The role of Mortalin on the malignant property of human cancer and molecular mechanism: therapeutic potential of oncolytic adenovirus expressing shRNA against Mortalin

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The role of Mortalin on the malignant property of human cancer and molecular mechanism: therapeutic potential of oncolytic adenovirus expressing shRNA against Mortalin

Directed by Professor Man-Wook Hur

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This certifies that the Doctoral Dissertation of Jihoon Ryu is approved.

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Most of all, I thank all my family for their role and support. I thank my parents for teaching me a value of education, encouraging me to have a higher education and supporting me during my study.

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ABSTRACT

Molecular mechanism of the role of Mortalin in human cancers: validation of its therapeutic potential by oncolytic adenovirus

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(Directed by Professor **Man-Wook Hur**)

Mortalin, a member of Hsp70 family proteins, is an essential chaperone present in various subcellular locations including plasma membrane, ER, cytosol and mitochondria. Functional significance of Mortalin F (full length, a.a., 679) in multiple subcellular locations remains unknown. In this thesis, I prepared stable cells expressing the Mortalin mutant lacking the signal peptide (Mortalin B, a.a., 637) and localizes mostly to the cytosplasm. In cancer cells, ectopic Mortalin B sequesters p53 in cytoplasm revealed strong cytoplasmic sequestration of p53. The cancer cells with Mortalin B overexpressed show

high telomerase activity and acquire malignant tumor phenotypes. In xenograft assays, the MCF7 cells overexpressing Mortalin B are highly tumorigenic and form aggressive malignant tumors in nude mice. When the MCF7-Mortalin B cells were injected via tail vein, they showed high degree of lung metastasis.

I prepared the oncolytic recombinant adenoviruses expressing shRNA Mortalin (Ad-ΔB7-shMot) to test anti-cancer therapeutic potential both *in vitro* and *in vivo.* The Ad-ΔB7-shMot virus shows selective cytotoxicity for cancer cells *in vitro.* While Mortalin F overexpression enhanced malignant properties of the MCF cancer cells in xenograft assays, Ad-ΔB7-shMot virus showed effective anti-tumor effect. The Ad-ΔB7-shMot virus caused apoptosis and suppressed micro-vessel formation. This study suggests that the Mortalin located outside of mitochondria may be related to malignant property of cancer cells. Because Mortalin F expression is upregulated in various tumors, recombinant adenovirus targeting Mortalin, Ad-ΔB7-shMot, might be useful tool for anti-cancer therapy.

Key words: Mortalin, mitochondria, p53, Malignancy, shRNA, oncolytic adenovirus, apoptosis, anti-cancer therapy

Molecular mechanism of the role of Mortalin in human cancers: validation of its therapeutic potential by oncolytic adenovirus

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I. INTRODUCTION

Mortalin/mthsp70/Grp75 is a member of heat shock protein 70 (Hsp70) family of protein.[1, 2] Mortalin is composed of 679 amino acids (75 kDa) and that has a high homology with other members of hsp70 family. It was first isolated as one of the proteins differentially expressed in mortal and immortal cells. Later, it was also detected in the cytoplasmic fraction of mouse embryonic fibroblast (MEF) and its mortal hybrids generated by fusion with immortal mouse fibroblasts. Because of its association with the cellular mortality, it was named 'Mortalin'. Consistent with its naming, overexpression of Mot-1 cDNA (Mortalin-1 cloned from normal mouse

fibroblasts) in NIH3T3 cells induced cellular senescence.[1] Biochemical and immunocytochemical experiments with anti-Mortalin-1 antibody revealed that the antibody is highly specific and does not cross-react to any other Hsp70 family proteins. Surprisingly, Mortalin was also found in mouse immortal cells. However, the cellular localization of Mortalin expression is different: pancytoplasmic in normal and perinuclear in immortal and cancer cells.[1, 3]

