Involvement of dual-strand of the miR-144 duplex and their targets in the pathogenesis of lung squamous cell carcinoma

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The prognosis of patients with advanced-stage lung squamous cell carcinoma (LUSQ) is poor, and effective treatment protocols are limited. Our continuous analyses of antitumor microRNAs (miRNAs) and their oncogenic targets have revealed novel oncogenic pathways in LUSQ. Analyses of our original miRNA expression signatures indicated that both strands of miR-144 (miR-144-5p, the passenger strand; miR-144-3p, the guide strand) showed decreased expression in cancer tissues. Additionally, low expression of miR-144-5p significantly predicted a poor prognosis in patients with LUSQ by The Cancer Genome Atlas database analyses (overall survival, \( P = 0.026 \); disease-free survival, \( P = 0.023 \)). Functional assays revealed that ectopic expression of miR-144-5p and miR-144-3p significantly blocked the malignant abilities of LUSQ cells, eg, cancer cell proliferation, migration, and invasion. In LUSQ cells, 13 and 15 genes were identified as possible oncogenic targets that might be regulated by miR-144-5p and miR-144-3p, respectively. Among these targets, we identified 3 genes (SLC44A5, MARCKS, and NCS1) that might be regulated by both strands of miR-144. Interestingly, high expression of NCS1 predicted a significantly poorer prognosis in patients with LUSQ (overall survival, \( P = 0.013 \); disease-free survival, \( P = 0.048 \)). By multivariate analysis, NCS1 expression was found to be an independent prognostic factor for patients with LUSQ patients. Overexpression of NCS1 was detected in LUSQ clinical specimens, and its aberrant expression enhanced malignant transformation of LUSQ cells. Our approach, involving identification of antitumor miRNAs and their targets, will contribute to improving our understanding of the molecular pathogenesis of LUSQ.

KEYWORDS
lung squamous cell carcinoma, microRNA, miR-144-3p, miR-144-5p, NCS1

Abbreviations: Ago2, Argonaute2; D2R, dopamine D2 receptor; DFS, disease-free survival; GEO, Gene Expression Omnibus; LUAD, lung adenocarcinoma; LUSQ, lung squamous cell carcinoma; MARCKS, myristoylated alanine rich protein kinase C substrate; miRNA, microRNA; NCS1, neuronal calcium sensor 1; NSCLC, non-small cell lung cancer; OS, overall survival; qRT-PCR, real-time quantitative RT-PCR; RCC, renal cell carcinoma; RISC, RNA-induced silencing complex; siRNA, small interfering RNA; SLC44A5, solute carrier family 44 member 5; TCGA, The Cancer Genome Atlas.
1 | INTRODUCTION

Lung cancer is the leading cause of cancer-related deaths worldwide among both men and women.1 Lung cancer includes 2 major clinicopathological categories: small cell lung cancer and NSCLC. The latter accounts for approximately 85% of all cases of lung cancer, and NSCLC tumors consist mainly of 3 subtypes: LUAD, LUSQ, and large cell carcinoma.2 In patients with LUAD, currently developed treatment strategies (eg, epidermal growth factor receptor tyrosine kinase inhibitors, inhibitors of anaplastic lymphoma kinase, and immune checkpoint inhibitors) have dramatically improved OS rates in patients.3-6 In contrast, in patients with LUSQ, the lack of early diagnostic tools and effective treatment protocols has resulted in a poor OS rate in patients with LUSQ.7,8 Therefore, identification of therapeutic target molecules is essential for achieving improved outcomes in patients with LUSQ.

MicroRNAs are a class of small, noncoding RNAs (19-22 nt in length). These molecules regulate gene expression by repressing translation or cleaving RNA transcripts in a sequence-dependent manner.9 Interestingly, a single miRNA species could regulate a vast number of protein-coding and noncoding RNA transcripts.10 In human disease cells, aberrantly expressed miRNAs trigger the failure of orderly and controlled RNA networks.11 Numerous studies have shown that aberrant expression of miRNAs and dysregulated RNA networks are deeply involved in human diseases, including cancer.12-16

Through our continuing work, we have identified antitumor miRNAs and their target oncogenic genes in LUSQ.17-22 Moreover, detailed analyses of our original miRNA expression signatures by RNA sequencing have revealed that passenger strands of miRNAs actually act as antitumor miRNAs and are involved in cancer pathogenesis.23-25 Additionally, our recent studies showed that both strands of the miR-144 duplex (ie, the passenger strand miR-144-5p and the guide strand miR-144-3p) are downregulated in bladder cancer and RCC tissues and act as antitumor miRNAs.26,27 The involvement of both strands of the miRNA duplex in cancer pathogenesis is a new concept for cancer research.

