in an NOD/SCID mouse. The results are shown in Figure 4A and Supplementary Figure 2A (Supplemental Digital Content 4, http://links.lww.com/JTO/A340). The main histopathology of the tumor was acinar-predominant adenocarcinoma showing a cribriform pattern with mucin production. This finding supports the proposal by Takeuchi et al.[4] that a cribriform acinar pattern is a distinctive histopathologic feature of kinase-fusion tumors. In the periphery of the tumor, a micropapillary component was also seen (Supplementary Figure 2B, Supplemental Digital Content 4, http://links.lww.com/JTO/A340). Immunohistochemically, the tumor was positive for RET, Napsin A, and TTF-1, but negative for thyroglobulin (Fig. 4C and 4D and Supplementary Figure 2C, Supplemental Digital Content 4, http://links.lww.com/JTO/A340), which confirmed that LC-2/ad originated from the primary lung adenocarcinoma and not from a thyroid tumor.

As far as we know, this is the first report to demonstrate that a patient-derived lung adenocarcinoma cell line harboring endogenous *RET* fusions is sensitive to vandetanib. This cell line may be a useful tool to address the following issues regarding *RET*-fusion genes. First, this cell line can be used for drug screening. We have not examined the sensitivities to the RET inhibitors other than vandetanib, but it will be interesting to test the sensitivity of LC-2/ad to sorafenib and sunitinib as well. The LC-2/ad xenografts can be also used for examination of the sensitivity in vivo. Second, this cell line may be used to study the intracellular signaling pathway of *RET* fusion in lung tumors. Third, vandetanib-resistant cell line may be generated from LC-2/ad and such a resistant subline would be invaluable in the study of the mechanisms of acquired resistance to vandetanib. Fourth, although the acinar-predominant pattern has been proposed as a morphologic feature of tumors harboring kinase fusions,[4] why kinase-fusion genes lead to such characteristic morphology is currently unclear. Thus, this cell line may provide a useful resource to study the molecular basis for the molecular–morphologic correlations in cancer.

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Involvement of miR-214 and miR-375 in Malignant Features of Non-Small-Cell Lung Cancer by Down-Regulating CADM1

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ABSTRACT

A tumor suppressor gene, *CADM1*, encoding an immunoglobulin superfamily cell adhesion molecule, is inactivated in various cancers, including non-small-cell lung cancer (NSCLC). Although promoter methylation is one of the mechanisms to suppress *CADM1* expression, about half of tumors lacking *CADM1* expression do not show methylation of the gene promoter. We herein investigated the possible involvement of microRNA (miRNA) in the down-regulation of *CADM1*. Using computational algorithms, miR-214 and miR-375 were identified as candidate miRNAs targeting *CADM1*. A luciferase reporter assay demonstrated that miR-214 and miR-375 repressed the promoter activity through 3'-UTR of *CADM1*. Quantitative RT-PCR analysis demonstrated that miR-214 and miR-375 was highly expressed in 21 (62%) and 17 (50%) cases of 34 primary NSCLCs. Notably, increased expression of miR-214 was preferentially observed in tumors with advanced pathological stages and in those lacking *CADM1* expression but were not associated with the promoter methylation, suggesting that miR-214-mediated silencing would be another mechanism to suppress *CADM1* expression. On the other hand, introduction of miR-214 or miR-375 into NSCLC cells decreased *CADM1* protein expression. Furthermore, overexpression of miR-214 enhanced anchorage-independent growth of NSCLC cells, A549, whereas transfection of miRNA inhibitors of miR-214 or miR-375 significantly suppressed the *in vitro* wound healing activity of HCC827 cells. These findings suggest that overexpression of miR-214 and miR-375 could participate in the malignant features of NSCLC through down-regulating *CADM1* and would provide a potential target for the treatment of a subset of NSCLC.