Mot-2, an isoform of Mortalin, was isolated from mouse immortalized cells, which shows perinuclear expression pattern. It differs from Mot-1 at two amino acid residues (V618M and R624G). Overexpression of Mot-2 in NIH3T3 cells caused a malignant cellular transformation. The two mouse Mortalin proteins have different functional characteristics described above. [4, 5]

Human cDNA encoding Mortalin was cloned and human Mortalin has characteristics similar to mouse Mortalin-2 and therefore was named hMot-2.[3] Only one kind of human Mortalin cDNA sequence is present. Subcellular localization of Mortalin expression in normal and immortal/transformed cells is different and may be determined by other subcellular factors interacting with Mortalin. Human Mortalin, like mouse Mot-2, was shown to increase cell proliferation, stimulate transformation of NIH3T3, extends life span of normal human fibroblasts and attenuates differentiation of HL-60 cells. [4, 6, 7]

As a mitochondrial protein, Mortalin has been shown to play roles in the mitochondrial import, oxidative stress response, regulation of mitochondrial

membrane potential, and apoptosis.[2, 8-13] Mortalin has been shown to play active role in intracellular transport, chaperonization, p53 functions, immune response and tumorigenesis.[4, 14-18] Loss of Mortalin function and distruption of mitochondrial homeostasis has been closely linked to age-pathologies including Alzheimer's and Parkinson's.[19-22] It was shown previously that Mortalin and p53 co-localize and they interact with each other in transformed cells, but not in normal cells, resulting in the exclusion from nucleus and inhibition of transcriptional activation of p53.[10, 23-25] Malignant transformation of NIH3T3, extension of life span of MRC-5 and attenuation of differentiation of HL-60 cells may be explained by inhibition of transcriptional activation of p53 target genes by overexpression of Mortalin (Mot-2). [4, 6, 7]

The expression profile analysis of Mortalin in normal and a variety of immortal and tumorigenic cell lines revealed bi-phasic pattern: an initial elevation (relative to a down-regulation during replicative senescence of human fibroblasts) and an up-regulation at a later stage of immortalization, which coincides with the acquisition of an invasive phenotype of immortalized cells.[26] Mortalin was identified as a prognostic marker for colorectal cancers by the proteomic analysis of cancer tissue arrays.[27] Global profiling of the cell surface proteome of cancer cells, including neuroblastoma, lung adenocarcinoma, colon adenocarcinoma, acute lymphoblastic leukemia and ovarian tumor cells, revealed its remarkable

abundance along with several other chaperone proteins, including Grp78, Hsp70, Hsp60 and Hsp27.[28] Proteomic analysis of chronic myeloid leukemia (CML) cells with a hyper-activated Bcr-Abl protein tyrosine kinase showed that Mortalin is one of the four major proteins that were highly expressed. Interestingly, there was no direct correlation between the protein expression and mRNA levels, suggesting increase in post-translational stability of Mortalin may be associated with the malignant phenotype.[29]

Anti-Mortalin molecules such as antisense RNA, ribozyme, siRNA, p53 binding-antagonist polypeptides, the chemicals that abrogated Mortalin-p53 interaction, and the relocation of p53 to cell nucleus resulted in growth arrest/apoptosis of cancer cells.[10, 13, 18, 30-34] The early recurrence of hepatocarcinoma in post-operative patients and liver cancer metastasis may be related with high Mortalin expression. These observations suggest that anti-Mortalin molecules may be useful not only as anti-cancer agents, but also as a therapeutic agents preventing cancer recurrence. [35]