Several cohort analyses by TCGA database have indicated that low expression of miR-144-5p predicts poor prognosis in patients with LUSQ (OS, \( P = 0.026 \); DFS, \( P = 0.023 \)). Thus, both strands of the miR-144 duplex are involved in LUSQ molecular pathogenesis. Here, we aimed to verify that miR-144-5p and miR-144-3p possess antitumor functions. We also sought to identify their molecular targets, thereby revealing new details of LUSQ pathogenesis.

2 | MATERIALS AND METHODS

2.1 | Clinical specimen collection, cell lines, and cell culture

The present study was approved by the Bioethics Committee of Kagoshima University Hospital (Kagoshima, Japan) (approval nos 26-164). Written prior informed consent and approval were obtained from all patients. In total, 30 LUSQ specimens and 20 noncancerous lung specimens were collected from patients who underwent thoracic surgery at Kagoshima University Hospital from 2010 to 2013. The pathological stages of LUSQ were classified according to the International Association for the Study of Lung Cancer TNM classification, 7th edition.28 The clinicopathological features of the patients are shown in Table 1. The procedure for RNA extraction from formalin-fixed, paraffin-embedded specimens was described in previous studies.19

In addition, we evaluated 2 LUSQ cell lines (EBC-1 and SK-MES-1), obtained from the Japanese Cancer Research Resources Bank (Osaka, Japan) and ATCC (Manassas, VA, USA), respectively. Cell culture, extraction of total RNA, and extraction of protein were carried out as described in our earlier reports.18,19,21

2.2 | Quantitative real-time RT-PCR

The procedure for qRT-PCR has been described previously.18,22,29 The expression levels of miRNAs were analyzed by TaqMan qRT-PCR assays (assay IDs 002148 and 002676; Applied Biosystems, Foster City, CA, USA). Data were normalized to the expression of RNU48 (assay ID 001006; Applied Biosystems). NCS1 expression levels were determined using TaqMan probes and primers (assay ID HS01179522_m1; Applied Biosystems), and GAPDH (assay ID HS99999905_m1; Applied Biosystems) was used for normalization.

| TABLE 1 | Characteristics of lung cancer and noncancerous cases |
| --- | --- |
| A. Characteristics of lung cancer cases | n (%) |
| Total number | 30 |
| Median age, years (range) | 71 (50-88) |
| Sex | n (%) |
| Male | 29 (96.7) |
| Female | 1 (3.3) |
| Pathological stage | n (%) |
| IA | 5 (16.7) |
| IB | 9 (30.0) |
| IIA | 2 (6.7) |
| IIB | 6 (20.0) |
| IIIA | 7 (23.3) |
| IIIB | 1 (3.3) |
| B. Characteristics of noncancerous cases | n |
| Total number | 20 |
| Median age, years (range) | 70.5 (50-88) |
| Sex | n |
| Male | 20 |
| Female | 0 |

Pathological stage of lung cancer was classified according to the International Association for the Study of Lung Cancer TNM classification, 7th edition.28
2.3 Transfection of LUSQ cells with mature miRNA and siRNA

The following mature miRNA species were used in this study: mir-Vana miRNA mimic, hsa-miR-144-5p (product ID MC12631; Applied Biosystems), hsa-miR-144-3p (product ID MC11051; Applied Biosystems), and negative control miRNA, anti-miR negative control #1 (catalog no. AM17010; Applied Biosystems). The following siRNAs were used: Stealth Select RNAi siRNA, si-NCS1 (P/N HSS118732 and HSS118734; Invitrogen, Carlsbad, CA, USA), and negative control miRNA/siRNA, anti-miR negative control #1 (catalog no. AM17010; Applied Biosystems). The transfection procedures were described previously.28

