厚生労働科学研究費補助金（第３次対がん総合戦略研究事業）
（総括・分担）研究報告書

細胞接着・運動性経路を標的としたATL細胞の浸潤、増殖抑制医薬品開発のための基礎研究

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研究要旨：
ATL で異所性発現を示す細胞接着分子 TSLC1/CADM1 に注目した医薬品の開発が妥当であるか、ATL 症例の病理検体を用いた実証的検討をめざし、秋田県内 ATL 症例の収集、TSLC1/CADM1 の免疫組織化学的検討を行った。

E. 研究目的

本研究の目的は、ATL の浸潤、増殖能を抑制する新規治療法の開発である。ターゲットとする分子あるいは経路が実際の ATL 症例で機能しているかを ATL 症例の病理検体を用いて検証する必要がある。とくに、本研究で着目する TSLC1/CADM1 系については、西南日本 ATL 症例ではすでにその異常がＴ細胞癌化に関与することが示されているが、日本海側東北地方のような、他の ATL 好発地域での症例でどうなのか、現在までのところ知見が得られていない。

そこで、研究分担者（後藤）は、秋田県内の ATL 症例を病理学的に検討するとともに、TSLC1/CADM1 異常がみられるかを検討し、本研究で提案される新規治療法が広く ATL 症例に有効であると予想されるか否かを検証することを目的として研究を行う。

また、 CADM1, Tiam1 等の発現を抑制する miRNA の同定と核酸医薬としての評価を総括研究者（村上）と協力して行った。

F. 研究方法

1. 秋田県 ATL 症例の病理学的検討：

秋田県内主要病院での 1990 年より 2010 年にかけての 20 年にわたる解剖例を参照し、その臨床病歴より ATL 病型を分類するとともに、各種の臨床病理学的因子を検索し、その特徴を明らかとする。また、病理解剖結果をもとに、ATL 進展の特徴を明らかとする。

2. 秋田県 ATL 症例での TSLC1/CADM1 異常の検討：

1.で同定された ATL 症例につき、病理ブロックより切片を作成し、TSLC1/CADM1 および関連する諸分子の免疫組織化学的検討

を行い、その発現状態と異常を明らかとする。また、3.で同定されるマイクロＲＮＡの測定を病理ブロックより行う。

3. CADM1 経路分子の発現を抑制するRNAの同定と核酸医薬としての評価：

総括研究者と協力し、CADM1 の発現を抑制する miRNA 候補分子は、２種のアルゴリズム（TargetScan、PicTar）を用いて同定した。候補 miRNA が CADM1 mRNA の 3`UTR 配列を直接標的とすることは、ルシフェラーゼ・レポータープラスミドと miRNA mimic を細胞に導入し、ルシフェラーゼの発現を抑制することで検証した。

G. 研究結果

1. 秋田県での ATL 症例の臨床病理学的解析：

昨年度に施行した２病院（秋田大学医学部附属病院および由利組合総合病院）に引き続き、解剖例の参照を新たに市立秋田総合病院、秋田組合総合病院、山本組合総合病院の３病院で行い、新たに１０例のＡＴＬ症例を見出した（総計１９例）。昨年度に確認した９例に同様、ATL 病型の分布は全国調査とほぼ類似の傾向を示した。

2. 秋田県 ATL 症例で TSLC1/CADM1 異常の検討：

昨年度の予備的検討として、秋田大学医学部附属病院および由利組合総合病院解剖例パラフィンブロックでは

保存状態のマーカーとなるタンパクに対する免疫組織化学の結果が良好であった。したがって免疫組織化学で信頼しうる結果が得られるものと考えられ、CADM1 免疫組織化学を施行した。検討した９例のうち８例では ATL 細胞に CADM1 の強発現をみたが（図１）、１例（８４歳男性、急性型）（図２）では CADM1 発現は陰性ないし弱陽性程度にとどまった。

