Critical Function for Nuclear Envelope Protein TMEM209 in Human Pulmonary Carcinogenesis

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Abstract

Therapeutic targets for more effective and less toxic treatments of lung cancer remain important. Here we report the identification of the integral nuclear envelope protein TMEM209 as a critical driver of human lung cancer growth and survival. TMEM209 expression was normally limited to testis, but we found that it was widely expressed in lung cancer, in which it localized to the nuclear envelope, Golgi apparatus, and the cytoplasm of lung cancer cells. Ectopic overexpression of TMEM209 promoted cell growth, whereas TMEM209 attenuation was sufficient to block growth. Mass spectrometric analysis identified the nucleoporin protein NUP205 as a TMEM209-interacting protein, stabilizing NUP205 and increasing the level of c-Myc in the nucleus. Taken together, our findings indicate that TMEM209 overexpression and TMEM209–NUP205 interaction are critical drivers of lung cancer proliferation, suggesting a promising new target for lung cancer therapy.

Introduction

Lung cancer is one of the leading causes of death in the worldwide (1). Many genetic alterations associated with development and progression of lung cancers have been reported and contributed to the better understanding of the molecular mechanisms of pulmonary carcinogenesis (2). However, despite some advances in the early detection and recent improvements in its treatment, the prognosis of the lung cancer patients is not much improved. Over the last few decades, several newly developed cytotoxic agents such as paclitaxel, docetaxel, gemcitabine, and vinorelbine have begun to offer multiple choices for treatment of patients with advanced lung cancer, but each of those regimens confers only a modest survival benefit compared with cisplatin-based therapies (3–5). In addition to these cytotoxic drugs, several molecular targeted agents, such as monoclonal antibodies against VEGF (i.e., bevacizumab/anti-VEGF) or epidermal growth factor receptor (EGFR; i.e., cetuximab/anti-EGFR) as well as inhibitors for EGFR tyrosine kinase (i.e., gefitinib and erlotinib) and anaplastic lymphoma kinase (i.e., crizotinib) were developed and are applied in clinical practice (6, 7).

Materials and Methods

Lung cancer cell lines and tissue samples

The human lung cancer cell lines used in this study were as follows: lung adenocarcinoma cell lines A549, LC319, PC14, NCI-H1373, and NCI-H1781; lung squamous cell carcinoma cell lines SKMES-1, LL61, NCI-H520, NCI-H1703, and NCI-H2170; small cell lung carcinoma cell lines DMS114, DMS273, SBC-3, and SBC-5, and a large cell carcinoma cell line LX1 (Supplementary Table S1). All cells were grown in

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monolayers in appropriate medium supplemented with 10% fetal calf serum and were maintained at 37°C in atmospheres of humidified air with 5% CO₂. Human small airway epithelial cells were grown in optimized medium purchased from Cambrex BioScience, Inc. Primary lung cancer tissue samples had been obtained with informed consent as described previously (10, 14). This study and the use of all clinical materials were approved by individual institutional ethical committees.

**Semi quantitative reverse transcription PCR**

Total RNA was extracted from cultured cells using the TRIzol reagent (Life Technologies, Inc.) according to the manufacturer’s protocol. Extracted RNAs were treated with DNase I (Nippon Gene) and reversely transcribed using oligo (dT) primer and SuperScript II. Semi quantitative reverse transcription PCR (RT-PCR) experiments were carried out with the following synthesized specific primers for TMEM209, NUP205, CDC25A, CDK1, or β-actin (ACTB) as follows: TMEM209, 5'-GCAGACTCACTAAAGTATCCCC-3' and 5’-CTCCATGGTGCTTTTAATGAAG-3'; NUP205, 5’-GAAAACCTCCTGGACATTTGAAGGA-3' and 5’-TGAGGATGGAACACTAGGGGAAG-3'; CDC25A, 5’-TGAGGATGAGGTGGGTTTTT-3' and 5’-GCCATCCCACCTTCTCTTTT-3'; CDK1, 5’-AACCATTTCCTCATGGGGAT-3' and 5’-TGGATGATTCAGTGCCATTT-3'; ACTB, 5’-GAGGTTGATACTTTGCTTCCG-3', and 5’-CAAGTCAGTGTACAGG-3' PCR reactions were optimized for the number of cycles to ensure product integrity within the logarithmic phase of amplification.