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**TABLE 2** Characteristics and immunohistochemical status of patients included in tissue microarray analysis

| A. Immunohistochemical status and characteristics of cases with lung squamous cell carcinoma |
|---|
| Patient no. | Grade | T | N | M | Pathological stage | Immunohistochemical staining intensity |
| 23 | 2 | 2 | 1 | 0 | IIB | (+) |
| 24 | 2 | 2 | 0 | 0 | IB | (++) |
| 25 | 2 | 1 | 0 | 0 | IA | (++) |
| 26 | 1 | 2 | 1 | 0 | IIB | (+) |
| 27 | 2 | 1 | 0 | 0 | IA | (++) |
| 28 | 1 | 3 | 0 | 0 | IIB | (++) |
| 29 | 1 | 2 | 0 | 0 | IB | (++) |
| 30 | 2 | 2 | 0 | 0 | IB | (++) |
| 31 | 3 | 2 | 0 | 0 | IB | (++) |
| 32 | 3 | 2 | 1 | 0 | IIB | (+) |
| 33 | 3 | 2 | 0 | 0 | IB | (+) |
| 34 | 3 | 2 | 1 | 0 | IIB | (++) |
| 35 | 2 | 1 | 1 | 0 | IIIA | (++) |
| 36 | 3 | 2 | 1 | 0 | IIA | (++) |
| 37 | 3 | 2 | 0 | 0 | IIB | (++) |
| 38 | 3 | 2 | 0 | 0 | IB | (++) |
| 39 | 3 | 2 | 1 | 0 | IIB | (++) |
| 40 | 3 | 2 | 0 | 0 | IB | (++) |
| 41 | 2-3 | 3 | 0 | 0 | IIB | (++) |
| 42 | 3 | 1 | 2 | 0 | IIIA | (+) |
| 43 | 3 | 2 | 0 | 0 | IB | (+) |
| 44 | 3 | 2 | 0 | 0 | IB | (+) |

| B. Immunohistochemical status of noncancerous cases |
|---|
| Patient no. | Immunohistochemical staining intensity |
| 69 | (+) |
| 70 | (−) |
| 71 | (−) |
| 72 | (+) |
| 73 | (+) |
| 74 | (+) |
| 75 | (+) |
| 76 | (++) |
| 77 | (+) |
| 78 | (+) |
| 79 | (+) |
| 80 | (+) |

Pathological stages of lung cancer were classified according to the International Association for the Study of Lung Cancer TNM classification.28
2.4 | Incorporation of miR-144-5p and miR-144-3p into RISC: Assessment by Ago2 immunoprecipitation

MicroRNAs were transfected into EBC-1 cells by reverse transfection. After a 48-hour incubation period, miRNAs were isolated by immunoprecipitation using a microRNA Isolation Kit for Human Ago2 (Wako, Osaka, Japan). We then assessed the expression of Ago2-conjugated miRNAs by qRT-PCR, as described in previous studies.

2.5 | Cell proliferation, migration, and invasion assays

Protocols for determining cell proliferation, migration, and invasion were described previously.

2.6 | Identification of putative target genes regulated by miR-144-5p and miR-144-3p in LUSQ cells

Gene expression analyses by oligo microarray and in silico analyses were used to identify putative target genes regulated by miR-144-5p and miR-144-3p. The microarray data were deposited in the GEO repository under accession number GSE115801. Putative target genes having binding sites for miR-144-5p and miR-144-3p were detected by TargetScanHuman version 7.2 (http://www.targetscan.org/vert_72/). The GEO database (GSE19188) was used for assessment of the association between target genes and expression of NSCLC clinical specimens. Identification of miR-144-5p and miR-144-3p target genes was carried out as described in Figure S1.

2.7 | Clinical database analysis

The clinical significance of miRNAs and their target genes in LUSQ was investigated with TCGA database (https://tcga-data.nci.nih.gov/tcga/). The gene expression and clinical data were retrieved from cBioPortal (http://www.cbioportal.org/) and OncoLnc (http://www.oncolnc.org) (data downloaded on April 28, 2018).