図１：TSLC1/CADM 強発現 ATL 症例

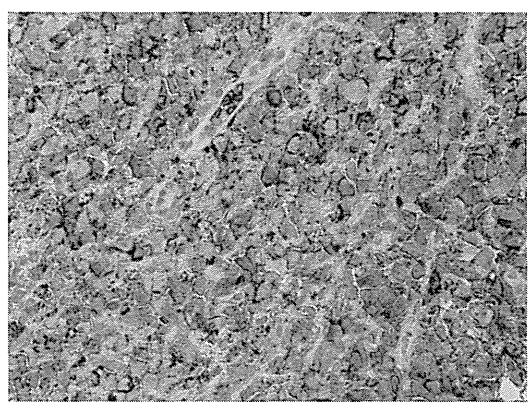
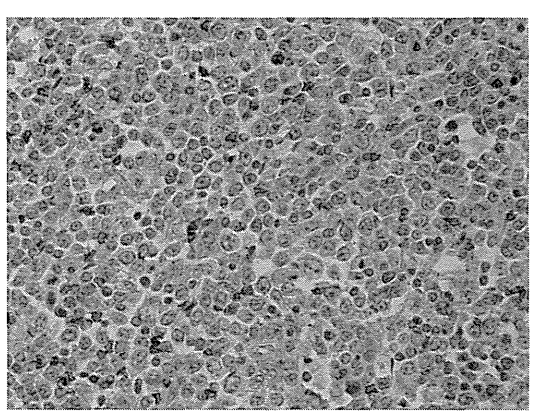


図２：TSLC1/CAD1 陰性-弱陽性 ATL 症例



以降のマイクロＲＮＡ解析の予備的検討として、これらの各症例の病理ブロックより RNA を抽出し、その収量および品質を評価した。半定量的 PCR を用いてのマイクロ RNA 測定を行うには十分な収量であり、品質も A260/A280（吸光度比）が 1.74 から 2.00 と適当であった。

3. CADM1 経路分子の発現を抑制するRNAの同定と核酸医薬としての評価：総括研究者と協力し、CADM13`UTR配列と直接結合し、その発現を抑制するmiRNA候補として、miR-214/199a-5pとmiR-375を同定した。

D．考察

CADM1については、ATLではCADM1が異所性に発現し、(Sasaki *et al, Blood*, 2005)、細胞内で、Tiam1分子と結合し、低分子量Gタンパク質RACを活性化し、ATL細胞の in vitro での運動性、浸潤性、血管内皮細胞や間質細胞への接着性を亢進することを報告しられている (Masuda *et al, JBC*, 2010)。ATL発症は九州を中心とする西南日本で多いことはよく知られており、CADM1に関する知見を含め、現在までの臨床的あるいは生物学的検討の成果の多くは西南日本の症例を基礎にしたものである。一方、東日本でも、日本海沿岸で散在性にATL発症の多い地域が存在する。にもかかわらず、こうした地域と西南日本例でのATLの異同はいまだ明らかではない。したがって、本研究で提案されているTSLC1/CADM1に注目した治療法が西南日本同様、東日本の日本海沿岸地域で行うことが妥当かどうかを判断するための生物学的根拠が十分であるとはいえない。

そこで本分担研究は、TSLC1/CADM1とその関連分子、あるいは TSLC1/CADM1 抑制性 miRNA が東日本の日本海沿岸（秋田県）で発生する ATL においても同様の異常あるいは傾向を示すかを実証するべく、行われている。まず、秋田県内各病院の病理解剖例の参照により、昨年度２病院で９例、今年度３病院で１０例 ATL 症例が同定された。この症例規模は上記の治療法の妥当性検討や病理学的研究一般のために十分な症例数が達成されたと考える。

病理解剖例を用いた分子病理学的解析として、TSLC1/CADM1 免疫組織化学を９例につき行った。１例を除き、ATL 細胞に TSLC1/CADM1 の強い発現がみられた（8例/9 例=88.9%）。この結果は、従来の臨床材料および細胞株を用いた検討の結果を裏付ける。同時に TSLC1/CADM1 系については、西南日本 ATL 症例も日本海側東北地方の ATL 症例も同様の傾向であり、TSLC1/CADM1 系に着目した治療戦略が ATL に対して一般性を有することがわかる。病理解剖例ブロックより、予備的にRNA を抽出し、その収量品質を検討した結果、miRNA 測定が安定して行えることが判明した。このことにより、ATL 症例における TSLC1/CADM1 高発現の背景解明など、分子病理学的理解が進展するものと期待される。