**Northern blot analysis**

Human multiple tissue blots (BD Biosciences, Clontech) were hybridized with 32P-labeled PCR products of TMEM209 and NUP205. The cDNA probes of TMEM209 and NUP205 were prepared by RT-PCR using following primers: TMEM209, 5’-AACACCTTAGATTTAGTTAG-3' and 5’-CTCCATGGTGCTTTTAATGAAG-3'; NUP205, 5’-GGGCCAAGAAGCCGACC-3' and 5’-ACTTTGCTTGAAGGCTAGG-3'. Prehybridization, hybridization, and washing were done according to the supplier’s recommendations. The blots were autoradiographed at −80°C for 14 days with intensifying BAS screens (Bio-Rad).

**Western blotting**

Whole cells were lysed with NP-40 buffer [150 mmol/L NaCl, 0.5% NP-40, 50 mmol/L Tris-HCl (pH 8.0)] containing Protease Inhibitor Cocktail Set III and Phosphatase Inhibitor Cocktail Set II (Calbiochem). Protein fractionation was carried out with NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo). Protein samples were separated by SDS-polyacrylamide gels and electroblotted onto Hybond-ECL nitrocellulose membranes (GE Healthcare Bio-Sciences). Blots were incubated with either of antibodies to TMEM209 (catalog no. HPA031678; ATLAS Antibodies), NUP205 (catalog no. HPA024574; ATLAS Antibodies), c-Myc (catalog no. sc-40; Santa Cruz), Flag (catalog no. F3165; Sigma), or ACTB (catalog no. A5316; Sigma). Antigen–antibody complexes were detected using secondary antibodies conjugated to horseradish peroxidase (GE Healthcare Bio-Sciences). Protein bands were visualized by enhanced chemiluminescence Western blotting detection reagents (GE Healthcare Bio-Sciences).

**Immunofluorescence analysis**

Cells were plated onto glass coverslips (Recort Dickinson Labware), fixed with 4% paraformaldehyde, and permeabilized with 0.1% Triton X-100 in PBS for 5 minutes at room temperature. Nonspecific binding was blocked by 5% Skim milk for 30 minutes at room temperature. Cells were then incubated for 60 minutes at room temperature with primary antibodies for anti-TMEM209 antibody (catalog no. HPA031678; ATLAS Antibodies), anti-c-Myc antibody (catalog no. sc-40; Santa Cruz) or anti-Golgi 58K Protein/Formiminotransferase Cyclodeaminase antibody (catalog no. G2404; Sigma) diluted in PBS containing 1% bovine serum albumin. After being washed with PBS, the cells were stained by Alexa Fluor 488–conjugated or Alexa Fluor 594–conjugated secondary antibody (Molecular Probes) for 60 minutes at room temperature. After another wash with PBS, each specimen was mounted with Vectashield (Vector Laboratories, Inc.) containing 4′,6-diamidine-2′-phenylindolendi-hydrochloride (DAPI) and visualized with Spectral Confocal Scanning Systems (TSC SP2 AOBS; Leica Microsystems).

**RNA interference assay**

To evaluate the biologic functions of TMEM209 and NUP205 in lung cancer cells, we used short interfering RNA (siRNA) duplexes against the target genes (Sigma). The target sequences of the synthetic oligonucleotides for RNA interference were as follows: control-1: [EGFP, enhanced GFP (eGFP) gene, a mutant of Aequorea victoria GFP], 5’-GAGGACAGCACAGUCUUC-3'; control-2 (LUC, luciferase gene from Photinus pyralis), 5’-CGUACGCGAAUACUCGA-3'; si-TMEM209-1, 5’-CUACGAAUUUGAUACU-3'; si-TMEM209-2, 5’-GUGU- GAAUAUUGUGG-3'; si-NUP205, and 5’-CUCCUUACCU- GUGGCGCUU-3'. Lung cancer cells, LC319, SBC-3, and SBC-5, were plated onto 10-cm dishes and transfected at subconfluent condition with either of the siRNA oligonucleotides (50 μmol/L) using 30 μL of Lipofectamine 2000 (Invitrogen) according to the manufacturers’ instructions. After 7 days of incubation, these cells were stained by Giemsa solution to assess colony formation, and cell numbers were measured by MTT assay; briefly, cell-counting kit-8 solution (Dojindo) was added to each dish at a concentration of 1/10 volume, and the plates were incubated at 37°C for additional 30 minutes. Absorbance was then measured at 490 nm, and at 630 nm as a reference, with a Microplate Reader 550 (Bio-Rad).