Keywords: *CADM1*; miR-214; miR-375; Non-Small-Cell Lung Cancer

1. Introduction

Lung cancer is the leading cause of cancer death in the world. More than 80% of lung cancer is categorized as non-small-cell lung cancer (NSCLC). We previously identified a tumor suppressor gene, *CADM1*/TSLC1, in NSCLC by functional cloning [1]. *CADM1* encodes an immunoglobulin superfamily cell-cell adhesion molecule and is expressed in various tissues, including the brain, testis, lung, kidney, and breast [2]. *CADM1* expression was frequently lost or reduced in concordance with tumor progression in NSCLC and various other cancers [3]. Moreover, loss or reduction of *CADM1* expression indicated worse clinical prognosis of the cases in lung adenocarcinoma, hepatocellular carcinoma, and esophageal squamous cell carcinoma [4-7]. However, the mechanisms underlying the loss of *CADM1* expression in cancers are not fully clarified yet. So far, a major postulated mechanism is promoter methylation of the *CADM1* gene through bi-allelic methylation or mono-allelic methylation associated with loss of the other allele. In fact, 44% of NSCLC as well as 30% - 60% of various other cancers showed promoter methylation of the *CADM1* gene [8]. However, considerable numbers of tumors lacking *CADM1* expression do not show promoter methylation of the *CADM1*, suggesting that additional unknown mechanisms would be involved in the suppression of *CADM1* expression.

MicroRNA (miRNA) is a group of small non-coding RNAs that suppress gene expression by interacting with the 3'-UTR of target mRNAs for translational supp-

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ression and mRNA degradation [9]. Numbers of miRNAs were identified to regulate cell proliferation, differentiation, and apoptosis by targeting different gene sets, thereby acting in an either oncogenic or tumor-suppressor manner. Several oncogenic and tumor-suppressor miRNAs have been implicated in the regulation of tumor progression in NSCLC, such as miR-21 and let-7 [10-12]. Therefore, it is tempting to speculate that suppression of *CADM1* expression could be mediated by miRNAs in NSCLC.

In the present study, we identified miRNAs, miR-214 and miR-375, to target 3'-UTR of *CADM1* and examined their functional roles in anchorage-independent cell growth and tumor cell migration/invasion in NSCLC cell lines. Furthermore, we analyzed the expression of miR-214 and miR-375 in primary NSCLC. Enhancement of the malignant phenotype of NSCLC by these miRNAs, as well as high incidence of their overexpression in primary NSCLC, suggests that miR-214 and miR-375 act as oncomiRs of NSCLC by suppressing *CADM1* expression.

2. Materials and Methods

2.1. Cell Lines, Transfection and Clinical Samples

A549 and Caco-2 cells were obtained from RIKEN Cell Bank (Ibaraki, Japan); HEK293 from the Health Science Research Resources Bank (Osaka, Japan); and NCI-H441 and HCC827 from The American Type Culture Collection (Manassas, VA). Cells were cultured according to the supplier's recommendation. Control siRNA (ON-TARGETplus siCONTROL Non-targeting pool, D-001810-10), siRNA against *CADM1* (siGENOME ON-TARGETplus Human IGSF4, J-016565-05), microRNA mimics (miRIDIAN Mimic Human hsa-miR-214, C-300569-07; miRIDIAN Mimic Human hsa-miR-375, C-300682-05) and microRNA inhibitors (miRIDIAN Hairpin Inhibitor Human hsa-miR-214, IH-300569-08; miRIDIAN Hairpin Inhibitor Human hsa-miR-375, IH-300682-07) were obtained from Thermo Fisher Scientific (Waltham, MA). Cells were transfected with 50nM of siRNA or miRNA using Lipofectamine™ LTX reagent (Invitrogen). We collected 34 cancer tissues and adjacent non-cancerous lung tissues from NSCLC patients who underwent surgical resection at the University of Tokyo Hospital (Tokyo, Japan) after receiving approval from the Institutional Ethics Review Committee and obtaining informed consent from all patients.

2.2. Real-Time Quantitative PCR (qRT-PCR)

Oligonucleotide primer sequences for real-time PCR are shown in **Table 1**. Total cellular RNA was extracted using an RNeasy Mini kit (QIAGEN Sciences, Germantown, MD) and first-strand cDNA was synthesized using a Transcriptor first-strand cDNA synthesis kit (Roche Diagnostics, Basel, Switzerland). Then, real-time PCR was carried out using Light Cycler® 1.5 with light cycler Taq Man master and universal probe of #21 for miRNAs (Roche Diagnostics). Expression of miRNAs was normalized to that of small nuclear RNA U6.

2.3. Immunoblot Analysis

Immunoblotting was carried out as described previously [2] using a rabbit anti-*CADM1* polyclonal antibody (pAb) (C-18) [13] or a goat anti-GAPDH pAb (V-18, Santa Cruz Biotechnology, Santa Cruz, CA) as a control. Quantification of signal intensities was performed using Image J Software Ver. 1.44.

2.4. Luciferase Assay

CADM1 3'-UTR of 2,848 bp was cloned by RT-PCR

Table 1. Primers for cloning of *CADM1* 3'-UTR and real-time PCR of miRNAs.

