Inhibition of Myc Effectively Targets KRAS Mutation-positive Lung Cancer Expressing High Levels of Myc

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Abstract. Myc is an oncogenic transcription factor that promotes tumorigenesis. Recently, a dominant negative form of Myc (Omomyc) was shown to cause regression of lung tumors in a mouse model of lung cancer caused by KRAS mutation, suggesting that Myc might be a potential therapeutic target to treat the KRAS lung cancer. However, it is not yet known whether Omomyc can also inhibit the growth of human lung tumors that carry a similar KRAS mutation. In the present study, we demonstrate that Omomyc induces cell death of KRAS-mutated human lung adenocarcinoma A549 cells in vitro and in vivo. However, Omomyc does not induce cell death in human lung adenocarcinoma H441 cells that also carry the KRAS mutation. Interestingly, A549 cells express high levels of Myc, while H441 cells do not. Co-expression of exogenous Myc with Omomyc in H441 cells induces cell death, indicating that Omomyc requires high levels of Myc to induce cell death in KRAS mutation-positive lung adenocarcinoma. Here, we show for the first time that KRAS mutation-positive lung cancer displaying high levels of Myc could be treated by inhibiting Myc transactivation function.

Introduction. KRAS is one of the two most commonly mutated oncogenes (KRAS, EGFR) in lung cancer. While small molecules specifically targeting mutated EGFR have been identified and clinically used, effective therapies including standard chemotherapy to target mutated KRAS have not been developed yet (1). Encouraged by the identification of effective EGFR-targeting small molecules, molecular mechanisms of KRAS mutation-positive lung oncogenesis have recently been sought in order to identify molecular targets to treat such lung cancer (2-4). One of the genes downstream of KRAS that is likely to be responsible for KRAS lung cancer pathogenesis is the Myc transcription factor, which is phosphorylated and stabilized by the KRAS pathway (5, 6). Stabilized Myc heterodimerizes with Max and activates transcription of genes involved in growth control and cancer (7). Soucek et al. have developed a dominant negative form of Myc (Omomyc) by deleting the transactivation domain in the N-terminus and mutating the DNA binding domain in the C-terminus of Myc. Omomyc inhibits Myc-mediated transcription by heterodimerizing with Myc and sequestering it from its DNA binding (8). In a transgenic mouse study, ubiquitous expression of Omomyc regressed lung tumors developed in a mouse model of KRAS driven lung cancer (9), suggesting that Myc is a dominant effector of KRAS mutation-positive lung cancer pathogenesis. In an in vitro study, Johnson et al. have shown that knockdown of Myc by siRNA reduces the cell number of A549 human lung cancer cells that carry the KRAS mutation (10), suggesting again that KRAS mutation-positive lung cancer pathogenesis is mediated by Myc. In the present study, our goal was to determine whether targeting Myc is effective to treat KRAS mutation-positive lung cancer.

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Materials and Methods

Cell lines and culture conditions. The human pulmonary adenocarcinoma cells A549 and H441 were obtained from the American Type Culture Collection (Manassas, VA, USA) and grown in high glucose Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal bovine serum. These cell lines were cultured in 10% CO₂ at 37°C. The lysates of human adult lung tissue were obtained from Novas Biologicals (Littleton, CO, USA).

Construction of the recombinant adeno viral vectors. The Omomyc gene was synthesized by PCR from pBp Omomyc (11) using specific primers: ttt ttacgcacaagagtcgattcgcgctttggg and ligation into pCMVShuttle. The hemagglutinin-tagged Omomyc (HA-Omomyc) gene was synthesized by PCR from pBP Omomyc using specific primers: ttt ttacgcacaagagtcgattcgcgctttggg and ligation into pCMVShuttle. The MYC gene was excised from pCMVSP6T MYC obtained from Human Science Research Resources Bank (Sennan, Osaka, Japan) and ligation into pCMVShuttle. Ad-Omomyc or Ad-Myc was generated by homologous recombination and plaque purified (12). Ad-Luc and Ad-GFP were kindly provided from Dr. Bingliang Fang (M.D. Anderson Cancer Center, Houston, TX, USA). The viral titer for each vector was determined by plaque assay and the optimal multiplicity of infection (MOI) was determined by infecting each cell line with Ad-CMV/GFP (13) and assessing the expression of GFP by flow cytometric analysis. In in vitro experiments, all cells were infected with the recombinant adenoviral vectors at a MOI of 5000 viral particles (vp) per cell.