Adenoviruses are the DNA viruses that cause a spectrum of respiratory diseases. Genetic modifications of E1A and E1B viral genes are often employed to achieve attenuation of viral proliferation in host cells. E1A and E1B bind and inactivate pRB and p53. Since cancer cells often show inactivation of pRB and/or p53 pathways and uncontrolled cell proliferation, adenovirus can be amplified in cancer cells.[36] Due to the ability of adenovirus

to replicate, to lysis the infected cell and to infect surrounding cells, adenovirus can be used for cancer therapy. For tumor cell specific replicating adenovirus can be prepared by viral coat protein modifications or placing the viral genes under the control of a cancer cell specific promoter.[37-39] Several oncolytic adenoviruses have been prepared by incorporating cytotoxic transgenes for enhancement of oncolytic capability. Others had previously generated recombinant adenovirus (YKL-1) with the gene encoding E1B (55 kDa form) deleted for tumor-specific replication, but oncolytic effects of YKL-1 were decreased compare to wild-type adenovirus.[40] To overcome this limitation, E1A- and E1B-double mutated oncolytic adenovirus (Ad-ΔB7) in which Rb binding site of E1A was mutated and the genes encoding both the E1B and E1B were deleted was generated. Ad-ΔB7 elicited markedly enhanced cancer cell-specific killing effect and high replication capability in a cancer cell-specific manner as compared to YKL-1. [41]

Mortalin can be a target of anticancer gene therapy, because Mortalin expression is related with tumor aggressiveness by cytoplasmic trapping of p53 and activation of telomerase. Using the recombinant oncolytic adenoviruses Ad-ΔB7 expressing shRNA Mortalin, I found that the recombinant Ad-ΔB7 expressing shRNA Mortalin induces potent apoptosis and inhibits angiogenesis. The recombinant adenovirus might be useful in anticancer gene therapy.

II. MATERIALS AND METHODS

1. Cells culture

Normal human fibroblasts (TIG-1 and WI-38), osteosarcoma (U2OS), breast carcinoma (MCF7) and fibrosarcoma (HT1080) were cultured in Dulbecco's modified Eagle's minimal essential medium (DMEM)-supplemented with 10% fetal bovine serum (FBS), penicillin, streptomycin and fungizone (Life Technologies, Inc.) at 37^{o}C in an atmosphere of 5% CO_2 and 95% air in a humidified incubator. Full-length Mortalin and its three deletion mutants (motF- full-length Mortalin, motA- N-terminal amino acid residues 1-180 and motB- amino acid residues 42-679)-overexpressing cells were generated by retrovirus driven expression. The cDNAs encoding mutants of Mortalin protein were cloned into the *Hin*dIII site of the vector pCX4neo (pCX4neo, provided by Dr. Akagi, Osaka, Japan). All constructs were designed to have myc tag at the carboxy-terminus. For production of retroviruses, Plat-E, an ecotropic murine leukemia virus (MuLV)-packaging cell line was transfected with the pVPack-GP (expression gal and pol) and pVPack VSVG (expressing env) vectors (Stratagene, CA) along with either pCX4neo/motF, pCX4neo/motA, or, pCX4neo/motB using FuGENE6 (Roche, Boehringer Mannheim, IN). After 48 h, culture supernatants were collected and filtered through 0.45 μm filters. The filtered supernatants (viral stock) were used for cell infection without freeze thawing. Cells (2 x 10^5 / well in 6-

well dishes) were treated with 8 μg/ml polybrene for 1 h at 37°C, following which they were incubated with 200-300 μl of filtered viral stock for 1 h followed by addition of 2 ml of DMEM and continued incubation at 37°C for further 48 h. The infected cells were selected in medium containing G418 (100-200 g/ml for normal and 500 g/ml for cancer cells) until stable expressing cell lines were obtained. The stably infected cells lines were examined for expression of full-length Mortalin and its three deletion mutants by Western blotting and immunostaining with myc antibody and were maintained in 100 g/ml G418-supplemented medium. For transfections of Mortalin-shRNA, plasmids 3 μg of plasmid DNA was used per 60% confluent cells in 6 cm dishes. Cells were selected in puromycin (2 g/ml) supplemented medium for 48 h. For oncolytic adenovirus infections, cells were plated in 48-well dishes and were infected with viruses at multiplicity of infection (MOI) ranging from 10-1000. The effect of viruses on cell growth was examined by crystal violet (0.5% in 50% methanol) staining and WST-based cell proliferation assay (Roche, Boehringer Mannheim, IN).