Target		Primer sequence (5' → 3')	PCR product size (bp)
CADM1 3'-UTR	F	5'-GCTAGCATCAGCCTTTTTGTTTCAATGAGG-3'	2848
	R	5'-ACTAGTCACTTTGTAACATTAATTTTTTTTTATTAAG -3'	
miR-214	RT	5'-GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCACCAGAGCCAACACTGCC-3'	62
	F	5'-CGGCGGACAGCAGGCACAGACA-3'	
miR-375	RT	5'-GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCACCAGAGCCAACTCACGC-3'	62
	F	5'-CGGCGGTTTGTTCGTTCGGCTC-3'	
miR Universal	R	5'-GTGCAGGGTCCGAGGT-3'	
U6	RT	5'-CGCTTCACGAATTTGCGTGTCAT-3'	72
	F	5'-GTGCTCGCTTCGGCAGCACATATAC-3'	
	R	5'-CCTTGCGCAGGGGCCATGCTAA-3'	

RT: primer for reverse transcriptase reaction; F: forward primer for PCR; R: reverse primer for PCR.

from RNA of Caco-2 cells using primers described in **Table 1**. The amplified fragment was inserted downstream of the Firefly luciferase gene of pGL3-Basic (Promega, Madison, WI). Then, a CMV promoter cleaved from pcDNA3.1/Hygro(+) (Invitrogen) was inserted upstream of the *luciferase* gene to obtain pCMV-GL3-*CADM1*-3'-UTR. HEK293 cells were transfected with 25 nM of microRNA mimic, 0.25 μg of pCMV-GL3-*CADM1*-3'-UTR, and 2.8 ng of pRL-TK (Promega). The luciferase activities of Firefly and Renilla were measured after 48 h with a dual luciferase reporter assay system (Promega) using a Lumat LB9507 luminometer (Berthold Technologies, Bad Wildbad, Germany).

2.5. Colony Formation Assay in Soft Agar

After 48 h of transfection, A549 cells were trypsinized and plated at 1×10^4 in 0.36% soft agar on the top of a base layer containing 0.5% agar and grown at 37˚C for 15 days. The numbers of colonies per dish with diameters over 100 μm formed in soft agar were counted under the microscope and normalized to those in a liquid culture for 7 days.

2.6. Wound Healing Assay

HCC827 cells transfected with 50 nM of microRNA inhibitors or 1 μg of the *CADM1* expression vector were cultured to obtain 90% confluency. A wound was created by scraping the cells using a P200 pipette tip and images were captured immediately (0 h) and at 10 h and 20 h. Migration of cells was assessed by measuring the average width of the wounds at three different points.

2.7. Immunohistochemical Analysis

Immunohistochemistry was performed using anti-*CADM1* polyclonal antibody, CC2, as described previously [5].

2.8. Methylation-Specific PCR (MSP)

Methylation-specific PCR of the *CADM1* promoter was performed as described elsewhere [14]. The primers used were: 5'-AGTGACGGAAATTTGTAACG-3' and 5'-AAAAACTCGAACTCCAAAAAACG-3' for the methylated DNA and 5'-AGTGATGGAAATTTGTAATG-3' and 5'-AAAAACTCAAACTCCAAAAAACA-3' for unmethylated DNA.

2.9. Statistical Analysis

Statistical differences were determined by the two-tailed Student's *t*-test with SigmaPlot software Ver. 11 (Systat Software, San Jose, CA). Fisher's exact tests were applied to test significant associations between the expression state of miRNAs and the clinicopathological characteristics of primary NSCLC. A p-value of <0.05 was considered as statistically significant.

3. Results

3.1. Identification of miRNAs Targeting *CADM1*

A couple of computational algorithms TargetScan [15] and PicTar [16], were used to identify a numbers of potential miRNAs targeting *CADM1*. The search program TargetScan identified miR-375 and miR-214 (**Table 2**) as the strongest candidate miRNA targeting *CADM1*, while another program, PicTar, identified miR-214 (**Table 3**). Database analysis showed that three and two predicted binding sites of miR-214 and miR-375 were present, respectively, in the 3'-UTR of the *CADM1* (**Figure 1(a)**). The seed sequences (SS) of miR-214 and miR-375 were well conserved between mouse and human, except for the third predicted seed sequences of miR-214-SS3 located about the 1200 bp from the starting site of the *CADM1* 3'-UTR (**Figure 1(b)**).

Table 2. Prediction of miRNAs targeting *CADM1* by TargetScan Human 5.2.