Immunoblot analysis. For endogenous MYC detection, A549 or H441 cells were lysed in ice cold lysis buffer [1% Triton X-100, 20 mM Tris-HCL (pH 8.0), 137 mM NaCl, 10% glycerol (v/v), 2 mM EDTA, 1 mM sodium orthovanadate (v/v) 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin]. For Omomyc detection, A549 or H441 cells were lysed in ice cold lysis buffer 36 hours after Ad-Omomyc Ad-Luc or phosphate buffered saline (PBS) treatment. Cell lysates were clarified by centrifugation (10 min at 15,000 x g at 4°C) and protein concentration was determined using the DC protein assay (BioRad, Hercules, CA, USA). Equal amounts of protein were separated on a sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel. The gel was electrophoretically transferred to a Hybond polyvinylidene fluoride (Hybond PVDF) transfer membrane (Amersham, Arlington Heights, IL, USA). The membrane was incubated with primary and secondary antibodies according to the Supersignal West Pico chemiluminescence protocol (Pierce, Rockford, IL, USA) to detect secondary antibody binding. Antibody specific for hemagglutinin and anti-β-actin antibody were obtained from Sigma (St. Louis, MO, USA) and antibody specific for human Myc was obtained from Novus Biological (Littleton, CO, USA). Secondary horseradish peroxidase-conjugated goat anti-rabbit antibody and anti-mouse antibody were obtained from Jackson Immunoresearch Laboratories (West Grove, PA, USA).

Cell viability assay. Cells were plated in 96-well plates at a density of 2x10⁵ cells/well 24 hours prior to infections and treated with PBS, Ad-Luc or Ad-Omomyc at a MOI of 5000 vp. Cell viability was evaluated at 0, 1, 2, 3, and 4 days following the adenoviral infection by MTS assay (CellTiter 96; Promega) according to the manufacturer’s protocol. Statistical significance was defined as *p<0.05 compared to control.

Flow cytometric analysis for apoptosis. A549 or H441 cells were plated in 24-well plates at a density of 1x10⁵ cells per well 1 day before the recombinant adenoviral vector infection. After 96 h, cells were harvested and washed once with PBS. Cells were resuspended in PBS containing 0.2% Triton X-100 and 1 mg/ml RNase for 5 min at room temperature and then stained with propidium iodide at 50 μg/ml to determine sub-G₀/G₁ DNA content using a FACSscan (Becton Dickinson, Becton Drive Franklin Lakes, New Jersey, USA). Doublets, cell debris, and fixation artifacts were gated out, and sub-G₀/G₁ DNA content was determined using Cell Quest Ver. 3.3 software (Becton Dickinson, Franklin Lakes, NJ, USA).

Inhibition and measurement of caspase-3 activity. Caspase-3 activity was determined using ApoAlert caspase colorimetric assay kits (Clontech, Palo Alto, CA, USA). The fold increase in protease activity was determined 96 h after adenoviral infections by comparing the levels of caspase activity in treated cells with that of control cells (PBS). For caspase inhibition, A549 cells were preincubated with 50 mM zVAD-fmk (BioVision, Inc., Mountain View, CA, USA) for 30 min prior to infection and caspase-3 activity was determined 96 h after treatments.

Local injection and intratracheal administration of adenoviral vector. The experimental protocols were approved by the Ethics Review Committee of Okayama University Graduate School of Medicine and Dentistry and Pharmaceutical sciences (Approval Number: 09067). Human lung cancer xenografts were established in 4-week-old female BALB/C nude mice (Charles River Laboratories Japan, Kanagawa, Japan) by subcutaneous inoculation of 4x10⁶ A549 cells into the dorsal flank. The mice were randomly assigned into six groups (n=6/group). After the tumors had reached a diameter of about 0.5 cm (approximately 6 days after tumor inoculations), each group of mice was injected with 100 μl solution containing PBS, 5x10¹⁰ vp of Ad-Luc or Ad-Omomyc into dorsal-flank tumor for 3 days. Animals were then observed closely and survival studies were performed. Tumors were measured 2 to 3 times a week, and tumor volume was calculated as ab²x0.5, where a and b were the large and small diameters, respectively. Body weight of mice treated with Ad-Omomyc, Ad-Luc was measured 15 and 30 days after inoculation of tumor.

Statistical analysis. All data were expressed as mean±standard error of the mean. Comparisons between groups were made using the two-tailed Student’s t-test. An analysis of variance (ANOVA) test, where appropriate, was used to identify statistical significance for multiple comparisons. Statistical significance was defined as p<0.05.