**Flow cytometry**

Cells were collected in PBS and fixed in 70% cold ethanol for 30 minutes. After treatment with 100 μg/mL of RNase (Sigma), the cells were stained with 50 μg/mL propidium iodide (Sigma) in PBS. Flow cytometry was analyzed by using FACSscan (Beckman Coulter). The cells selected from at least 20,000 un gated cells were analyzed for DNA content.

**Cell growth assay**

We cloned the entire coding sequence of TMEM209 into the appropriate site of COOH-terminal Flag-tagged pCAGGS.
plasmid vector. COS-7 and SBC-3 cells transfected either with plasmid expressing Flag-tagged TMEM209 or mock plasmid were grown for 7 days in Dulbecco’s Modified Eagle’s Medium (COS-7) or RPMI (SBC-3) containing 10% fetal calf serum in the presence of appropriate concentrations of genetin (G418). Cell viability was evaluated by MTT assay.

**Coimmunoprecipitation and matrix-assisted laser desorption/ionizing—time of flight mass spectrometry mapping of TMEM209-associated proteins**

Cell extracts from a lung cancer SBC-5 cell, which was transfected with TMEM209 expression vector or mock vector, were precleared by incubation at 4°C for 1 hour with 80 μL of protein G–agarose beads in a final volume of 200 μL of immunoprecipitation buffer (0.5% NP-40, 50 mmol/L Tris-HCl, 150 mmol/L NaCl) in the presence of Protease Inhibitor Cocktail Set III (Calbiochem). After centrifugation at 1,000 rpm for 5 minutes at 4°C, the supernatants were incubated at 4°C with anti-Flag M2 agarose (catalog no. A2220; Sigma) for 1 hour. The beads were then collected by centrifugation at 5,000 rpm for 1 minute and washed 6 times with 1 mL of immunoprecipitation buffer. The washed beads were resuspended in 30 μL of Laemmli sample buffer and boiled for 5 minutes, and the proteins were separated using 5% to 20% gradient SDS-PAGE gel (Bio-Rad). After electrophoresis, the gel was stained with SilverQuest (Invitrogen). Protein bands specifically found in extracts from the cells that were transfected with TMEM209 vector were excised and served for matrix-assisted laser desorption/ionization—time-of-flight mass spectrometry analysis (AXIMA-CFR plus; Shimadzu Biotech).

**Immunoprecipitation assay**

Cell extracts from lung cancer SBC-5 cells were precleared by incubation at 4°C for 1 hour with 80 μL of protein G–agarose beads in a final volume of 200 μL of immunoprecipitation buffer (0.5% NP-40, 50 mmol/L Tris-HCl, 150 mmol/L NaCl) in the presence of Protease Inhibitor Cocktail Set III (Calbiochem). After centrifugation at 1,000 rpm for 5 minutes at 4°C, the supernatants were incubated at 4°C with normal rabbit IgG (catalog no. sc-2027; Santa Cruz) or anti-TMEM209 antibody (catalog no. 06-1020; Millipore) for overnight. The beads were then collected by centrifugation at 5,000 rpm for 1 minute and washed 6 times with 1 mL of immunoprecipitation buffer. The washed beads were resuspended in 30 μL of protein G–agarose beads in a final volume of 200 μL of immunoprecipitation buffer (0.5% NP-40, 50 mmol/L Tris-HCl, 150 mmol/L NaCl) in the presence of Protease Inhibitor Cocktail Set III (Calbiochem) for 1 hour. The beads were then collected by centrifugation at 5,000 rpm for 1 minute and washed 6 times with 1 mL of immunoprecipitation buffer. The washed beads were resuspended in 30 μL of Laemmli sample buffer and boiled for 5 minutes, and the proteins were separated using 5% to 20% gradient SDS-PAGE gel (Bio-Rad). After electrophoresis, the gel was stained with SilverQuest (Invitrogen). Protein bands specifically found in extracts from the cells that were transfected with TMEM209 vector were excised and served for matrix-assisted laser desorption/ionization—time-of-flight mass spectrometry analysis (AXIMA-CFR plus; Shimadzu Biotech).