2. Immunocytochemistry.

Cells or transfected cells with Mortalin-shRNA were plated on glass coverslips. Briefly, cells were washed with cold phosphate-buffered saline (PBS) and fixed with pre-chilled methanol:acetone (1:1) for 5 mins on ice. Fixed cells were washed with PBS, permeabilized with 0.2% Triton X-100 in

PBS and incubated in blocking buffer (2% BSA in PBS) containing the appropriate primary antibodies at 4^{o}C overnight. Cells were washed extensively in PBS with 0.2% Triton X-100 before incubation with the fluorochrome - conjugated secondary antibodies (Alexa-488-conjugated goat anti-rabbit and anti-mouse or Alexa-594-conjugated goat anti-rabbit and anti-mouse (Molecular Probes, OR)) for another 30 mins. After six washes in PBS with 0.2% Triton X-100, cells were overlaid with a coverslip with Fluoromount (Difco, MI). The cells were examined on a Carl Zeiss microscope; the images were analyzed by Metamorph software.

3. Western blotting and immunoprecipitation.

Cell lysates were prepared in Nonidet P-40 lysis buffer, separated on a SDS-polyacrylamide gel was electroblotted onto a nylon membrane (Millipore, Bedford, MA) using a semidry transfer blotter (Biometra, Tokyo). Membranes were blocked in 0.2% Tween 20 in TBS containing 3% BSA. After blocking, membranes were incubated with antibodies against Mortalin, myc (Santa Cruz, CA), α-tubulin (Sigma, MO), hTERT (Santa Cruz, CA), β-actin (Chemicon International, CA). After probing with the first antibodies as indicated, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody (Santa Cruz, CA). Finally, the blots were developed using chemiluminescence (GE Healthcare, UK) and visualized using Lumino Image Analyzer equipped with CCD camera (LAS3000-mini,

Fuji Film). Mitochondrial fractions were prepared using the Qproteome mitochondria isolation kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Washed cells were suspended with lysis buffer for disrupting the plasma membrane. After incubation for 10 min, lysate were centrifuged at 1000 ×g for 10 min at 4^{o}C and then the supernatant was harvested as cytosolic fraction. Pellet containing nuclei, cell debris and unbroken cells was resuspended with ice-cold Disruption Buffer. Resuspended pellet was centrifuged at 1000 ×g for 10 min at 4^{o}C and then the supernatant was harvested as microsomal fraction. The resulting pellet was centrifuged at 8000 ×g for 10 min at 4^{o}C for mitochondrial fraction by density gradient purification. After centrifugation, the band towards the bottom of the tube was harvested as mitochondria fraction. For co-immunoprecipitation, cell lysates (300 µg protein) in 300 µl Nonidet P-40 lysis buffer were incubated at 4^{o}C for 1-2 h with anti-myc or anti-hTERT. Immunocomplexes were separated by incubation with Protein-A/G Sepharose, and Western blotting was performed with the indicated antibodies using procedure described above.

4. Cell transformation assays

Cell transformation assay was performed by using Cell Transformation Detection Kit (CHEMICON, CA) according to the manufacturer's instructions. Base agar solution (0.8% agar in culture media) and Top agar solution (0.4% agar in culture media) were prepared and pre-incubated at 37^{o}C. Plates (24

well) were coated with Base agar solution and warmed-up for 5 min in a 37^{o}C incubator. Equal number of MCF7, MCF7-motF and MCF7-motB cells (1250 cells per well of 24 well plate) were mixed with Top agar solution. Mixture of cells and Top agar solution was placed on the top of base agar layer. Cells were fed with cell culture media for 2 times per week and incubated at 37^{o}C with 5% CO_2. On day 28, formed colonies were stained with Cell Stain Solution. Colony forming activity is expressed as the mean number of colonies in 5 fields from different three independent experiments.