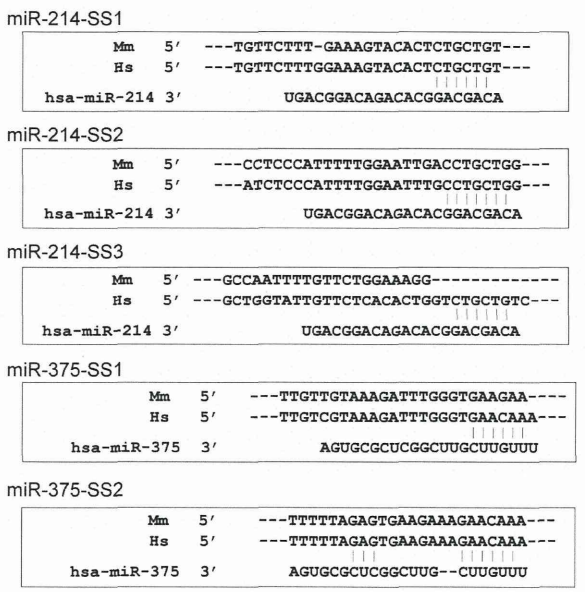
miRNA	Aggregate P_{CT}[a]
miR-375	0.50
miR-214/761	0.42
miR-124/506	0.37
miR-129/129-5p	0.33
miR-205	0.30
miR-148/152	0.28
miR-101	0.27
miR-138	0.26
miR-200bc/429	0.22
miR-208/208ab	0.19

[a]P_{CT}, Preferentially conserved targeting.

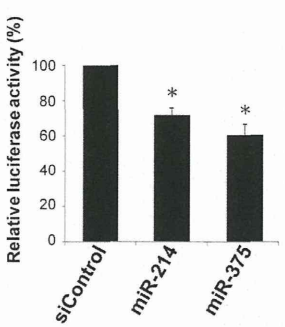
Table 3. Prediction of miRNAs targeting *CADM1* by PicTar.

miRNA	PicTar Score
miR-214	10.25
miR-182*	8.57
miR-144	3.94
miR-199a*	3.94
miR-101	3.60
miR-190	2.47
miR-186	2.47
miR-10b	2.29
miR-10a	2.09
miR-195	1.87

(a)



(b)



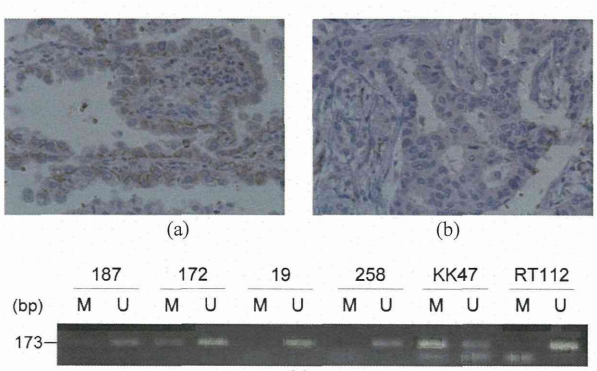
(c)

Figure 1. Targeting *CADM1* by miR-214 and miR-375. (a) Predicted binding sites for the seed sequences of miR-214 and miR-375 in the 3'-UTR of *CADM1*; (b) Sequence alignments of miR-214 and miR-375 with 3'-UTR of *CADM1* as indicated in A. The species abbreviations are Hs: *Homo sapiens*; Mm: *Mus musculus*; (c) Suppression of the luciferase activity of *luciferase-CADM1* 3'-UTR reporter gene by miRNA mimics of miR-214 and miR-375. Data are the mean ± SEM of four independent experiments in duplicate. *$p < 0.01$.

To determine whether miR-214 and miR-375 target the 3'-UTR of the *CADM1* mRNA, we performed a luciferase assay using a reporter construct bearing the 3'-UTR of the *CADM1* at the downstream of the *luciferase* gene. Co-transfection of the reporter plasmid with a miRNA mimic, miR-214 or miR-375, into HEK293 cells decreased the luciferase activity to 72% and 60% of that from siControl-transfected cells, respectively. It indicates that the 3'-UTR of the *CADM1* is a target of miR-214 and miR-375 (**Figure 1(c)**).

