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Effect of cancer/testis antigen NY-SAR-35 on the proliferation, migration and invasion of cancer cells

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Abstract. NY-SAR-35 is a cancer/testis (CT) antigen that was identified by serological analysis of recombinant complementary DNA expression libraries. The gene encoding NY-SAR-35 is located on the X chromosome and is aberrantly expressed in a number of cancer types and germ cells, such as those in the testes, but not in normal tissue. It has been reported that treatment with a demethylating agent induced the expression of NY-SAR-35 in several types of cancer cells. However, the function of NY-SAR-35 in cancer remains undetermined. In present study, the role of NY-SAR-35 in human lung adenocarcinoma (SK-LC-14) and hepatocellular carcinoma (SNU-449) cells was investigated following stable transfection of the NY-SAR-35 gene. NY-SAR-35 was observed to be expressed in the cytoplasm of the cells. In addition, the bromodeoxyuridine incorporation assay and immunofluorescence staining for proliferating cell nuclear antigen and Ki-67 demonstrated that proliferation was increased in cells transfected with NY-SAR-35. In addition, the trypan blue exclusion assay indicated that NY-SAR-35 increased cancer cell viability. Furthermore, NY-SAR-35 increased the migration and invasion of the cells. These results indicate that NY-SAR-35 increases cancer cell viability, proliferation, migration and invasion.

Introduction

Cancer/testis (CT) antigens are a heterogeneous group of >200 proteins with an eponymous expression pattern of being restricted to tumor cells of different histological origins, and to germ cells in the testes and placenta (1-4). CT antigens are classified as those that are encoded on the X chromosome, known as CT-X antigens, and those that are not, which are named non-X CT antigens (5). In total, >50% of all the identified CT antigens to date are CT-X antigens, which are frequently multicopy genes (5). The genes encoding non-X CT antigens, however, are distributed throughout the genome and are typically single-copy genes (5,6). Despite >200 CT antigens have been categorized, the function of the majority of CT antigens in gametogenesis and carcinogenesis remains unclear (2,7,8).

The CT antigen NY-SAR-35, also referred to as CT antigen 37 or fragile X mental retardation 1 neighbor, was identified using the serological analysis of recombinant complementary DNA (cDNA) expression libraries (SEREX) method and is encoded by a gene located on the X chromosome (9). NY-SAR-35 is a 255-amino acid multi-pass membrane protein with a predicted molecular mass of 29.2 kDa and a trefoil (P-type) domain (9). The P-type domain is a three-looped clover leaf-shaped domain of ~38 amino acids in length, in which the loops are held together by highly conserved disulfide bonds (10-12).

NY-SAR-35 has been identified to be expressed in a number of cancer types, including melanoma, sarcoma, and lung, breast and ovarian cancer (9). Despite the aberrant expression of NY-SAR-35 in a range of malignancies, it is not expressed in certain cancer types, including colon and renal cancer. Analysis of the methylation status of the NY-SAR-35 gene indicated that its expression is regulated by methylation of its promoter region (13).

Hepatocellular carcinoma and lung cancer are the most common tumor types worldwide and the leading causes of cancer-associated mortality in Korea (14-17). A number of germline genes are overexpressed during the development of these malignancies (5,6,18-23), although whether NY-SAR-35 serves a role in their tumorigenesis remains undetermined. The present study hypothesized that NY-SAR-35 may function in oncogenesis. The aim of the current study was to use *in vitro* cell models lacking the NY-SAR-35 gene to assess the function of NY-SAR-35 in hepatocellular carcinoma and lung cancer. In particular, the current study aimed to determine whether NY-SAR-35 expression affects cell proliferation, migration and invasion in cancer cells.

Materials and methods

Cell culture. Human hepatocellular carcinoma SNU-449 and lung adenocarcinoma SK-LC-14 cells were obtained from the Korean Cell Line Bank (Cancer Research Institute, Soul National University, Seoul, Korea) and the American Type

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Culture Collection (Manassas, VA, USA), respectively. Cells were maintained in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.,), 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. The cells were cultured at 37˚C in a humidified atmosphere containing 5% CO_2.