5. Chemotaxis Assay.

Chemotaxis assays were performed on MCF7, MCF7-motF and MCF7-motB cells. Cells at 60-70% confluency were washed with cold PBS, trypsinized and resuspended in DMEM supplemented with 0.5% BSA (Sigma, MO) at 2×10^5 cells/ml. 2×10^4 cells were plated in 12 mm-pored Transwell inserts (Costar, MA) and the invasion assay was performed following the manufacturer's instructions. Fibronectin from human plasma (Sigma, MO) was used as chemo-attractant. Cells that moved through the transwell were fixed with 4% formaldehyde in PBS and stained with crystal violet (0.2% in PBS).

6. Telomere repeat amplification protocol (TRAP) assay

TRAP assay was performed using the TeloTAGGG Telomerase PCR

ELISA kit (Roche, Boehringer Mannheim, IN) according to the manufacturer's instructions. Cell lysates were prepared in lysis reagent from 2×10^5 cultured cells. After centrifugation at 16,000 × g for 20 min at 4℃, the supernatant was collected. Cell lysates were mixed with reaction mixture containing biotin-labeled P1-TS primer, P2 primer, telomerase substrate and Taq polymerase. The mixture was incubated at 25°C for 30 min in a final volume of 50 μl, and was then subjected to PCR for 30 cycles (94°C for 30s, 50°C for 30s and 72°C for 90s). The PCR products were mixed with denaturation buffer and incubated at 25°C for 10min. Denatured mixtures were mixed with hybridization buffer including DIG-labeled detection probe complementary to telomeric repeat sequence and then the mixture was moved to streptavidin-coated micro-plate. After incubation at 37°C for 2 h, amplification product detected with an anti-digoxigenin antibody conjugated with peroxidase at wavelength of 450 nm. Telomerase activity in the sample was calculated as the ratio of the sample to the absorbance value of the positive control supplied with the kit, and represented as relative telomerase activity (RTA). All assays were performed in triplicate.

7. Colony forming assay.

Cells were transfected with Mortalin shRNA plasmid as described above. Equal number of control and puromycin-selected transfected cells (1000 cells per 10 cm dish) was plated in triplicates. Cells were maintained until the

appearance of colonies with regular change of medium. Colonies were fixed in methanol, stained with 10% GIEMSA solution, photographed and counted.

8. Generation of Mortalin-targeting oncolytic adenoviruses.

For construction of siRNA expression plasmid vectors, tetracycline inducible U6 promoter vector was used.[4, 5] Target sites for shRNA (#596-GCGTCTCATTGGCCGGCGATA; #696-GATGCTGGCCAGATATCTGGA; #906-GACTTTGACCAGGCCTTGCTA; #007-GCAACAAGCTGAAAGAAGA; #008-GCCAGAAGGACAACATATG; #009-GAATGAGGCTAGACCTTTA) were selected using an algorithm (http://www.igene-therapeutics.co.jp). Replication-competent oncolytic adenoviruses were prepared as described below[42]. Six mot- oncolytic adenoviruses (Ad-ΔB7-shMot3, Ad-ΔB7-shMot4, Ad-ΔB7-shMot5, Ad-ΔB7-shMot7, Ad-ΔB7-shMot8 and Ad-ΔB7-shMot9) with the siRNA target sequences were generated that incorporated the above target sequences, #596, #696, #906, #007, #008 and #009 respectively. Typically, to generate Mortalin targeting shRNA, the DNA fragments for the expression of shRNA targeting human Mortalin were generated by annealing the sense oligonucleotide 5′-gatccc GCAACAAGCTGAAAGAAGA ttcaagagaTCTTCTTTCAGCTTGTTGCttttttggaaa-3′ and its cognate antisense oligonucleotide 5′- agcttttccaaaaaaGCAACAAGCTGAAAGAAGA ttcaagagaTCTTCTTTCAGCTTGTTGCgg-3′ for shRNAMot7 or the sense