**Protein synthesis and proteasome inhibitors**

Protein synthesis inhibitor, cycloheximide (Calbiochem) was dissolved in ethanol and added in culture medium at 100 μg/mL. Proteasome inhibitor, MG132 (Synonym: Z-Leu-Leu-Leu-al; Sigma) was dissolved in dimethyl sulfoxide and added in culture medium at 20 μmol/L.

**Quantitative real-time PCR**

Quantitative real-time PCR was conducted with the SYBR Green I Master Kit on a LightCycler 480 (Roche) according to the manufacturer’s recommendations. Each experiment was done in triplicate. GAPDH was used for normalization of expression levels. cDNAs as templates were synthesized as described above. For quantitative RT-PCR reactions, specific primers for all human TMEM209, CDK25A, CDK1, and GAPDH were designed as follows: TMEM209, 5′-TCGCCCCGCTAGTGTTAT-3′ and 5′-CCACATGTGAGGGTACCG-3′; CDK25A, 5′-ATCTCTTACACAGAGCGAAGA-3′ and 5′-CCTGTGTACGTCTATCTCTT-3′; CDK1, 5′-TGGATCTGAAAGATACTGGAATTCTA-3′ and 5′-CAATCCCCGTAGGATTGTGG-3′; GAPDH, 5′-GCAAATTCATGCGACCCTC-3′ and 5′-TGCCCCACTTGATTGTCGG-3′.

**Result**

**TMEM209 expression in lung cancers and normal tissues**

To identify novel target molecules for the development of therapeutic agents and/or diagnostic biomarkers of lung cancer, we had previously carried out gene expression profile analysis of 120 lung carcinomas using cDNA microarray containing 27,648 genes or expressed sequence tags (9–14). We identified TMEM209 that showed 3-fold or higher level of expression in the majority of 120 lung cancer samples examined and confirmed its transactivation by semiquantitative RT-PCR experiments in 10 of 15 additional lung cancer tissues and in 12 of 15 lung cancer cell lines (Figs. 1A and 1B). We also confirmed, by Western blotting analysis, high levels of TMEM209 (63 kDa) expression in lung cancer cell lines using anti-TMEM209 antibody (Fig. 1C). To examine the subcellular localization of endogenous TMEM209 in cancer cells, we carried out immunocytochemical analysis of lung cancer SBC-5 cells that overexpressed the endogenous TMEM209 protein using anti-TMEM209 antibody. TMEM209 was detected on the nuclear envelope and the Golgi apparatus and weakly in cytoplasm (Fig. 1D). Northern blot analysis with a TMEM209 cDNA as a probe identified a 3.5-kb transcript specifically in the testis among 16 normal human tissues examined (Fig. 1E).

**Inhibition of growth of lung cancer cells by siRNA against TMEM209**

To assess whether TMEM209 is essential for growth or survival of lung cancer cells, we transfected synthetic oligonucleotide siRNAs against TMEM209 into lung adenocarcinoma LC319 and small cell lung cancer SBC-5 cells in which TMEM209 was highly expressed. The protein levels of TMEM209 in the cells transfected with si-TMEM209-#1 or #2 were significantly decreased in comparison with cells transfected with either of control siRNAs (Figs. 2A and B). We also observed significant decrease in the number of colonies and the number of viable cells measured by MTT assay (Figs. 2C–F). On the other hand, we examined the effects of these siRNAs on the lung cancer SBC-3 cells in which endogenous TMEM209 was hardly detectable. MTT assay revealed that the viability of cells treated with TMEM209 siRNAs (#1, 2) was equivalent to that treated with either of control siRNAs (si-EF1p or LUC), indicating that suppression of cancer cell viability of cells treated with TMEM209 siRNAs (#1, 2) was likely to be off-target effects (Fig. 2G). To further assess the
knockdown effect of TMEM209, we carried out flow cytometric analysis and found the G1 arrest of SBC-5 cells transfected with siRNA against TMEM209 (Fig. 2H).