Total RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was extracted from the cells using the RNeasy Mini kit (Qiagen, Inc., Valencia, CA, USA) according to the manufacturer's protocol. Subsequently, cDNA was synthesized from 1 μg total RNA using 5 Units Moloney murine leukemia virus reverse transcriptase (M-MLV) in 5X M-MLV buffer (both Promega Corporation, Madison, WI, USA). The reverse transcriptase and reaction buffer were incubated at 37˚C for 50 min, prior to RT. RNA and reverse transcriptase were subsequently incubated with 100 pmol random primer (Takara Biotechnology Co., Ltd., Dalian, China) and 1 μl mixed dNTPs (10 mM; Solgent Co., Ltd., Seoul, Korea) at 65˚C for 5 min, and immediately transferred to ice. The NY-SAR-35 primer sequences used were as follows: Forward, 5'-CTTGGTGCGATCAGCCTTAT-3' and reverse, 5'-TTGATGCATGAAAACAGAAC-3'. The GAPDH primer sequences used were as follows: Forward, 5'-GTTTAC ATGTTCCAATATGATTCCAC-3' and reverse, 5'-TCATAT TTGGCAGGTTTTTCTAGAC-3'. PCR amplification was performed using the 2X TOPsimple™ DyeMIX-Tenuto kit (Enzynomics, Daejeon, Korea) and the following thermocycling conditions: Denaturation for 5 min at 94˚C; 35 cycles of 30 sec at 94˚C, 30 sec at 55˚C, and 1 min at 72˚C; and a 10 min final extension at 72˚C. PCR products were analyzed by 1.2% agarose gel electrophoresis and visualized using ethidium bromide. The complementary DNA templates were normalized using GAPDH.

Construction of stable cell lines. To generate cells stably expressing NY-SAR-35, the open reading frame of the NY-SAR-35 gene was cloned into the pcDNA3.1/V5-HisA mammalian expression vector (Invitrogen; Thermo Fisher Scientific, Inc.), which has a C-terminal fusion tag (V5 and 6-His epitopes) as well as *Eco*RI and *Xho*I restriction sites. Subsequently, cells were seeded into 6-well plates at a density of $2.5x10^5$ cells/well and transfected with 1 μg cloned pcDNA3.1/V5-HisA-NY-SAR-35 using Lipofectamine LTX Reagent (Thermo Fisher Scientific, Inc.). Transfected cells were selected by supplementing their culture medium with 1 mg/ml G418 (Sigma-Aldrich; Merck Millipore, Darmstadt, Germany) and then being maintained in culture medium containing 0.3 mg/ml G418. Untransfected cells were used as the control.

Cell viability assay. A total of $5x10^5$ cells were seeded into 100-mm culture plates and cultured for 4 days in standard culture medium supplemented with either 1 or 10% FBS. To test the effect of growth factor withdrawal on the proliferation of NY-SAR-35 transfectants, the cells were trypsinized and the trypan blue solution was added on day 4, and incubated for 5 min at room temperature. Subsequently, samples were counted using a hemocytometer and the ratio of viable/dead cells was determined.

Bromodeoxyuridine (BrdU) incorporation assay. Cell proliferation was measured through BrdU incorporation using the Cell Proliferation ELISA BrdU kit (Roche Diagnostics GmbH, Manheim, Germany), according to the manufacturer's protocol. A total of 20,000 cells/well were grown in 96-well plates for 2 days at 37˚C and labeled with 10 μM BrdU for 2 h, prior to fixation and DNA denaturation. Subsequently, anti-BrdU peroxidase-conjugated fragment-antigen binding fragments and substrate were added to the medium, and the optical density at 450 nm was determined using an ELISA reader (BioTek Instruments, Inc., Winooski, VT, USA) and a reference wavelength of 690 nm.

