled phagocytosis of acute lymphoblastic leukemia cells by macrophages *in vitro* and inhibited tumor engraftment *in vivo* (13). These findings suggest that the rapid rejection of xenograft cells by recipient macrophages could have resulted from the critical role of *CD47* in self protection. These studies support the idea that miR-133a contributes to anti-tumorigenesis in ESCC through the regulation of *CD47* expression. To the best of our knowledge, this is the first study describing the clinical significance of *CD47* and its regulation by miR-133a in ESCC.

The expression of *CD47* is significantly correlated with lymph node metastasis, the main cause of death in most cancer patients. In particular, lymph node metastasis leads to poor prognosis after surgical resection in ESCC patients (34).

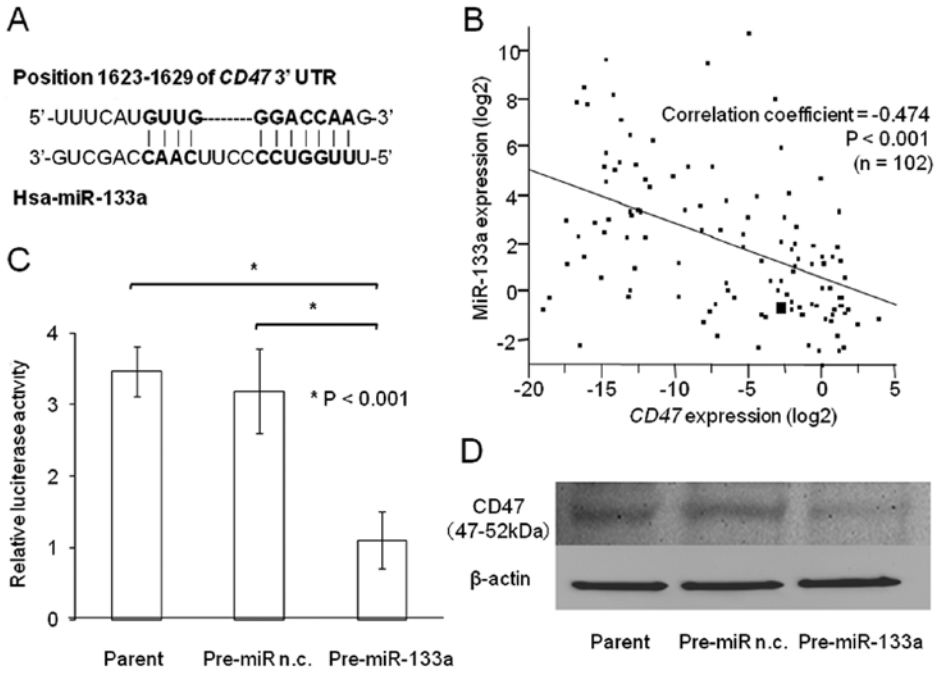

Figure 3. Regulation of *CD47* expression by miR-133a. (A) The miR-133a-binding site in the *CD47* 3'-UTR. Putative conserved target sites in the *CD47* 3'-UTR were identified with the TargetScan database. (B) Inverse correlation between miR-133a and *CD47* expression in ESCC. High levels of miR-133a were associated with low *CD47* expression (P<0.001). miR-133a and *CD47* expression were examined by real-time PCR analysis and normalized to RNU6B and β-actin, respectively. (C) Luciferase assay in TE-8 cells. miR-133a transfectants showed lower luciferase activity than control and parent TE-8 cells (*P<0.001). The error bar represents the SD from 8 replicates. Left, *CD47*-3'-UTR luciferase vector only; middle, *CD47*-3'-UTR luciferase vector +Pre-miR n.c.; right, *CD47*-3'-UTR luciferase vector +Pre-miR-133a. (D) Western blots indicated that *CD47* protein expression is decreased by the ectopic expression of miR-133a. Left, parent; middle, Pre-miR n.c.; right, Pre-miR-133a.