3.2. Up-Regulation of miR-214 and miR-375 in Human Primary NSCLC

Next, we examined the expression of miR-214 and miR-375 by qRT-PCR in 34 pairs of cancerous and adjacent non-cancerous lung tissues from primary NSCLC. Overexpression of miR-214 and miR-375 was defined by more than two fold expression in tumors compared with the adjacent non-cancerous region. According to this criteria, miR-214 and miR-375 were overexpressed in 21 (62%) and 17 cases (50%) of primary NSCLC, respectively (**Table 4**). When expression of *CADM1* was analyzed in the same series of samples by immunohistochemistry, loss of *CADM1* expression was observed in 20 of 34 (59%) primary NSCLC, as representative images were shown in **Figures 2(a)** and **(b)** [5]. Overexpression of miR-214 and miR-375 was observed in similar incidence (55% and 50%, respectively) in tumors lacking *CADM1* expression. However, when focused on the pathological stages of the tumors lacking *CADM1* expression, miR-214 was overexpressed in all 5 tumors with pathological stages II and III but only in 6 of 14 (43%) tumors with pathological stage I ($P < 0.05$). These findings suggest that overexpression of miR-214 is involved in the suppression of *CADM1* expression in the



(c)

Figure 2. Expression and promoter methylation state of *CADM1* in primary NSCLC. (a) and (b) Representative images of immunohistochemical analysis of *CADM1*. Membranous staining of *CADM1* in a well-differentiated lung adenocarcinoma (a); Loss of *CADM1* in a moderately differentiated lung adenocarcinoma (b); Magnification ×40 (objective); (c) Representative results of methylation-specific PCR of the *CADM1* promoter. U and M indicate unmethylated and methylated DNA, respectively. Samples 187, 172, 19, and 258, derived from primary NSCLC. Bladder cancer cell lines, KK47 with hypermethylation and RT112 without methylation of the *CADM1* promoter, were used as controls.

Table 4. Overexpression of miR-214 and miR-375 and clinicopathological characteristics of primary NSCLCs.

	No. of tumors examined			No. of tumors lacking *CADM1* expression		
	Total	Overexpression of miR-214[a]	Overexpression of miR-375[a]	Total	Overexpression of miR-214[a]	Overexpression of miR-375[a]
	34	21 (62)	17 (50)	20	11 (55)	10 (50)
Age						
<65	16	11 (67)	7 (44)	8	6 (75)	3 (38)
≧65	18	10 (56)	10 (56)	12	5 (42)	7 (58)
Sex						
Male	17	8 (47)	8 (47)	12	6 (50)	7 (58)
Female	17	13 (76)	9 (53)	8	5 (63)	3 (38)
Histology						
Adenocarcinoma	25	16 (64)	13 (52)	12	7 (58)	6 (50)
Squmos cell carcinoma	9	5 (56)	4 (44)	8	4 (50)	4 (50)
Pathological stage [b]						
I	24	13 (54)	12 (50)	15	6 (40) *	6 (40)
II and III	10	8 (80)	5 (50)	5	5 (100) *	4 (80)

P-value was calculated using Fisher's exact test. *$p < 0.05$; [a]High expression was defined by >2-fold expression compared with the adjacent noncancerous region; [b]According to the TNM pathological classification.

late stage of tumor progression. On the other hand, any other clinicopathological factors examined were not significantly correlated with overexpression of miR-214 or miR-375.

We next examined the status of promoter methylation of *CADM1*, which is one of the major mechanisms to suppress *CADM1* expression. Methylation-specific PCR demonstrated that, among 20 NSCLCs lacking *CADM1* expression, 14 (70%) tumors showed promoter methylation of the *CADM1* gene, supporting the previous findings that promoter methylation is an important mechanism to inactivate *CADM1* in NSCLC (**Figure 2(c)**) [1,17]. However, 6 (30%) tumors lacking *CADM1* expression did not show promoter methylation of the *CADM1*. It should be noted that, among the 6 tumors, overexpression of miR-214 and miR-375 was detected in 5 (83%) and 1 (17%) tumors, respectively (**Table 5**). In these tumors, overexpression of miR-214 or miR-375 would be causally involved in the down-regulation of *CADM1*.

3.3. Biological Functions of miR-214 and miR-375 in Malignant Features of NSCLC Cells

We then examined the effect of miR-214 and miR-375 on the expression of *CADM1*. For this purpose, each mature miRNA-mimic of these miRNAs was transfected into NSCLC cell lines, NCI-H441 and A549, and the introduction of an excess amount of each miRNA was confirmed by qRT-PCR (data not shown). These cells were used as recipient cells because they expressed significant amounts of *CADM1* protein and did not show methylation of the gene promoter. Introduction of miR-375 or miR-214 reduced *CADM1* expression in both NCI-H441 and A549 cells relative to that in cells transfected with siControl (**Figure 3**).