Immunofluorescence microscopy. Cells were grown at a density of $2x10^5$ cells/dish in 35 mm coverglass-bottom dishes at 37˚C for 24 h, and were washed with PBS and fixed with 4% paraformaldehyde. Subsequently, cells were blocked with 3% FBS in PBS and incubated with anti-proliferating cell nuclear antigen (PCNA; 1:150; BD Biosciences, Franklin Lakes, NJ, USA; cat. no. 555566) or anti-Ki-67 (1:150; BD Biosciences; cat. no. 556003) primary mouse antibodies at 37˚C for 1 h. Cells were washed three times with PBS and then incubated with fluorescein isothiocyanate-coupled secondary goat antibodies (1:500; BD Biosciences; cat. no. 554001) at 4˚C for 30 min in the dark. Cells were visualized and images were captured using a confocal microscope (Olympus Corporation, Tokyo, Japan).

Cell migration and invasion assays. To measure cell migration, transwell chamber assays were performed using a Corning BioCoat Matrigel Invasion Chamber (BD Biosciences, Franklin Lakes, NJ, USA). The lower surface of the filters was coated with 1% gelatin. Cells were suspended in serum-free RPMI-1640 medium and added to the upper chamber at a density of $5x10^4$ cells/insert. Culture medium containing 10% FBS was added to the lower chamber and the cells were incubated at 37˚C for 18 h. The number of cells that migrated to the lower side of the upper chamber was counted following staining with crystal violet. To measure cell invasion, the BioCoat Matrigel Invasion chambers (BD Biosciences) were used. The process described above was performed, with the exception that the cells were incubated for 24 h. Inserts were then stained with crystal violet and the number of invading cells was counted. Three fields of cells on the lower side of the chambers were counted, and the migration and invasion of cells were expressed in percentage values compared with those of the mock control cells.

Statistical analysis. Values are presented as the mean ± standard deviation of three independent experiments. Differences were analyzed using the Student's t-test. The analysis was performed using the SPSS statistical package (version 14.0; SPSS Inc., Chicago, IL, USA). $P<0.05$ were considered to indicate a statistically significant difference.

Results

Expression and localization of NY-SAR-35 in stably transfected SNU-449 and SK-LC-14 cells. To assess the role of NY-SAR-35 in cancer, NY-SAR-35-positive human hepatocellular carcinoma (SNU449/NY-SAR-35) and lung