E．結論

秋田県内病院の ATL 解剖例につき、本研究目的を達成するに十分な症例の収集を終える（19 例）とともに、免疫組織化学的に TSLC1/CADM1 高発現が検討症例中の88.9%と極めて高率に見られることが示された。

F．健康危険情報

特になし。

G．研究発表

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H. 知的財産権の出願・登録状況

（予定を含む。）

1. 特許取得：なし。

2. 実用新案登録：なし

3. その他：なし

III. 研究成果の刊行に関する一覧表

別紙４

研究成果の刊行に関する一覧表レイアウト

書籍

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IV. 研究成果の刊行物・別刷

Journal of Cancer Therapy, 2012, *, **-**
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Scientific Research

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 cases (50%) 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 inhibitor, 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 immunogloblin 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

suppression 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

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'	2,848
	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; F, forward primer for PCR; R, reverse primer

(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 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 25nM 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 50nM 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

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

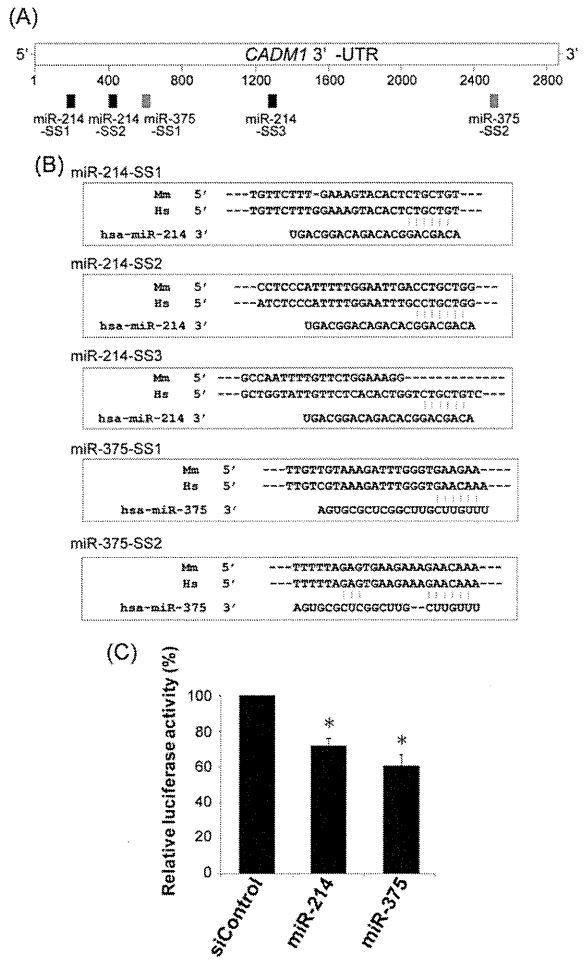

Fig. 1. Targeting CADM1 by miR-214 and miR-375. (A) Predicted binding sites for the seed sequences of miR-214 and miR-375 in the 3'-UTR of *CADM1*. (B) Sequence alignments of miR-214 and miR-375 with 3'-UTR of *CADM1* as indicated in A. The species abbreviations are Hs: *Homo sapiens*; Mm: *Mus musculus*. (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.

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

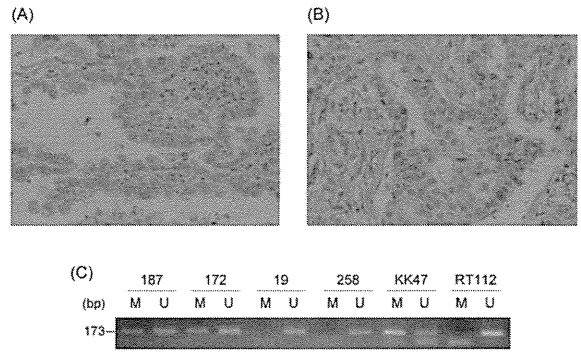

Fig. 2. Expression and promoter methylation state of CADM1 in primary NSCLC cells. (A and B) Representative images of immunohistochemical analysis of CADM1. Membranous staining of CADM1 in a well-differentiated lung adenocarcinoma (A). Loss of CADM1 in a moderately differentiated lung adenocarcinoma (B). Magnification x40 (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.