Then, the biological functions of these miRNAs were examined *in vitro* assays related to the tumor suppressive activities of *CADM1*. Since A549 is known to have a moderate activity of colony formation in soft agar [18], we examined the effect of these miRNAs on colony formation in soft agar using A549 as recipient cells. As shown in **Figures 4(a)** and **(b)**, introduction of miR-214 significantly enhanced the colony formation of A549 cells. In addition, miR-375 slightly promoted colony formation, although not in a statistically significant manner. These results suggest that miRNA-mediated suppression of *CADM1* expression enhances anchoring-independent growth of NSCLC cells, although biological activities by other target molecules of each miRNA could also modify the degree of colony-forming ability in each cell.

Next, we examined the effect of the inhibitors of miR-214 and miR-375 on *CADM1* expression by introducing these inhibiters into a NSCLC cell line, HCC827. As shown in **Figures 5(a)** and **(b)**, introduction of the inhibitor of miR-214 or miR-375 significantly enhanced the protein expression of *CADM1*. Since HCC827 cells showed sheet-like morphology with significant migration ability, we examined whether miRNA-mediated suppression of *CADM1* could affect the collective cell migration of HCC827 cells. For this purpose, we transfected these

Table 5. Overexpression of miR-214 and miR-375 and expression and promoter methylation state of *CADM1* in primary NSCLCs.

	No. of tumors examined			No. of tumors lacking *CADM1* expression		
	Total	Overexpression of miR-214[a]	Overexpression of miR-375[a]	Total	Overexpression of miR-214[a]	Overexpression of miR-375[a]
Methylation state						
Methylated	17	9 (53)	10 (59)	14	6 (43)	9 (64)
Unmethylated	17	12 (71)	7 (41)	6	5 (83)	1 (17)

[a] High expression was defined by >2-fold expression compared with the adjacent noncancerous region.

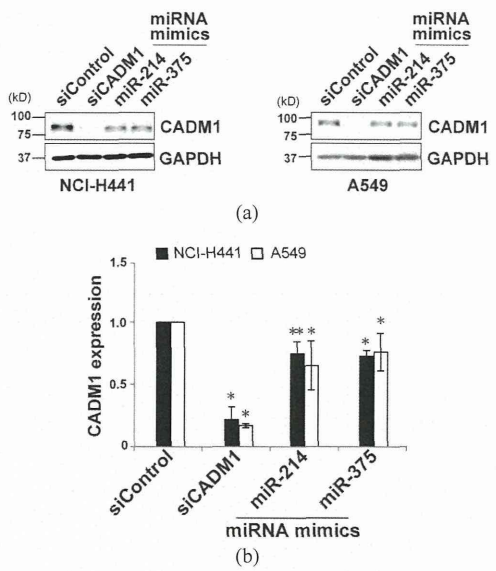


Figure 3. Effects of miRNA mimics and inhibitors of miR-214 and miR-375 on *CADM1* expression in NSCLC cells. Immunoblotting (a) and its quantification (b) of NCI-H441 (left) and A549 cells (right) transfected with siControl, si-*CADM1*, or miRNA mimics as indicated; (b) Intensities of *CADM1* were normalized to those of GAPDH in NCI-H441 (black) and A549 (white) cells. Relative intensities to siControl-transfected cells are shown. Data are the mean ± SD of four independent experiments. $^{**}p < 0.05$; $^{*}p < 0.01$.

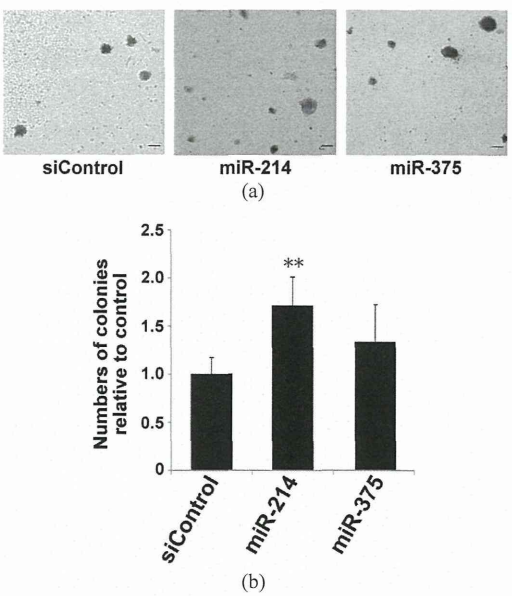


Figure 4. Soft agar colony formation of A549 cells transfected with miRNA mimics of miR-214 or miR-375. (a) Representative images of A549 cells with miRNA mimics as indicated. Bars, 200 μm; (b) Numbers of relevant colonies are shown relative to those of control cells. Data are the mean ± SD of three independent experiments. $^{**}p < 0.05$.