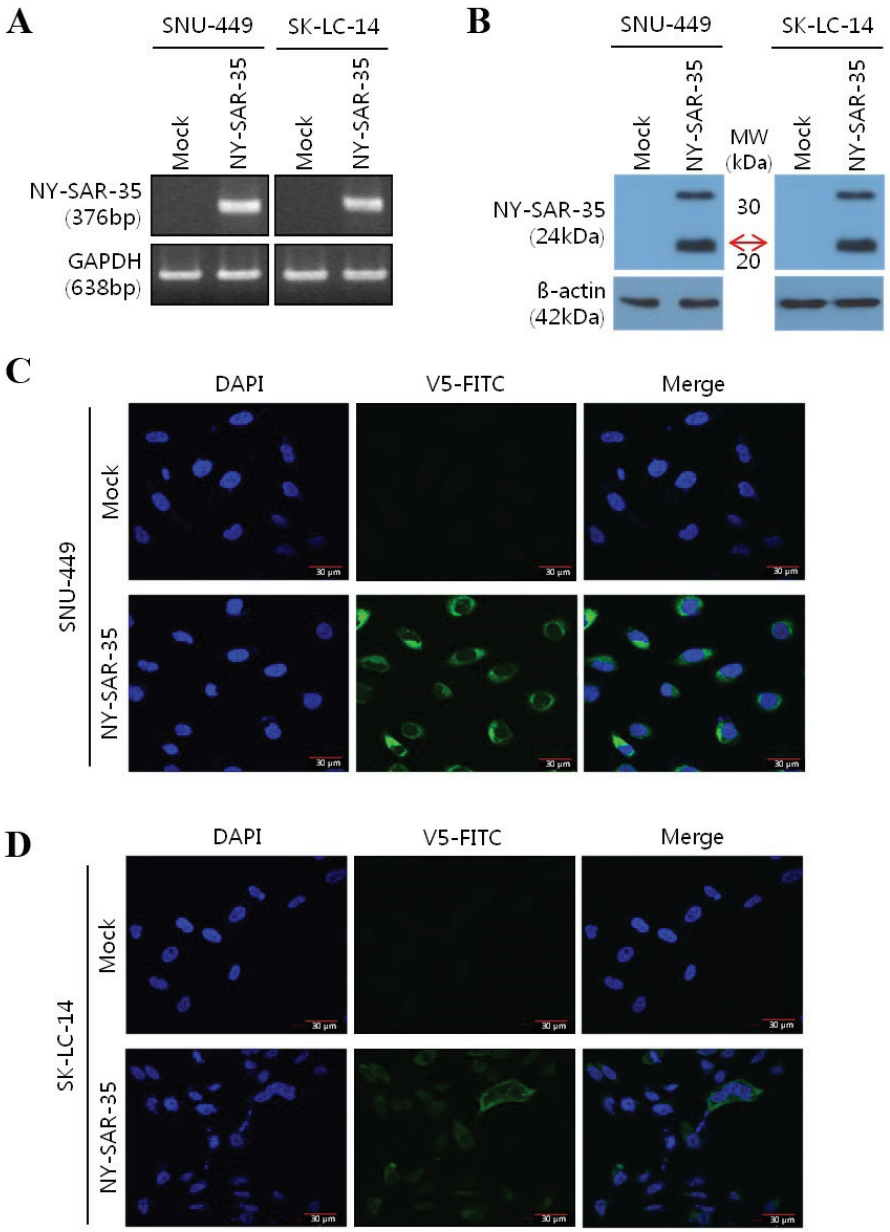

Figure 1. Expression and localization of NY-SAR-35 in stably transfected SNU-449 and SK-LC-14 cells. (A) Reverse transcription-polymerase chain reaction analysis of NY-SAR-35 messenger RNA. (B) Western blot analysis of NY-SAR-35 protein expression. Red arrows indicate the molecular weight of the NY-SAR-35 protein bands (24 kDa). Immunofluorescence of the V5 epitopes antibody (green) and DAPI (blue) in NY-SAR-35 stably transfected (C) SNU-449 and (D) SK-LC14 cells (magnification, x600). FITC, fluorescein isothiocyanate.

adenocarcinoma (SK-LC-14/NY-SAR-35) cell lines, which do not naturally express NY-SAR-35, were established through stable transfection. Expression of NY-SAR-35 messenger RNA and protein in the cells was confirmed using RT-PCR (Fig. 1A) and western blot analysis (Fig. 1B), respectively. The subcellular localization of the NY-SAR-35 was analyzed using immunofluorescence miscopy. This revealed that NY-SAR-35 was located in the cytoplasm of the cells (Fig. 1C and D).

NY-SAR-35 increases SK-LC-14 and SNU-449 cell viability and proliferation. Expression of NY-SAR-35 in SNU-449 cells cultured in medium containing 1 or 10% FBS significantly increased cell viability by a mean of 1.3- (P=0.049) and 1.9-fold (P=0.036), respectively, compared with that of mock cells (Fig. 2A). However, no significant difference in viability was noticed between SK-LC-14/NY-SAR-35 and mock cells (Fig. 2B). In addition, BrdU incorporation assays determined that cell proliferation was significantly increased in SNU449/NY-SAR-35 (P=0.002) and SK-LC-14-NY-SAR-35 cells (P=0.021) compared with that of mock cells (Fig. 2C and D). Furthermore, the effect of NY-SAR-35 expression on SNU449 and SK-LC-14 cell proliferation was analyzed by immunofluorescence staining for PCNA (Fig. 3A) and Ki-67 (Fig. 3B). SNU449/NY-SAR-35 and SK-LC-14/NY-SAR-35 cells had increased PCNA and markedly increased Ki-61 staining compared with those in the mock cells, indicating that NY-SAR-35 stimulates cancer cell proliferation.