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* (**Fig. 1A**). 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 1,200 bp from the starting site of the *CADM1* 3'-UTR (**Fig. 1B**).

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 (**Fig. 1C**).

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

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.

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 **Figs. 2A and 2B** [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 suggests that overexpression of miR-214 is involved in the suppression of CADM1expression in the 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 (**Fig. 2C**) [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%)

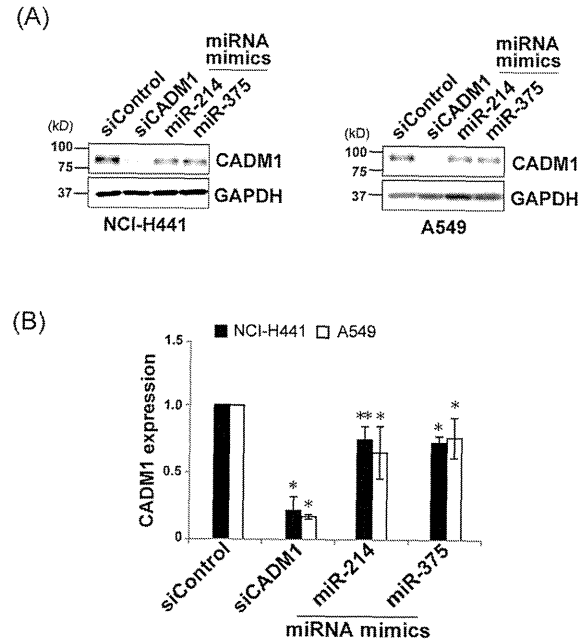

Fig. 3. Effects of miRNA mimics and inhibitors of miR-214 and miR-375 on CADM1 expression in NSCLC cells. Immunoblotting (A) and its quantification (B) of NCI-H441 (left) and A549 cells (right) transfected with siControl, siCADM1, or miRNA mimics as indicated. (B) Intensities of CADM1 were normalized 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.

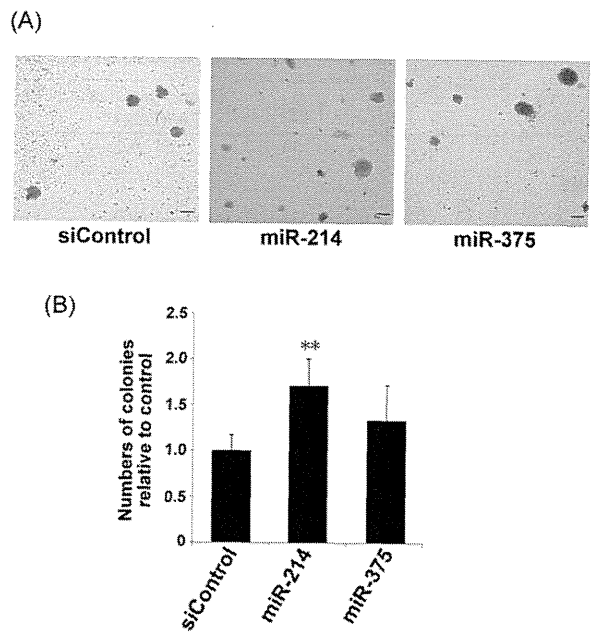

Fig. 4. Soft agar colony formation of A549 cells transfected with miRNA mimics of miR-214 or miR-375. (A) Representative images of A549 cells with miRNA mimics as indicated. Bars, 200 μm. (B) Numbers of relevant colonies are shown relative to those of control cells. Data are the mean ± SD of three independent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

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 miRNA-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 cells 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 (**Fig. 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 **Figs. 4A and 4B**, 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.