Growth-promoting effect of TMEM209
To further clarify a potential role of TMEM209 in carcinogenesis, we constructed plasmid vector (pCAGGS vector) expressing TMEM209 with a Flag tag at a C-terminal (TMEM209-Flag). We then transfected TMEM209-Flag vector or mock plasmid into COS-7 and SBC-3 cells, in which endogenous TMEM209 was expressed at a very low level and carried out cell growth assay. We detected the growth-promoting effect of cells overexpressing TMEM209, compared with those transfected with mock vector (Figs. 2I–L).

Interaction of TMEM209 with NUP205
To elucidate the function of TMEM209, we screened a protein(s) that interacts with TMEM209 in cancer cells. Lysates of lung cancer SBC-5 cells, which were transfected with Flag-tagged TMEM209 expression vector or mock vector, were extracted and immunoprecipitated with anti-Flag M2 agarose. The protein complex was stained with SilverQuest on the SDS-PAGE gel (Supplementary Fig. S1A). A 205-kDa band, which was observed in cell lysates transfected with TMEM209 vector, but not in those with mock vector, was successfully characterized by peptide sequencing to be a human NUP205 (Nucleoporin 205 kDa). We subsequently confirmed the interaction between endogenous TMEM209 and endogenous NUP205 in normal human tissues detected by Northern blot analysis. ADC, adenocarcinoma; SCC, small cell carcinoma; LCC, large cell carcinoma; SAEC, small airway epithelial cells; IB, immunoblotting.

Figure 1. TMEM209 expression in lung cancers and normal tissues. A, expression of TMEM209 in clinical samples of non-small cell lung carcinoma and small cell lung carcinoma (SCLC) and normal lung tissues analyzed by semiquantitative RT-PCR. Appropriate dilutions of each single-stranded cDNA generated from mRNAs of lung cancer samples were prepared, using β-actin (ACTB) expression as a quantitative control. B, expression of TMEM209 in lung cancer cell lines examined by semiquantitative RT-PCR. C, expression of TMEM209 protein in lung cancer cell lines examined by Western blot analysis. D, subcellular localization of endogenous TMEM209 protein in lung cancer SBC-5 cells. E, expression of TMEM209 in normal human tissues detected by Northern blot analysis. ADC, adenocarcinoma; SCC, small cell carcinoma; LCC, large cell carcinoma; SAEC, small airway epithelial cells; IB, immunoblotting.
including DNAs, RNAs, and proteins between these 2 compartments. NPCs are the gateways that facilitate this transport across the nuclear envelope in co-operation with soluble transport receptors and play a crucial and essential role in cellular event (16). We found NUP205 expression in lung cancers, but not in normal tissues such as lung, liver, kidney, heart, and brain (Fig. 3B). Northern blot analysis with NUP205 as a probe identified a 6.3-kb transcript in testis among 16 tissues examined, indicating that both TMEM209 and NUP205 are likely to be cancer testis antigens (Fig. 3C). To assess the functional relationship between TMEM209 and NUP205, we examined the NUP205 protein level after inhibition of TMEM209 expression by siRNA treatment in SBC-5 cells. We transfected siRNA oligonucleotides against TMEM209 (si-#1 or si-#2), si-EGFP, or si-LUC into SBC-5 cells and at 24 hours after treatment with siRNAs, incubated SBC-5 cells in growth medium supplemented with protein synthesis inhibitor cycloheximide and monitored endogenous NUP205 protein levels in cells transfected with si-TMEM209 or si-EGFP. We observed the knockdown of TMEM209 transcription in SBC-5 cells transfected with si-TMEM209 (si-#1 or si-#2), si-EGFP, or si-LUC. H, cell-cycle population change in SBC-5 cells after treatment of siRNA against TMEM209. I and J, transient expression of TMEM209 in COS-7 and SBC-3 cells detected by Western blot analysis. K and L, assays showing the growth-promoting effect of transient introduction of TMEM209 in COS-7 and SBC-3 cells. Assays were done in triplicate and in triplicate wells. IB, immunoblotting.