NY-SAR-35 increases SK-LC-14 and SNU-449 cell migration and invasion. The migratory and invasive capacities of

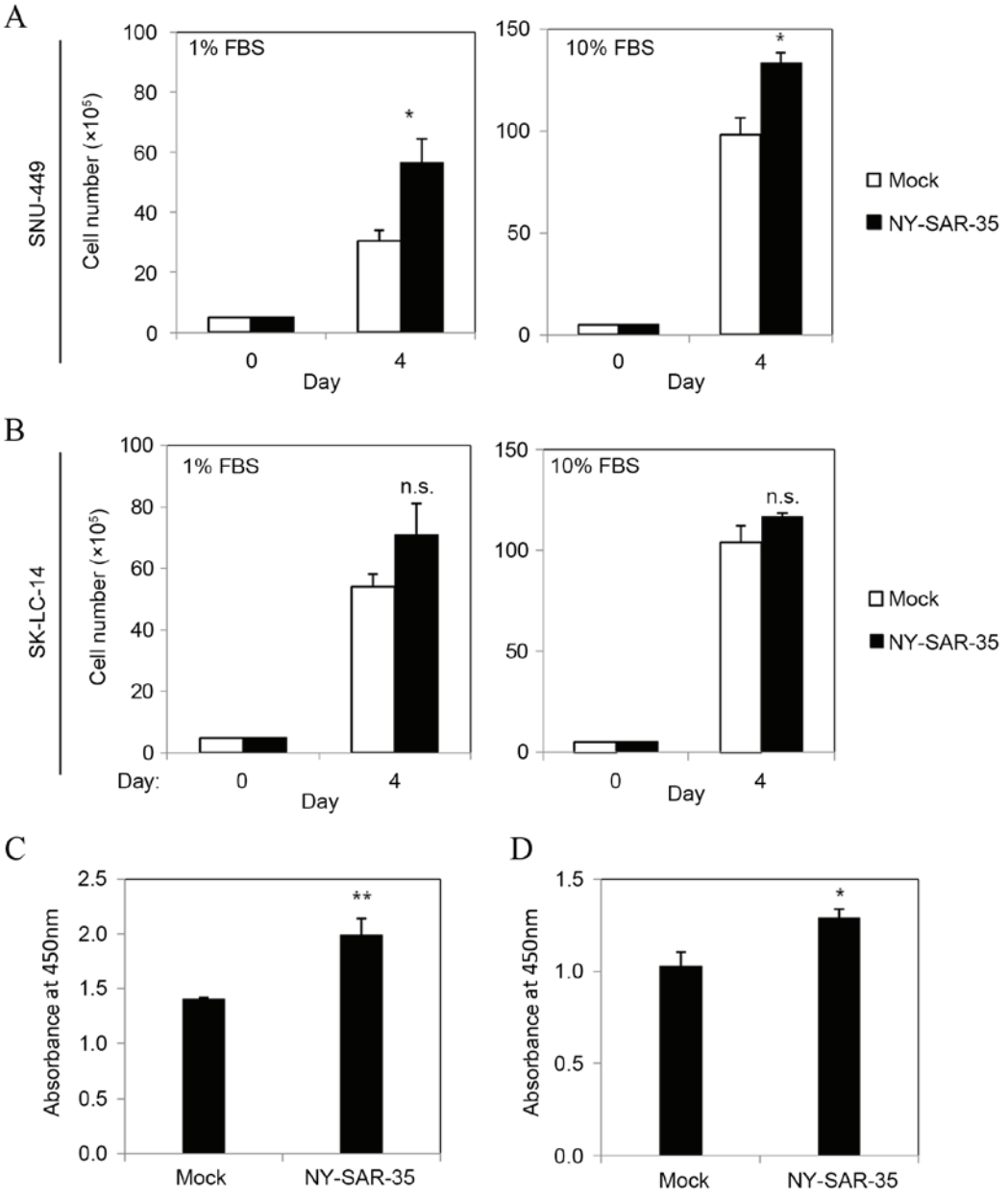

Figure 2. NY-SAR-35 increases the viability and proliferation of SNU449/NY-SAR-35 and SK-LC-14/NY-SAR-35 cells. Trypan blue exclusion assay of the viability of (A) SNU449/NY-SAR-35 and (B) SKLC14/NY-SAR-35 cells cultured in medium containing 1 or 10% FBS. Results are presented as the mean ± standard deviation of three independent experiments. BrdU incorporation assay was used to determine the proliferation of (C) SNU449/NY-SAR-35 and (D) SKLC14/NY-SAR-35 cells. *P<0.05, **P<0.01 vs. mock cells. FBS, fetal bovine serum; n.s., no significant difference.