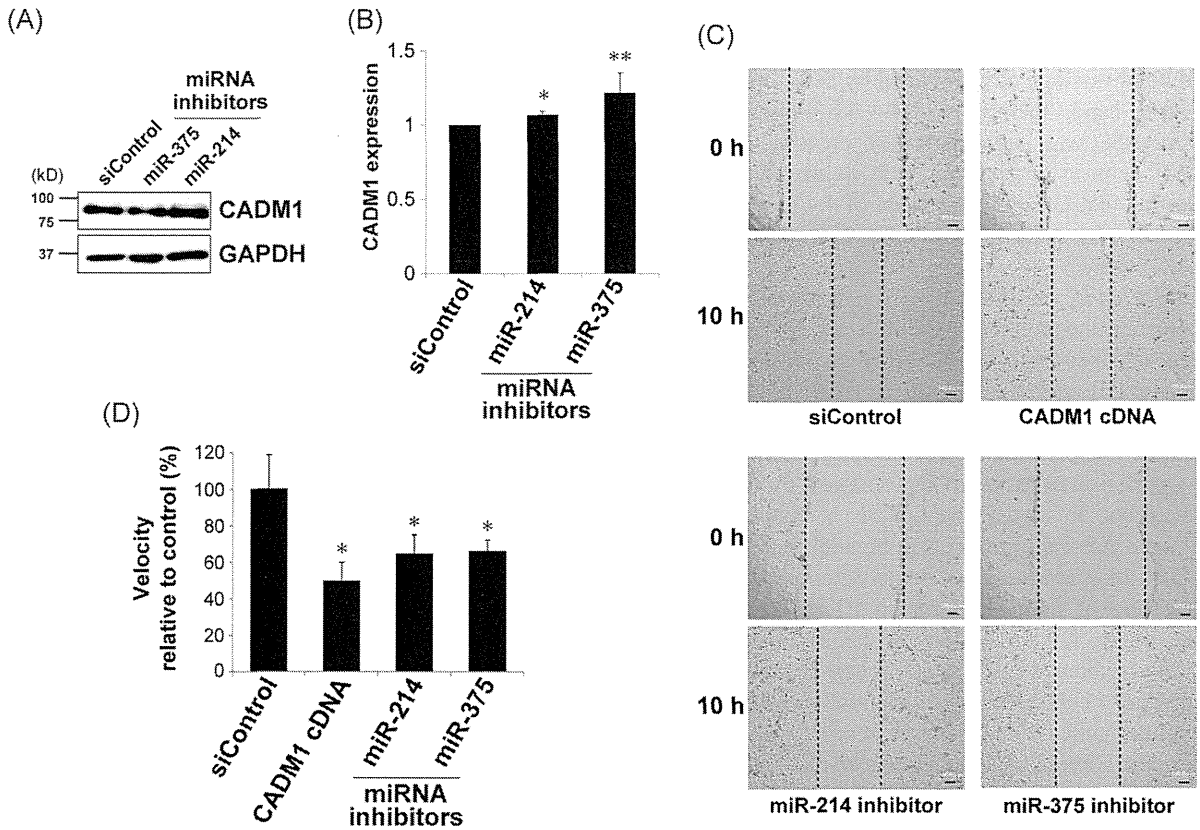

Fig. 5. Collective cell migration activity analyzed by wound-healing assay using HCC827 cells transfected with expression vector of *CADM1* or miRNA inhibitors of miR-214 or miR-375. Immunoblotting (A) and its quantification (B) of cells transfected with siControl, siCADM1, or miRNA inhibitors as indicated. (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. **, $p<0.05$; *, $p<0.01$.

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 **Fig. 5A and 5B**, 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 inhibitors of miRNAs as well as an expression vector of CADM1 into HCC827 cells and assessed their wound-healing activity. As shown in **Figs. 5C and 5D**, 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.

Using *in vitro* assays, miR-214 consistently conferred a malignant phenotype to NSCLC cells, including anan-chorage-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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