2.8 | Plasmid construction and dual-luciferase reporter assay

Wild-type or deletion-type sequences targeted by miR-144-5p and miR-144-3p were inserted into the psiCHECK-2 vector (C8021; Promega, Madison, WI, USA).
After cotransfecting miRNA and the constructed vector into EBC-1 and SK-MES-1 cells, firefly and Renilla luciferase activities were measured using a dual Luciferase assay kit (Promega). The procedure was described previously.18,20,22

**FIGURE 2** Functional assays of miR-144-5p and miR-144-3p in lung squamous cell carcinoma cell lines. A, Cell proliferation was determined by XTT assays 72 hours after transfection with miR-144-5p and miR-144-3p. B, Cell migration was measured by wound healing assays. C, Cell invasion was determined by Matrigel invasion assays. Cell proliferation, migration, and invasion were significantly suppressed in miR-144-5p and miR-144-3p transfectants compared with those in mock and control transfectants. *P < 0.001

2.9 | Western blot analysis and immunohistochemistry

The procedures for western blotting and immunohistochemistry were described previously.21,22 Membranes were immunoblotted with monoclonal anti-NCS1 Abs (1:1000 dilution; ab129166; Abcam, Cambridge, UK) and monoclonal anti-GAPDH Abs (1:20 000 dilution; MAB374; EMD Millipore, Billerica, MA, USA).

Immunohistochemistry was carried out with a VECTASTAIN Universal Elite ABC Kit (catalog no. PK-6200; Vector Laboratories, Burlingame, CA, USA) according to the manufacturer's protocol. Primary rabbit mAbs against NCS1 (ab129166; Abcam) were used at a 1:100 dilution at 4°C overnight. The characteristics of patients included in the tissue microarray are described in Table 2. The procedure for immunohistochemistry was described previously.18,22,34
TABLE 3 Common putative target genes regulated by miR-144-5p and miR-144-3p in lung squamous cell carcinoma cells

| Entrez gene ID | Gene symbol | Description | SK-MES-1 miR-144-5p transfectant Log₂ ratio | SK-MES-1 miR-144-3p transfectant Log₂ ratio | GSE19188 Log fold-change | TCGA database 5-y OS P value |
|---------------|-------------|-------------|---------------------------------------------|---------------------------------------------|--------------------------|-----------------------------|
| 23413         | NCS1        | Neuronal calcium sensor 1 | -1.04                                      | -1.04                                      | 1.31                     | 0.013                       |
| 204962        | SLC44A5     | Solute carrier family 44 member 5 | -1.28                                      | -1.96                                      | 3.07                     | 0.325                       |
| 4082          | MARCKS      | Myristoylated alanine rich protein kinase C substrate | -1.53                                      | -1.04                                      | 1.44                     | 0.872                       |

Lower and upper percentiles of The Cancer Genome Atlas (TCGA) database were both 50.

GSE, Gene Expression Omnibus dataset results; OS, overall survival.

2.10 | Statistical analysis

All data were analyzed using SPSS version 23 software (IBM SPSS, Chicago, IL, USA). Differences between 2 groups were analyzed by Mann-Whitney U tests, and those between multiple groups were examined by one-way ANOVA and Tukey tests for post-hoc analysis. Differences between survival rates were analyzed by Kaplan-Meier survival curves and log-rank statistics. Univariate and multivariate analyses for OS using TCGA database were carried out by Cox proportional hazards regression analyses.

3 | RESULTS

3.1 | Downregulation of miR-144-5p and miR-144-3p in LUSQ clinical specimens and their clinical significance

The expression levels of miR-144-5p and miR-144-3p were markedly decreased in LUSQ tissues in comparison with those in noncancerous tissues (P < 0.001; Figure 1A,B). Positive correlations between the expression of miR-144-5p and miR-144-3p were confirmed by Spearman’s rank tests (r = 0.912 and P < 0.001; Figure 1C). In 2 cancer cell lines, EBC-1 and SK-MES-1, the expression levels of miR-144-5p and miR-144-3p were extremely low (Figure 1A,B).