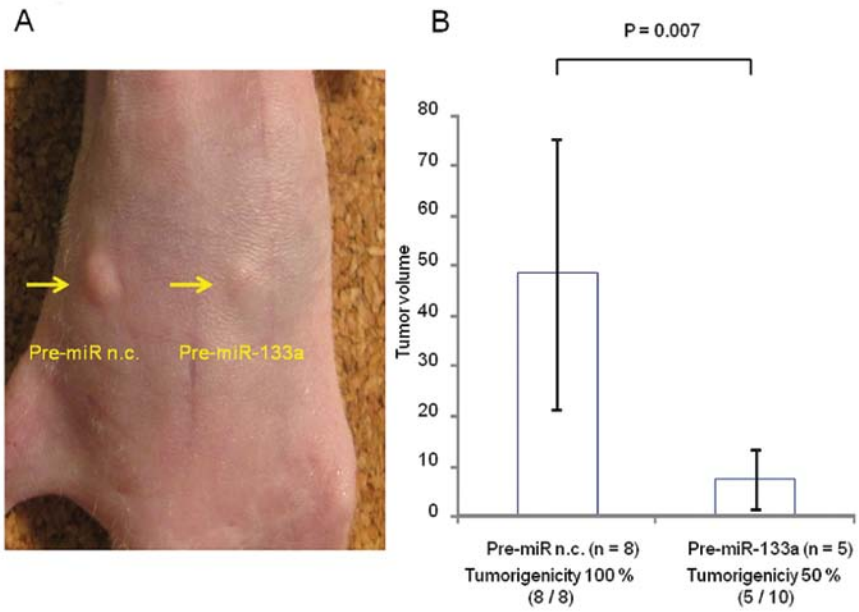

Figure 4. miR-133a inhibits tumorigenicity and tumor growth *in vivo*. (A) TE-8 cells transfected with Pre-miR-133a or Pre-miR negative control (n.c.) were injected subcutaneously into female BALB/c nude mice. The representative image was taken on Day 7. Left, Pre-miR n.c. transfectant; right, Pre-miR-133a transfectant. (B) Comparison of tumor size in nude mouse transplanted with TE-8 cells transfected with Pre-miR-133a or Pre-miR n.c. 14 days after subcutaneous injection. The incidence of tumors (tumorigenicity) is shown below. The bar represents the mean ± SD.

However, tumor immunosurveillance is a well-established mechanism for the regulation of tumor progression (11), and macrophages in the lymph node sinus are the front-line endogenic defense against metastasis (35). Asano *et al* showed that macrophages phagocytose dead tumor cells transported via lymphatic flow and subsequently cross-present tumor antigens to $CD8^+$ T cells, resulting in activation of the anti-tumor immunity system (36). Therefore, ESCC characterized by high CD47 expression may have the ability to evade phagocytosis, and after acquiring invasiveness, to participate

in the promotion of lymph node metastasis. Multivariate Cox proportional hazard regression analysis revealed that *CD47* expression had a significantly worse prognostic impact (P=0.045) on survival of ESCC patients independent of tumor depth (P=0.042) and lymph node metastasis (P=0.019). Novel biomarkers that are capable of precisely identifying high risk ESCC patients may provide the tools for determining appropriate therapeutic strategies. Our data indicate that *CD47* could be of value as a novel prognostic marker for the identification of aggressive ESCC.

Although miRNAs show signs of being a new class of targets for therapeutic applications (37), the development of effective and safe approaches for sequence-specific antagonism of miRNAs *in vivo* remains a significant scientific and therapeutic challenge (38,39). On another front, *CD47* was identified as a therapeutic antibody target in acute myeloid leukemia, non-Hodgkin's lymphoma, bladder cancer and acute lymphoblastic leukemia (12,13,40-42). However, until now, the regulation of *CD47* expression is not well understood. The identification of miR133a-mediated *CD47* regulation might provide a promising new therapeutic strategy in treating ESCC.

In conclusion, our data indicate that *CD47* is an independent prognostic marker that is regulated by miR-133a, which may function as a tumor suppressor in ESCC. This correlation could provide new insight into the mechanism of cancer progression and a promising candidate for a novel therapeutic target in ESCC.

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