Figure 2. Growth effect of TMEM209 expression. A and B, expression of TMEM209 by the treatment with si-TMEM209 (si-#1 or si-#2) or control siRNAs (si-EGFP or si-LUC) in LC319 cells and SBC-5 cells analyzed by Western blot analysis. C and D, viability of LC319 cells and SBC-5 cells evaluated by MTT assay by the treatment with si-TMEM209 (si-#1 or si-#2), si-EGFP, or si-LUC. All assays were done in triplicate and in triplicate wells. E and F, colony formation assays of LC319 cells and SBC-5 cells transfected with si-TMEM209 (si-#1 or si-#2) or control siRNAs, G, viability of SBC-3 cells evaluated by MTT assay after treatment with si-TMEM209 (si-#1 or si-#2), si-EGFP, or si-LUC. H, cell-cycle population change in SBC-5 cells after treatment of siRNA against TMEM209. I and J, transient expression of TMEM209 in COS-7 and SBC-3 cells detected by Western blot analysis. K and L, assays showing the growth-promoting effect of transient introduction of TMEM209 in COS-7 and SBC-3 cells. Assays were done in triplicate and in triplicate wells. IB, immunoblotting.

Nuclear c-Myc levels may be regulated by TMEM209–NUP205 complex

Previous reports for large-scale mapping of human protein–protein interactions by mass spectrometry suggested NUP205 to interact with c-Myc, an oncogenic transcription factor (17, 18). Therefore, we investigated the interaction between NUP205 and c-Myc in lung cancer cells using lysates of SBC-5 cells, which were transfected with Flag-tagged c-Myc...
expression vector or mock vector. Immunoprecipitation of the cell lysates with anti-Flag M2 agarose and subsequent immunoblotting with anti-NUP205 antibody confirmed their interaction (Supplementary Fig. S1B). Because NUP family members are known to regulate nucleocytoplasmic transport of macromolecules, we assessed the effect of TMEM209–NUP205 complex on c-Myc protein localization by fractionating cell lysates to cytoplasm and nucleus. Suppression of TMEM209 or NUP205 expression by siRNAs against TMEM209 or NUP205 seemed to reduce the levels of nuclear c-Myc protein (Supplementary Fig. S2A and B). Considering that c-Myc protein stability is strictly regulated by ubiquitin–proteasome system (18), we then treated SBC-5 cells, which had been transfected with siRNAs against TMEM209 or NUP205, with proteasome inhibitor MG132. The amount of the whole c-Myc protein was not changed, whereas the level of cytoplasmic c-Myc protein was elevated and that of nuclear c-Myc was reduced in the cells treated with si-TMEM209 or si-NUP205 (Fig. 4A). These data suggested that TMEM209 and NUP205 are involved in regulation of the nuclear transport of c-Myc. Furthermore, we examined the effects of other nuclear proteins, STAT3 and p65, which were reported to translocate from the cytoplasm to the nucleus in human cancer cells. The total amounts of STAT3 and p65 proteins as well as those in the nucleus were reduced in cells transfected with si-TMEM209 or si-NUP205 (Supplementary Fig. S3). The data implied that TMEM209–NUP205 complex is associated with the nuclear import of not only c-Myc but also some nuclear proteins. To further examine whether c-Myc transcription activity could be inhibited after the knockdown of TMEM209, we measured the expression levels of representative c-Myc target genes, CDC25A and CDK1, which are highly expressed in lung cancers and are reported to be involved in carcinogenesis (Supplementary Fig. S4). Suppression of TMEM209 by siRNAs reduced the expression levels of CDC25A and CDK1 in LC319 and SBC-5 cells, as detected by quantitative real-time PCR (Fig. 4C). These data indicated that overexpression of TMEM209–NUP205 complex proteins may prompt the nuclear transport of c-Myc and result in...
overexpression of oncogenic c-Myc target genes, such as \( \text{CDC25A} \) and \( \text{CDK1} \).

**Discussion**

Recent advances in the study of the biologic mechanisms underlying cancer development have caused the paradigm shift in designing and developing a new type of therapeutic drug, termed "molecular targeted drug," that selectively interferes with molecules or pathways involved in tumor growth and/or progression. Inactivation of growth factors and their receptors in tumor cells as well as the inhibition of oncogenic pathways or specific functions in cancer cells constitutes the main rationale of novel cancer treatments (19). Molecular targeted cancer therapies are expected to treat cancer cells more selectively than normal cells, thus to be less harmful to normal cells, to reduce side effects, and to improve quality of life of cancer patients. Intensive studies to screen molecular targets for development of novel drugs identified a number of possible candidates that can be applicable for novel lung cancer therapies. However, suppression of some of such molecules also caused serious adverse reactions \textit{in vivo} because of expression of molecules in certain types of normal tissue and/or off-target effects of compounds on nontarget molecules. Hence, the specificity of molecules in cancer cells as well as the selectivity of compounds to a certain target should be critical to develop drugs with high efficacy and minimum toxicity.