Results

Ad-Omomyc suppresses the growth of A549 but not H441 lung adenocarcinoma cells. Previously, a congenic mouse was created by crossing a ubiquitously expressing dominant negative Myc (Omomyc) mouse with a KRAS mutation-
positive lung cancer model mouse. In the presence of Omomyc, lung tumors caused by the expression of mutated K//RAS regressed in the congenic mouse, indicating that Omomyc caused tumor cell death of K//RAS mutation-positive lung cancer (9). In order to determine whether Omomyc does the same in human K//RAS mutation-positive lung cancer, we constructed a replication-deficient adenovirus vector containing Omomyc (Ad-Omomyc) (Figure 1A) and infected A549 and H441 human lung adenocarcinoma cells carrying the K//RAS mutation. As shown in Figure 1B, HA-tagged Omomyc expression was detected in both A549 and H441 cells infected with Ad-Omomyc. Unexpectedly, Ad-Omomyc significantly reduced cell viability only in A549 cells but not H441 cells (Figure 1C). Efficiency of the adenovirus infection was more than 80% in both A549 and H441 cells as indicated by infection of Ad-GFP (Figure 1D). These results indicate that Omomyc does not suppress the growth of all types of lung cancer cells with K//RAS-mutation.

**Ad-Omomyc induces apoptosis in A549 cells but not H441 cells.** In order to determine the mechanism by which Omomyc reduces cell viability in A549 cells (Figure 1), we measured the subG0/G1 DNA content in A549 and H441 cells after Ad-Omomyc infection by performing propidium iodide (PI) staining and flow cytometric analysis. As shown in Figure 2A, Ad-Omomyc increased apoptosis in A549 cells after 96 h infection (18.49%) while it did not in H441 cells (3.49%). Apoptosis was also confirmed by caspase-3 activation assay. As expected, Ad-Omomyc increased the activity of capase-3 in A549 cells, and the increased caspase-3 activity by Ad-Omomyc was inhibited by the general Caspase inhibitor zVAD-fmk (Figure 2B). These results indicate that Ad-Omomyc increases caspase-3 activity, and in turn induces apoptosis and suppression of cell growth.

**Omomyc requires high levels of MYC expression to induce cell death in the K//RAS mutation-positive lung cancer cells.** Previously, Omomyc was shown to induce apoptosis in C2C12 myoblast cells when it was co-expressed with exogenous Myc (14). In order to identify the mechanism by which Omomyc induces apoptosis only in A549 cell but not H441 cells, we looked at the expression of endogenous Myc in A549 and H441 cells. Interestingly, A549 cells express endogenous Myc at high levels but H441 cells do not (Figure 3A). In order to determine whether Omomyc failed to induce apoptosis in H441 cells because of the lack of sufficient levels of Myc, we constructed an adenovirus vector containing wild type Myc (Ad-Myc) and infected H441 cells with Ad-Omomyc in the presence or absence of Ad-Myc. As shown in Figure 3B, when H441 cells were infected with both Ad-Omomyc and Ad-Myc, significant apoptosis was observed (25.8%), indicating that Ad-Omomyc infection in the presence of Ad-Myc can become cytotoxic also towards H441 cells (Figure 3C). Accordingly, caspase-3 activity was increased in both Ad-Omomyc and Ad-Myc-infected H441 cells, and the increased caspase-3 activity was inhibited by the general caspase inhibitor zVAD-fmk (Figure 3D). These results indicate that Omomyc requires high levels of Myc to induce apoptosis in human K//RAS mutation-positive lung cancer cells.

**Omomyc suppresses tumor growth in lung cancer xenografted mice.** In order to determine whether inhibiting MYC transactivation function using Omomyc is also effective in reducing tumor growth in K//RAS mutation-positive lung cancer in vivo, we tested Ad-Omomyc in an A549 subcutaneous xenograft mouse model. As shown in Figure 4A, intratumorally injected Ad-Omomyc significantly reduced the tumor burden caused by A549 cells in this subcutaneous xenograft model. We did not significant observe body weight loss by the intratumoral injection of Ad-Omomyc (Figure 4B), indicating that inhibiting the Myc function using Ad-Omomyc is not significantly toxic to the mouse overall. These results suggest that inhibiting Myc function may be a promising approach for treating K//RAS mutation-positive lung cancer expressing high levels of Myc.