oligonucleotide 5′-gatccc GCCAGAAGGACAACATATG ttcaagagaCATATGTTGTCCTTCTGGCttttttggaaa-3′ and its cognate antisense oligonucleotide 5′- agcttttccaaaaaaGCCAGAAGGACAACATATG ttcaagagaCATATGTTGTCCTTCTGGCgg-3′ for shRNAMot8. The 19-nucleotide Mortalin target sequences are indicated in uppercase letters, whereas the 9-nucleotide hairpin and the sequences necessary for the directional cloning are depicted in lowercase letters. After annealing, the fragments were inserted into the *Bam*HI and *Hind*III site of a pSP72-E3-U6 adenovirus shuttle vector resulting in pSP72-E3-shMot7 and pSP72-E3-shMot8.[43] To generate oncolytic adenoviruses expressing Mortalin specific shRNA, the pSP72ΔE3-shMot7 and pSP72ΔE3-shMot8 shuttle vector were linearized with *Xmn*I, and were co-transformed with *Spe*I-treated E1A- and E1B-double mutant replicating adenovirus total vector, pdl-ΔB7, into *Escherichia coli* BJ5183 for homologous recombination, generating pΔB7-shMot7 and pΔB7-shMot8 adenoviral vectors, respectively.[7] To verify the respective homologous recombinants, the plasmid DNAs purified from overnight *E. coli* culture were digested with *Hind*III, and the digestion pattern was analyzed. The proper homologous recombinant Ad plasmid DNAs were then digested with *Pac*I and transfected into 293A cells to generate Ad-ΔB7-shMot7 and Ad-ΔB7-shMot8 adenoviruses. E1-deleted replication-incompetent Ad (Ad-ΔE1) and E1A- and E1B-double mutant replicating

adenovirus (Ad-ΔB7) were also prepared. All the viruses were propagated in 293A cells, purified by CsCl density purification, dissolved in storage buffer (10 mM Tris-HCl (pH 8.0, 4% sucrose, 2 mM $MgCl_2$), and stored at -80℃. Viral particle (v. p.) numbers were calculated from measurements of optical density at 260 nm (OD_{260}), where 1 absorbency unit is equivalent to 10^{12} viral particles per milliliter, and infectious titers (plaque forming unit (PFU) per milliliter) were determined by limiting dilution assay on 293 cells; the multiplicity of infection (MOI) was calculated from infectious titers.

9. Cytotoxic effect assay.

MCF7-motF and MCF7 cells (8×10^3) were plated onto 96-well plates at about 40-60% confluence, and then infected with Ad-ΔE1-E3, Ad-ΔB7, Ad-ΔB7/shMot3, Ad-ΔB7/shMot4, Ad-ΔB7/shMot7, Ad-ΔB7/shMot8 or Ad-ΔB7/shMot9 at MOIs of 50–1000. Cell killing effect was monitored daily by microscopy. After 6 days of incubation, plates were gently washed for removing dead cells and living cells on the plates were stained with 0.5% crystal violet in 50% methanol.

10. Tumor xenograft assay.

Balb/c nude mice (4 weeks old, female) were bought from Nihon Clea (Japan). MCF7, MCF7-motF and MCF7-motB cells (1×10^6 suspended in 0.3 ml of growth medium) were injected subcutaneously into the flank of

nude mice. Tumor formation was monitored for the next month. The assay was regarded as positive if tumors appeared and grew progressively. For experimental metastasis assay, MCF7, MCF7-motF and MCF7-motB cells were injected intravenously into the lateral tail vein of Balb/c nude mice. Two weeks after injection, lungs were harvested from the mice. The metastatic tumor nodules on the surface of lungs were observed and counted.