inhibitors of miRNAs as well as an expression vector of *CADM1* into HCC827 cells and assessed their wound-healing activity. As shown in **Figures 5(c)** and **(d)**, the velocity of HCC827 cells transfected with inhibitors of miR-214 and miR-375 significantly decreased to 64% and 66% of that of siControl-transfected cells, respectively, with values that are almost equivalent to those seen in HCC827 transfected with *CADM1* (50%). These results suggest that *CADM1* is one of target genes of miR-214 and miR-375 for their enhancement in collective cell migration of an NSCLC cell, HCC827.

4. Discussion

In this study, using a reporter assay, suppression of *CADM1* expression by miR-214 was demonstrated for the first time. Moreover, miR-375-mediated *CADM1* suppression is consistent with the previous report that *CADM1* is one of the 10 targets of miR-375 in pancreatic islet cells derived from miR-375-deficient mice [19]. It is noteworthy that *CADM1* gives a pair of transcript with the molecular size of 4.4 kb and 1.6 kb by Northern blot analysis [1], but that the shorter transcript does not contain the sequences in its 3'-UTR targeted by miR-214 or miR-375. In normal lung, as well as many other tissues, the transcript of 4.4 kb is predominantly expressed and could be physiologically regulated by these miRNAs. On the other hand, the ratio of 4.4 kb-transcript to 1.6 kb-transcript was significantly decreased in several NSCLC cells in comparison with normal lung tissues, suggesting that *CADM1* transcript of 4.4 kb is selectively degraded by overexpressed miR-214 and miR-375 in NSCLC cells.

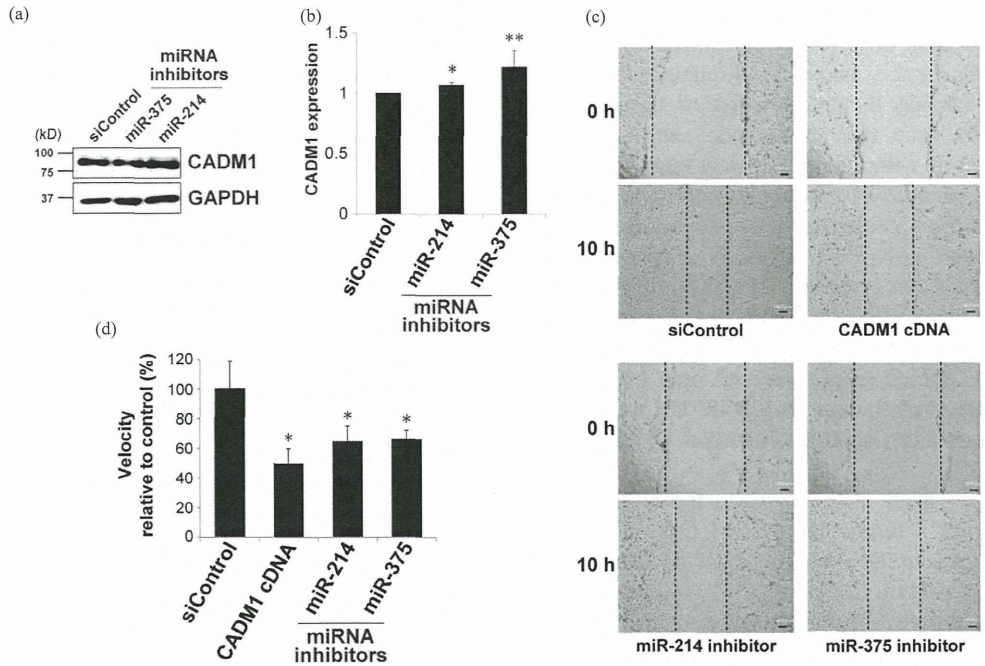


Figure 5. Collective cell migration activity analyzed by wound-healing assay using HCC827 cells transfected with expression vector of *CADM1* or miRNA inhibitors of miR-214 or miR-375. Immunoblotting (a) and its quantification (b) of cells transfected with siControl, si*CADM1*, or miRNA inhibitors as indicated; (b) Intensities of *CADM1* were normalized to those of GAPDH. Data are the mean ± SD of four independent experiments; (c) Representative image of HCC827 cells with miRNA inhibitors indicated at 0 h and 10 h after scratching. The dashed lines indicated the edge of the gap. Bars, 100 μm; (d) Velocity of relevant cells is shown relative to that of control cells. Data are the mean ± SEM of two independent experiments in triplicate. $^{**}p < 0.05$; $^{*}p < 0.01$.