**Discussion**

K//RAS mutation is detected in 10-30% of lung adenocarcinoma (15). Currently, there is not an effective therapy for K//RAS mutation-positive lung cancer. Thus, the molecular mechanisms of the K//RAS mutation-positive carcinogenesis have been actively investigated to identify molecular pathways that might be targeted by small molecules. Recently, Singh et al. reported that lung cancer cell lines harboring K//RAS mutations can be divided into two groups (4). One group requires K//RAS to maintain cell viability (K//RAS-dependent) while the other group does not (K//RAS-independent). Interestingly, the K//RAS-dependent group expresses epithelial cell markers while the K//RAS-independent group expresses mesenchymal cell markers. One of the K//RAS-dependent cell lines is the H441 lung adenocarcinoma cells and one of the K//RAS-independent cell lines is A549 lung adenocarcinoma cell, both used in our study. In Singh et al.’s study (4), Syk tyrosine kinase was identified as a downstream target for the K//RAS-dependent cells (H441 type). A small molecule targeting Syk effectively suppressed cell growth of the K//RAS-dependent cells (H441 type) but not that of the K//RAS-independent cells (A549 type). Singh et al. further reported that poorly differentiated tumors expressing mesenchymal genes like the ‘A549 type’ lung adenocarcinoma are generally more drug resistant and are also associated with poorer prognosis. In the present study, we determined that K//RAS-independent A549 cells expressed high levels of the oncogenic
Figure 1. Induction of cell death induced by Ad-Omomyc in A549 pulmonary adenocarcinoma cells. A: Schematic representation of Ad-Omomyc. B: Detection of Omomyc induced by Ad-Omomyc. Cells were treated with PBS, Ad-Luc or Ad-Omomyc at an MOI of 5000 vp; 36 hours after infection, expression of Omomyc was analyzed by immunoblot. C: Effect on cell viability induced by Ad-Omomyc infection in vitro. D: Phase-contrast photomicrographs of A549 and H441 pulmonary adenocarcinoma cells infected with Ad-Luc and Ad-Omomyc. Cell morphology was evaluated 96 hours after infection. Photomicrographs were taken at a magnification of x100.
Figure 2. Detection of apoptosis induced by Ad-Omomyc in A549 pulmonary adenocarcinoma cells. A: Flow cytometric analysis of apoptosis induced by Ad-Omomyc. Cells were infected with PBS, Ad-Luc or Ad-Omomyc for 96 hours at an MOI of 5000 vp per cell and sub-G0/G1 DNA content was measured by propidium iodide staining and flow cytometric analysis. B: A549 cells were treated with PBS, Ad-Luc or Ad-Omomyc with or without 50 μM zVAD-fmk for 72 hours before determining caspase-3 activity. Triplicate experiments were performed for each cell treatment; data represent the mean-fold increase±S.E. **p<0.01, compared to the PBS control.
Figure 3. Double infection by Ad-Myc and Ad-Omomyc induced apoptosis in H441 pulmonary adenocarcinoma cells. A: Detection of endogenous Myc expression in A549 and H441 pulmonary adenocarcinoma cells by immunoblot analysis. β-Actin is shown as control. B: Flow cytometric analysis of apoptosis induced by Ad-Myc and Ad-Omomyc. Cells were infected with Ad-Myc and Ad-Omomyc for 96 h at an MOI of 5000 vp per cell and sub-G0/G1 DNA content was measured by propidium iodide staining and flow cytometric analysis. C: Phase-contrast photomicrographs of H441 pulmonary adenocarcinoma cells infected with Ad-Myc and Ad-Omomyc. Cell morphology was evaluated 96 hours after infection. Photomicrographs were taken at a magnification of ×100. D: H441 cells were treated with PBS, Ad-Luc and Ad-Omomyc alone or in combination with or without 50 mM zVAD-fmk for 72 h before determining caspase-3 activity. Triplicate experiments were performed for each treatment; data represent the mean fold increase±S.E. **p<0.01, Compared to the PBS control.
transcription factor Myc while KRAS-dependent H441 cells did not (Figure 3A). Inhibition of the Myc transactivation function by a dominant negative form of Myc (Omomyc) effectively induced cell death in A549 cells (Figures 1 and 2), suggesting that inhibiting Myc transactivation function might be used to suppress tumor growth in ‘KRAS-independent’ lung adenocarcinoma with KRAS mutation that are generally difficult to treat.

There are two kinds of small molecules that might be used to target Myc. One inhibits Myc–Max heterodimerization suppressing Myc-mediated transcription (16, 17). The other inhibit the activity of the Myc promoter, which in turn suppresses mRNA expression of the Myc gene itself (18). Such compounds could both be useful for treating ‘Myc-expressing’ KRAS mutation-positive lung adenocarcinoma that are likely to be ‘KRAS-independent mesenchymal like’ lung cancer. Our data suggest that determining the type of the KRAS mutation-positive lung cancer using Myc expression as a marker may be critical for selecting the treatment to be used.

In summary, we have determined that there are at least two types of KRAS mutation-positive lung cancer and lung cancer with little or no expression of Myc. Inhibition of the Myc transactivation function using Omomyc induces cell death in the Myc-overexpressing KRAS mutation-positive lung cancer cells in vitro and in vivo, indicating that Myc can be targeted to treat this type of lung cancer. Appropriate categorizing of the types of KRAS mutation-positive lung cancer will be a critical step in determining their treatment.

Conflict of interest

None declared.

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References


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