11. Antitumor efficacy in mice xenograft model.

To assess the antitumor effect of the Mortalin-specific shRNA-expressing oncolytic adenoviruses, MCF7/mot xenografts were established subcutaneously by injecting 1×10^7 cells into the abdomen of 6- to 8-week-old female nude mice (Charles River Japan Inc., Japan). Once the tumors reached to 90~120 mm^3 in volume, mice were randomized into four groups and the viruses (Ad-ΔB7, Ad-ΔB7-shMot7, or Ad-ΔB7-shMot8) at 1×10^8 plaque-forming units (PFU) in 30 ul of PBS were injected directly into the tumors five times, every other day. PBS only-injected group was used as a control group. Tumor growth was measured three times weekly by a caliper until the end of the study by measuring the length and width of the tumor. Tumor volume was estimated on the basis of the following formula: volume = $0.523Lw^2$. All studies were conducted in the Yonsei University College of Medicine, an Association for Assessment and Accreditation of Laboratory Animal Care-associated animal facility, according to institutional regulations.

12. Evaluation of tumor xenograft by immunohistochemistry.

Paraffin block slides were used to examine down-regulation of Mortalin expression in tumors. Tumor tissue was fixed in IHC zinc fixative (formalin-free) (BD Biosciences PharMingen, CA), embedded in paraffin, and cut into 3-μm sections (Wax-it, Canada). Representative sections were stained with H&E, and examined by light microscopy. To detect microvessel density, slides were deparaffinized in xylene and then processed for CD31 staining as described previously.[43] The most vascular area of tumors was identified on low power (× 100) and vessels were counted in ten high-power fields (× 200). The data are presented as mean ± SE of three tumors per group. For detecting Mortalin expression in tumor tissue, slides were stained with anti-Mortalin antibody.[44] The same paraffin slides were also used to identify apoptotic cells in tumors by detecting cleaved deoxyribonucleic acids *in situ* with the TUNEL method (ApopTag kit). All slides were counterstained with Meyer's hematoxylin.

13. Statistical analysis.

The data are expressed as mean ± standard error (SE), and the significance of differences between group means was determined by the Mann-Whitney test (nonparametric rank sum test) using Stat View software (Abacus Concepts, Inc., CA). Differences were considered significant when $P < 0.05$.