Using *in vitro* assays, miR-214 consistently conferred a malignant phenotype to NSCLC cells, including an anchorage-independent growth ability to A549 cells and a collective cell migration activity to HCC827 cells. These effects could be explained by the functions of *CADM1* as a tumor suppressor. However, these results also suggest a partial implication of other targets by miR-214 in these assays. In this connection, it is noteworthy that PTEN and AP-2 are reported to be additional targets by miR-214. In ovarian cancer, it was demonstrated that the introduction of miR-214 targeted PTEN and activated Akt pathways for the survival of cancer cells [20]. Another study showed that Twist1, a key transcription factor involved in epithelial-mesenchymal transition (EMT), upregulated miR-214 expression, resulting in the proliferation and survival of ovarian cancer cells [21]. In melanoma, miR-214 was shown to participate in tumor progression by targeting a transcription factor, AP-2, and modulating various proteins implicated in cell invasion and blood vessel extravasation [22]. These findings suggest that miR-214 could act as an oncomiR of NSCLC and that *CADM1*, as well as additional targets of miR-214, would cooperatively regulate the malignant features of NSCLC.

On the other hand, miR-375 confers the malignant phenotype to NSCLC cells in rather restricted manner. Although the miR-375 inhibitor significantly suppressed the collecting cell migration activity of HCC827 cells, the introduction of miR-375 only slightly enhanced the colony formation activity of A549 cells in soft agar. Considering that *CADM1* consistently suppresses the malignant phenotype in these cells, the target genes of miR-375 other than *CADM1* appear to be deeply involved in these features of NSCLC cells. In fact, the activities of miR-375 are controversial in terms of tumor progression or suppression. In the pancreas, gene-deficient mice of miR-375 demonstrated that miR-375 was essential for the growth of islet cells, where *CADM1* was one of the possible targets of this miRNA [19]. In breast cancer, miR-375 was shown to be upregulated to promote cell growth by targeting RASD1, an activator of G-protein signaling [23]. In contrast, miR-375 targeted PDK1, and 14-3-3zeta and suppressed growth of gastric cancer cells [24,25]. These findings suggest that the functions of miR-375 in tumor progression or suppression are dependent on the cell types and the sets of target genes and that miR-375 has a restricted activity as an oncomiR in NSCLC.

In addition to the cell biological analyses, we examined the expression of miR-214 and miR-375 in 34 primary NSCLCs in this study. As discussed above, it is necessary to consider the patho-biological significance of these miRNAs from two viewpoints: one, as an independent oncomiR with multiple distinct target molecules, and the other, as miRNA that targets a tumor suppressor, *CADM1*. The high incidences of overexpression of miR-214 (62%) and miR-375 (50%) in primary NSCLC strongly suggest that both miR-214 and miR-375 act as oncomiRs in human NSCLC. MiR-214 was upregulated in primary ovarian cancer [20], whereas higher expression of miR-375 was associated with poor prognosis of esophageal adenocarcinoma [26]. MiR-375 was also reported as a candidate sputum biomarker of lung adenocarcinoma [27].

On the other hand, with regard to the ability of miRNAs to target a tumor suppressor, *CADM1*, overexpression of these miRNAs could provide a novel molecular mechanism to suppress *CADM1* expression in NSCLC in addition to the methylation of the gene promoter. In fact, among the tumors lacking *CADM1* expression, miR-214 overexpression is observed in much higher incidence in tumors without the *CADM1* methylation in comparison with tumors with the *CADM1* methylation. Furthermore, the high incidence of overexpression of miR-214 in advanced NSCLC with pathological stages II and III ($P < 0.05$). It suggests that disruption of miRNA expression could be a rather late event in multistage tumorigenesis of NSCLC.

5. Conclusion

We demonstrate a novel mechanism to suppress the expression of a tumor suppressor *CADM1* by miRNAs, miR-214 and miR-375, in NSCLC. Overexpression of miR-214 and miR-375 in more than half of primary NSCLC, as well as the enhancement in cell motility and anchorage-independent cell growth of NSCLC cells by miR-214 or miR-375 through suppression of *CADM1* expression, suggests that miR-214 and miR-375 would provide potential targets for the treatment of a subset of NSCLC.

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