By analyses using TCGA database, low expression of miR-144-5p significantly predicted poor prognosis compared with high expression of miR-144-5p (5-year OS, P = 0.026; 5-year DFS, P = 0.023; Figure 1D). Low expression of miR-144-3p also tended to predict poor prognosis compared with high expression of miR-144-3p in LUSQ patients (5-year OS, P = 0.072; 5-year DFS, P = 0.072; Figure 1E).

3.2 | Ectopic expression of miR-144-5p and miR-144-3p suppressed cancer cell proliferation, migration, and invasion in LUSQ cells

To verify the functional roles of miR-144-5p and miR-144-3p in LUSQ, EBC-1 and SK-MES-1 cells were transfected with mature miR-144-5p and miR-144-3p sequences. Cell proliferation assays showed significant inhibition of cell growth in miR-144-5p and miR-144-3p transfectants (Figure 2A). Moreover, cell migration and Matrigel invasion were significantly inhibited by miR-144-5p or miR-144-3p transfection (Figures 2B,C). In migration and invasion analyses, miR-144-5p had stronger antitumor effects than miR-144-3p.

3.3 | Incorporation of miR-144-5p and miR-144-3p into RISC in LUSQ cells

To verify whether the miR-144-5p passenger strand could be incorporated into the RISC in LUSQ cells, we undertook immunoprecipitation for Ago2, which is essential for formation of the RISC, in cells transfected with either miR-144-5p or miR-144-3p. Isolated Ago2-bound miRNAs were analyzed by qRT-PCR to examine the interactions between miR-144-5p and miR-144-3p and Ago2. A conceptual diagram of this analysis is shown in Figure 3A.

In EBC-1 cells, miR-144-5p transfectants showed higher expression levels of miR-144-5p than did mock transfectants or miR-control or miR-144-3p (P < 0.001) transfectants (Figure 3B). Similarly, after miR-144-3p transfection, we detected miR-144-3p using Ago2 immunoprecipitation (P < 0.001; Figure 3C).

3.4 | Identification of putative target genes regulated by miR-144-5p and miR-144-3p in LUSQ cells

Next, we aimed to identify miR-144-5p and miR-144-3p target genes. To this end, we undertook a combination of in silico and genome-wide gene expression analyses, as shown in Figure S1. Using TargetScanHuman database analysis, 1785 and 3776 putative target genes were found to have binding sites for genome-wide gene expression analyses, as shown in Figure S1. Using TargetScanHuman database analysis, 1785 and 3776 putative target genes were found to have binding sites for
Overall, 3 putative target genes (NCS1, SLC44A5, and MARCKS) were coordinately regulated by both miR-144-5p and miR-144-3p (Table 3). The TCGA database analysis showed that high NCS1 expression significantly predicted a poor prognosis in patients with LUSQ (5-year OS, \( P = 0.013 \); 5-year DFS, \( P = 0.048 \); Figure 4A). Subsequently, we focused on NCS1 and validated the functional significance of LUSQ cells.
FIGURE 5 Direct regulation of NCS1 by miR-144-5p and miR-144-3p in lung squamous cell carcinoma cell lines. A, Expression levels of NCS1 mRNA 72 hours after transfection with miR-144-5p and miR-144-3p using GAPDH as an internal control. B, NCS1 protein expression by western blot analysis 72 hours after transfection with miR-144-5p and miR-144-3p in EBC-1 and SK-MES-1 cell lines. GAPDH was used as a loading control. C, Putative miR-144-5p binding sites in the 3′-UTR of NCS1 mRNA. Dual luciferase reporter assays using vectors encoding putative miR-144-5p target sites in the NCS1 3′-UTR (position 666-672) for both wild-type and deleted regions. Normalized data were calculated as Renilla/firefly luciferase activity ratios. D, Putative miR-144-3p binding sites in the 3′-UTR of NCS1 mRNA. Dual luciferase reporter assays using vectors encoding putative miR-144-3p target sites in the NCS1 3′-UTR (position 841-847) for both wild-type and deleted regions. Normalized data were calculated as Renilla/firefly luciferase activity ratios. *P < 0.001
3.5 Direct regulation of NCS1 by miR-144-5p and miR-144-3p in LUSQ cells

We then examined whether NCS1 mRNA and NCS1 protein expression were suppressed by restoration of miR-144-5p and miR-144-3p in LUSQ cells. NCS1 mRNA and NCS1 protein levels were significantly decreased by miR-144-5p or miR-144-3p transfection compared with those in mock- or miR-control-transfected cells (Figure 5A,B).