NY-SAR-35-transfected SK-LC-14 and SNU-449 cells were examined using transwell assays. This demonstrated that the migration of SK-LC-14/NY-SAR-35 and SNU449/NY-SAR-35 cells was significantly increased by 2.2- (P=0.00000137) and 6.2-fold (P=0.0000976), respectively, compared with that of mock cells (Fig. 4). In addition, cell invasion was increased by 2.5- (P=0.0044) and 1.9-fold (P=0.0024) in each cell line, compared with that in mock cells (Fig. 4). These results indicate that NY-SAR-35 increases the migration and invasion of cancer cells.

Discussion

During carcinogenesis, numerous genes that are typically expressed during the embryonic developmental stage are re-expressed in cancer cells, including a number of proto-oncogenes and CT antigens (2,8,24-27). CT antigens are aberrantly expressed in variable proportions of a wide range of different types of tumor; however, not in normal tissues, excluding germ cells. As these cells do not express major histocompatibility class I complexes, cluster of differentiation (CD)8+ T cells are not able to recognize CT antigens expressed on these cells, suggesting that CT antigens expressed in tumors are targets for vaccine-based immunotherapy. SEREX-derived CT antigens have been demonstrated to induce CD8+ cytotoxic T lymphocytes (CTLs), and a positive association was observed between serum positivity for immunoglobulin G antibody and induction of CD8+ CTLs against several CT antigens, specifically NY-ESO-1 (2,25). Due to these features