To screen more appropriate molecular targets for the drug development, we had analyzed the whole-genome expression profiles of 120 clinical lung cancer samples using cDNA microarray data containing 27,648 genes or ESTs (10–14) and investigated loss-of-function phenotypes by RNA interference systems (20–46). On the basis of this approach, we found TMEM209 to be overexpressed in the majority of clinical lung cancer cases as well as lung cancer cell lines, although its expression was hardly detectable in normal tissues, except the testis. Furthermore, we showed that the knockdown of

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**Figure 4.** Nuclear levels of c-Myc may be regulated by TMEM209–NUP205 complex. A, attenuation of nuclear levels of c-Myc protein in SBC-5 cells transfected with si-TMEM209 or si-NUP205. B, the downregulation of c-Myc target genes, \( \text{CDC25A} \) and \( \text{CDK1} \), in LC319 and SBC-5 cells after the knockdown of TMEM209 expression with siRNAs. IB, immunoblotting.

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TMEM209 expression resulted in inhibition of cancer cell growth, whereas transient expression of TMEM209 resulted in the significant promotion of cell growth. The data suggested that TMEM209 plays indispensable roles in the growth of lung cancer cells, indicating that TMEM209 could serve as a target for the development of anticancer agents for lung cancer, although further analysis of TMEM209 including mutational screening and/or epigenetic alteration of this gene should be required to fully address the significant role of this gene in pulmonary carcinogenesis.

TMEM209 is a 63-kDa transmembrane protein that contains a NPC component domain in its N terminus. Some proteins containing this domain are known to be components of the NPC. One member of this family is nucleoporin POM34 (budding yeast) that is thought to have a role in anchoring peripheral NUP family proteins into the pore and mediating pore formation (47). Our study also showed that TMEM209 interacted with NUP205, a component of NPC. NUP205 was identified as a component of NPC and to interact with NUP93 and NUP53 that are involved in the integrity of the NPC in Xenopus (48). To date, there is no report describing the involvement of TMEM209–NUP205 complex in human carcinogenesis. We also found overexpression of NUP205 in lung cancer cells and similarly to TMEM209, its expression was scarcely detectable in normal tissues except testis, suggesting that the TMEM209–NUP205 complex could be expressed specifically in lung cancer cells and testis. We also showed that TMEM209 regulated the NUP205 protein stability by its interaction. It was reported that NUP93 and NUP53 could interact with and stabilize NUP205 in Hela cells (49). Further analysis is necessary to verify the detailed relationship between the TMEM209–NUP205 complex and other NUP proteins, but it is likely that TMEM209 protein is indispensable for the function of NPC in cancer cells.

Our data also indicated that TMEM209–NUP205 complex could play important roles in nuclear levels of c-Myc and then influence to the c-Myc transcriptional activity. In Drosophila, Nup93 is able to preferentially interact with the phosphorylated and activated form of MAD (Human SMAD1 homolog) and could be directly involved in the nuclear import of MAD (50). One can speculate that in the process of shuttling molecules from cytoplasm to nucleus, the nuclear import of some oncogenic factors, including c-Myc protein, may be supported by the TMEM209–NUP205 complex. In fact, the amounts of STAT3 and p65 (a subunit of NF-kB) proteins were significantly reduced by the loss of TMEM209, indicating that the TMEM209–NUP205 complex is likely to be involved in nuclear transport of various nuclear proteins in addition to c-Myc.

In summary, human TMEM209 has an essential role in the growth of lung cancer cells through its interaction with NUP205 and regulation of the nuclear transport of c-Myc. Our data indicate that TMEM209 may be a good molecular target for the development of novel treatment for lung cancer.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: T. Fujitomo, Y. Daigo, Y. Nakamura
Development of methodology: T. Fujitomo, Y. Daigo, K. Ueda
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): T. Fujitomo, Y. Daigo, K. Ueda
Analysis and interpretation of data (e.g., statistical analysis, bios-statistics, computational analysis): T. Fujitomo, Y. Daigo, K. Ueda, Y. Nakamura
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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): Y. Daigo
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