Next, we carried out luciferase reporter assays in EBC-1 and SK-MES-1 cells to validate the direct binding of miR-144-5p and miR-144-3p to NCS1 mRNA. The TargetScanHuman database projected the existence of binding sites in the 3′-UTR of NCS1 (miR-144-5p, position 666-672; miR-144-3p, position 841-847; Figure 5C,D). Accordingly, we undertook luciferase reporter assays with vectors that contained either the wild-type or deletion-type 3′-UTR of NCS1. Our results showed that the luminescence intensities were markedly reduced by transfection with miR-144-5p or miR-144-3p and the vector harboring the wild-type 3′-UTR of NCS1. In contrast, transfection with the deletion-type vector did not reduce the luminescence intensities in EBC-1 or SK-MES-1 cells (Figure 5C,D). These findings indicated that both strands of the miR-144 duplex bound directly to the 3′-UTR of NCS1.

**FIGURE 6** Effects of NCS1 silencing in lung squamous cell carcinoma cell lines. A, mRNA expression of NCS1 72 hours after transfection with si-NCS1 using GAPDH as an internal control. B, NCS1 protein expression by western blot analysis 72 hours after transfection with si-NCS1-1 and si-NCS1-2 in EBC-1 and SK-MES-1 cell lines. GAPDH was used as a loading control. C, Cell proliferation was identified by XTT assays 72 hours after transfection with si-NCS1-1 and si-NCS1-2. D, Cell migration was measured by wound healing assays. E, Cell invasion was determined by Matrigel invasion assays. *P < 0.001
3.6 | NCS1 knockdown inhibited cell proliferation, migration, and invasion in LUSQ cells

We examined the effects of NCS1 knockdown in EBC-1 and SK-MES-1 cells using 2 types of si-NCS1 oligos: si-NCS1-1 and si-NCS1-2. Both siRNAs effectively downregulated NCS1 mRNA and NCS1 protein expression (Figure 6A, B).

Cancer cell proliferation, migration, and invasive abilities were markedly inhibited by si-NCS1 transfection compared with those in mock or control EBC-1 and SK-MES-1 cells (Figure 6C-E).

3.7 | Expression of NCS1 in LUSQ clinical specimens and its clinical significance

Immunohistochemical analyses of LUSQ clinical specimens indicated that NCS1 protein was strongly expressed in LUSQ cells, but showed infrequent and weak expression in normal lung cells (Table 2 and Figure 7).

Finally, univariate and multivariate Cox hazard regression analyses were used to evaluate the clinical significance of NCS1 expression for OS in patients with LUSQ. Multivariate analysis showed that NCS1 expression was an independent predictive factor for OS (hazard ratio = 1.508, \( P = 0.007 \); Figure 8).

4 | DISCUSSION

In our previous studies, we showed that both strands of the miR-145 duplex (the guide strand miR-145-5p and the passenger strand miR-145-3p) were significantly downregulated in cancer tissues. These miRNAs were also found to have antitumor functions, and their targets were involved in NSCLC pathogenesis.\(^{20,35}\) Thus, research focusing on both strands of the miRNA duplex is important for improving outcomes in cancer.