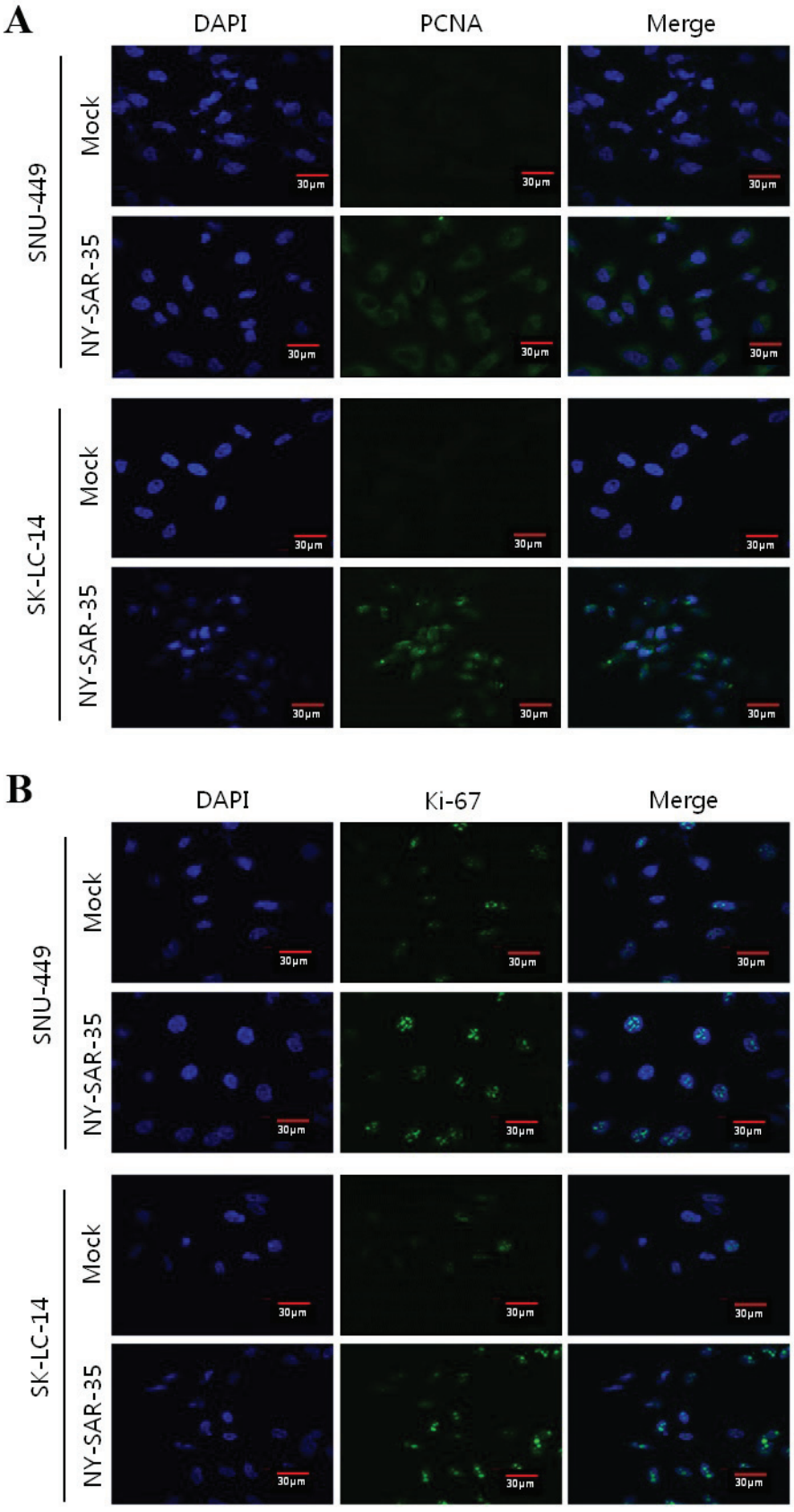

Figure 3. Expression of PCNA and Ki-67 in SNU449/NY-SAR-35 and SK-LC-14/NY-SAR-35 cells. Immunofluorescence microscopy of (A) PCNA and (B) Ki-67 (both green). DAPI (blue) staining was used to visualize the cell nuclei. Magnification, x600. PCNA, proliferating cell nuclear antigen.

of CT antigens, numerous studies have analyzed their potential use in cancer immunotherapy (1,3). NY-SAR-35 was identified as encoding a CT antigen by SEREX (9), and another study suggested that the NY-SAR-35 gene is

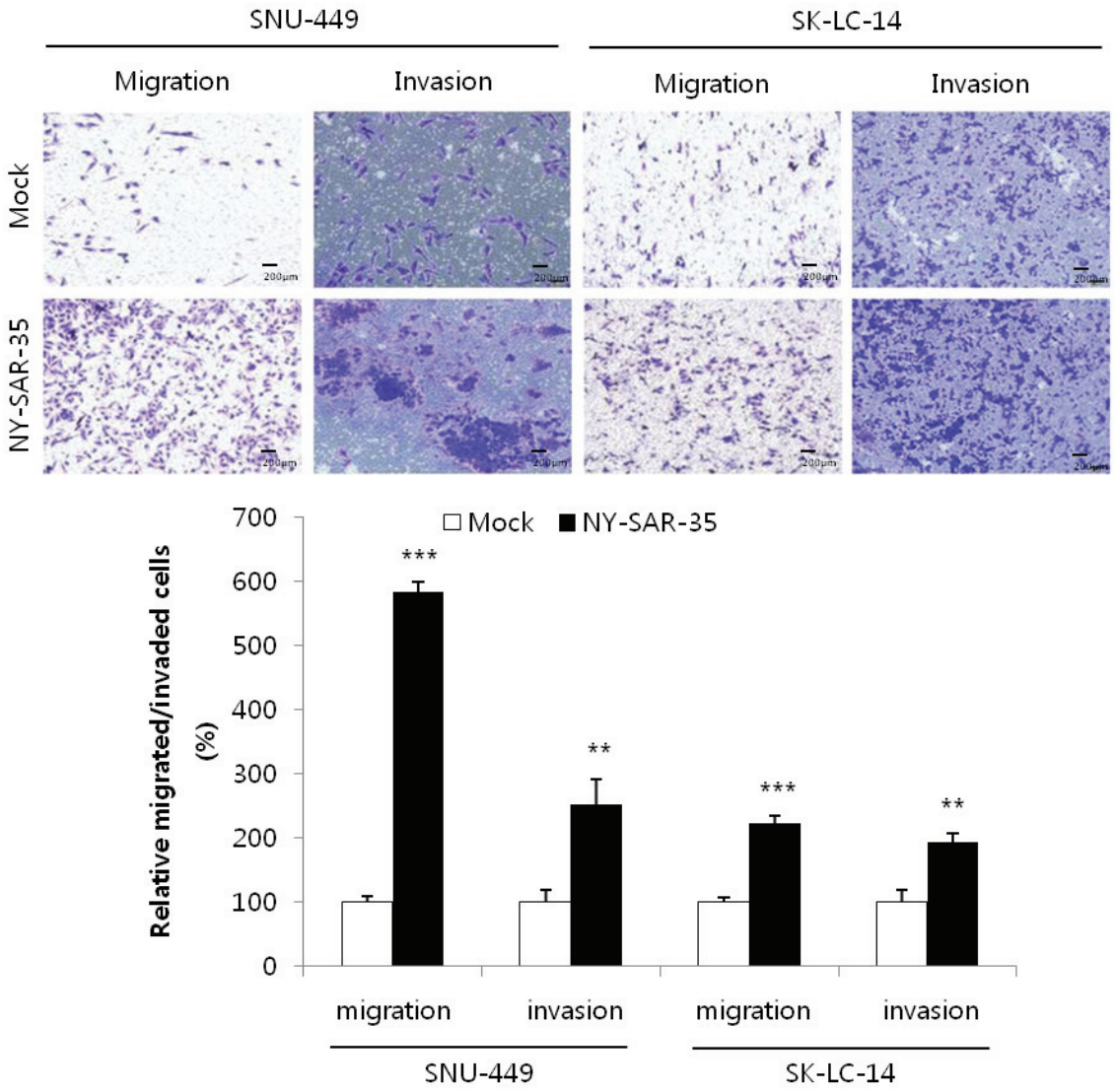

Figure 4. NY-SAR-35 increases the migration and invasion of SNU449/NY-SAR-35 and SK-LC-14/NY-SAR-35 cells. Representative images and quantification of transwell migration and invasion assays following crystal violet staining. Magnification, x100. **P<0.01, ***P<0.001 vs. mock cells.

subject to epigenetic regulation (13). However, the role of NY-SAR-35 in carcinogenesis remains unclear. The results of the present study demonstrated that NY-SAR-35 expression promoted the viability, proliferation, migration and invasion of hepatocellular carcinoma and lung adenocarcinoma cells, suggesting that NY-SAR-35 promotes carcinogenesis.

Immunofluorescence microscopy in the current study identified increased PCNA and markedly increased Ki-67 levels in SK-LC-14/NY-SAR-35 and SNU-449/NY-SAR-35 cells. PCNA is increased in late G_1 and S phases of the cell cycle, and is correlated with the rate of DNA synthesis and cellular proliferation (28). In addition, Ki-67 is associated with cell proliferation and is detected throughout the cell cycle (29,30). The differences observed between the extent of increase of PCNA and Ki-67 levels suggest that these antigens may be differentially affected by NY-SAR-35 expression, which may be due to the fact that they are expressed differently in different cell types (31). Furthermore, NY-SAR-35 expression significantly increased the migration and invasion of hepatocellular and lung carcinoma cells in the present study. This further suggests that NY-SAR-35 promotes cancer progression.

In conclusion, the results of the present study demonstrate that NY-SAR-35 increases cancer cell viability, proliferation, migration and invasion. These results are similar to those observed in human embryonic kidney 293 cells (32). However, the mechanisms underlying the effect of NY-SAR-35 in cancer have not been determined. The results of the current study indicate that this area warrants further study. In addition, further investigation of the effects and mechanisms of NY-SAR-35 may elucidate the functions of other CT antigens, and may provide novel approaches for cancer diagnosis and therapy.

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