In this study, we found that both miR-144-5p and miR-144-3p had tumor-suppressing effects in LUSQ cells and controlled several oncogenes. In an analysis of previous studies of the functional significance of the miR-144 duplex, the antitumor function of miR-144-3p was reported in several type of cancers.\(^{36-41}\) For example, expression of miR-144-3p suppressed cancer cell proliferation in glioblastoma and hepatocellular carcinoma through targeting c-MET and SGK3, respectively.\(^{36,37}\) In laryngeal squamous cell carcinoma, miR-144-3p inhibited the cancer cell epithelial-mesenchymal transition phenotype through targeting ETS-1.\(^{38}\) These data indicated that miR-144-3p was a pivotal tumor suppressor, and that downregulation of miR-144-3p enhanced cancer cell aggressiveness. Recent studies reported that some long noncoding RNAs (metastasis-associated lung adenocarcinoma transcript 1 [MALAT1] and taurine upregulated 1 [TUG1]) acted as competing endogenous RNAs and that their overexpression attenuated the expression of miR-144-3p in cancer cells.\(^{39,40}\) Another study showed that interleukin-1β affected miR-144-3p at the transcriptional level and that interleukin-1β levels were significantly higher in patients with LUAD and LUSQ.\(^{41}\)
In contrast to miR-144-3p analyses, few reports have examined the functional significance of miR-144-5p in cancer cells. Analyses of miRNA signatures by RNA sequencing showed that both strands of miR-144-5p and miR-144-3p were frequently downregulated in several cancers.26,27 In our studies, we focused on both strands of the miR-144 duplex and investigated the antitumor functions and targets of the duplex in cancer cells.26,27 Our previous studies showed that both strands of the miR-144 duplex had antitumor functions in bladder cancer and RCC and that both miRNAs coordinately targeted CCNE1/CCNE2 and SDC3, respectively.26,27 This study is the third paper reporting the antitumor functions of both strands of the miR-144 duplex in cancer cells. Importantly, low expression of miR-144-5p and miR-144-3p significantly predicted short survival in patients with RCC. Moreover, high expression of the oncogenic genes controlled by these miRNAs also predicted short survival in patients with RCC, suggesting that the miR-144 duplex and its targets were deeply involved in RCC pathogenesis.27

In this study, 3 genes (NCS1, SLC44A5, and MARCKS) were found to be coordinately controlled by both miR-144-5p and miR-144-3p in LUSQ cells. MARCKS is a major substrate of protein kinase C.42 Recent studies have shown that MARCKS plays a pivotal role in cancer development and progression.42 Moreover, in lung cancer, aberrant MARCKS expression was detected in clinical specimens, and MARCKS expression was implicated in this disease.43,44 Among the 3 targets, we focused on NCS1 because its aberrant expression significantly predicted poor prognosis in patients. The NCS1 protein is a member of the NCS family, which harbors a functional Ca\(^{2+}\) binding domain and N-terminally myristoylated site.45,46 Previous studies have reported that NCS1 is a multifunctional protein involved in exocytosis, neurite outgrowth, neuroprotection, axonal regeneration, and nuclear Ca\(^{2+}\) regulation.45,46 Furthermore, NCS1 interacts with various proteins to control their functions.45,46 For example, NCS1 interacts with D2R and inhibits D2R-mediated signaling pathways.47 Interestingly, activation of D2R-mediated signaling suppresses lung cancer aggressiveness.48 Moreover, aberrant expression of NCS1 could interfere with D2R-mediated signaling and might be involved in LUSQ cell progression.

Our functional assays showed that inhibition of NCS1 by siRNA suppressed cancer cell migration and invasion in LUSQ cells. Moreover, in multivariate Cox proportional hazards regression analysis, expression of NCS1 predicted poor prognosis in patients with LUSQ. Another study showed that overexpression of NCS1 promoted cell invasion and migration in breast cancer cells and that NCS1 overexpression was associated with poor prognosis in these patients.49 These findings indicate that aberrantly expressed NCS1 is involved in cancer cell aggressiveness. Thus, NCS1 could be a novel diagnostic and therapeutic target for patients with LUSQ.

In conclusion, genes coordinately controlled by the miR-144 duplex (miR-144-5p and miR-144-3p) were found to be related to LUSQ pathogenesis. Our findings describing the involvement of the passenger strand miR-144-5p are the first report of this phenomenon in LUSQ pathogenesis. NCS1 expression was directly regulated by the miR-144 duplex in LUSQ cells. Aberrantly expressed NCS1 enhanced LUSQ cell aggressiveness. Thus, elucidation of antitumor miRNAs controlling RNA networks could provide novel prognostic markers and therapeutic targets for this disease.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.
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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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