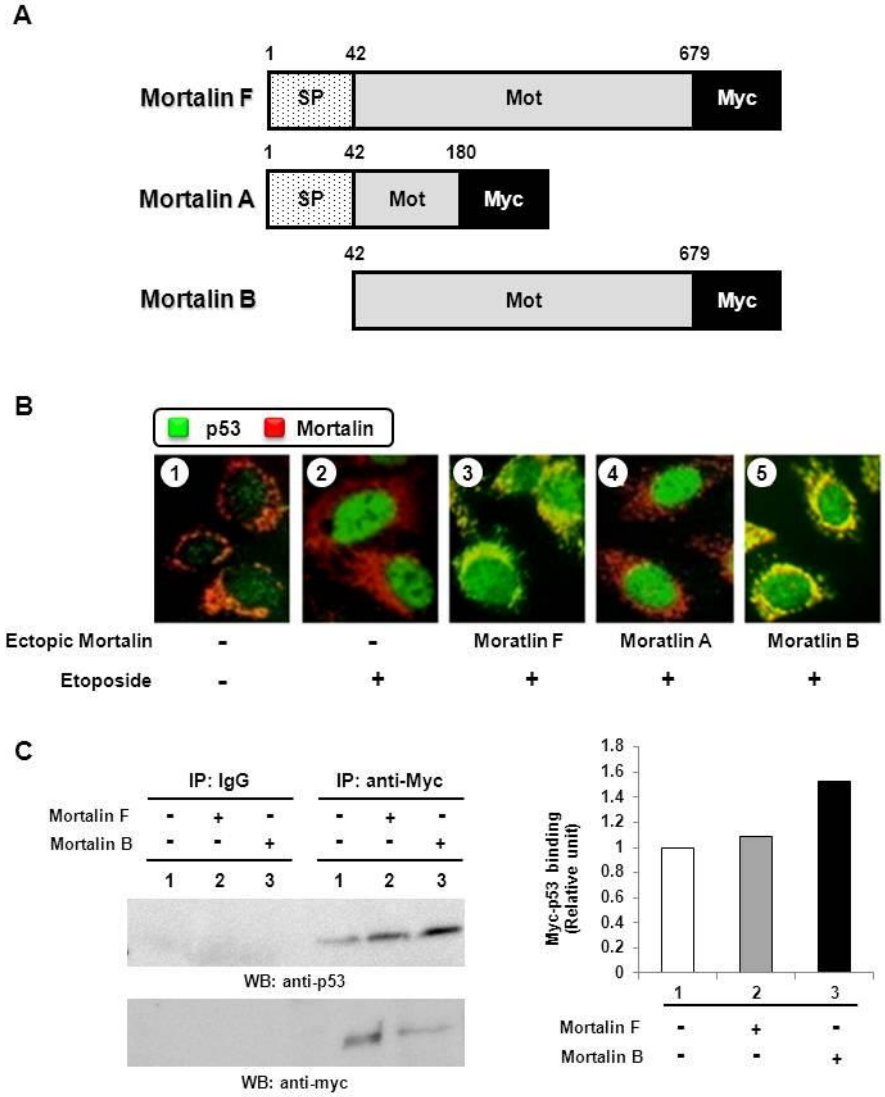
III. RESULT

1. Non-mitochondrial Mortalin inhibits nuclear localization of p53 in cytoplasm.

I prepared the retroviral constructs expressing a full length Mortalin (Mortalin F) and its two deletion mutants (Mortalin A, B), as described in Materials and Methods. Mortalin A lacks the p53 binding region of Mortalin and Mortalin B lacks the mitochondria signal peptide of Mortalin (Fig. 1A). I investigated whether Mortalin and its two deletion mutants affect the cellular localization of p53 in the U2OS cells transfected with the expression vectors and treated with etoposide. In the control cells, p53 is detected only in nucleus. Once cells are treated with etoposide, intense p53 is detected in nucleus. While ectopic Mortalin F and Mortalin B affect p53 localization and p53 is detected in both cytoplasm and nucleus, Mortalin A lacking p53 interacting domain do not affect p53 localization (Fig. 1B). In the U2OS cells with ectopic Mortalin B, more cytoplasmic p53 is detected compared to the cells with ectopic Mortalin F, indicating that cytoplasm specific expression of Mortalin B results in more p53 trapping in cytoplasm, as indicated by intense staining yellow color.

Accordingly, I investigated whether ectopic Mortalin F and Mortalin B interact with and sequester p53 in cytoplasm. Co-immunoprecipitation of the

whole cell lysates of MCF7, MCF7-Mot F, and MCF7-Mot B cells show that Mortalin forms a protein complex with the p53. In agreement with Fig. 1B, Mortalin B forms a more strong complex with p53 than Mortalin F.


A
1
42
679
Mortalin F
SP
Mot
Myc
1
42
180
Mortalin A
SP
Mot
Myc
42
679
Mortalin B
Mot
Myc
B
p53
Mortalin
1
2
3
4
5
Ectopic Mortalin
-
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Moratlin F
Moratlin A
Moratlin B
Etoposide
-
+
+
+
+
C
IP: IgG
IP: anti-Myc
Mortalin F
Mortalin B
1 2 3 1 2 3
WB: anti-p53
WB: anti-myc
Myc-p53 binding (Relative unit)
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
1
2
3
Mortalin F
Mortalin B

Figure 1. Mortalin decreases nuclear translocation of p53 in the U2OS cells treated with etoposide. (A) Schematic representation of Mortalin and Mortalin mutants. SP, mitochondria targeting signal peptide; Mot, Mortalin; Myc, Myc-tag). (B) Immunocytochemical analysis of cellular localizations of p53 and Mortalin in the U2OS cells. The cells with or without ectopic Mortalin were treated with etoposide and stained for p53 and Mortalin expression. p53 was stained with mouse anti-p53 antibody followed by incubation with an Alexa 488-conjugated secondary antibody (green) and Mortalin was stained with rabbit anti-Mortalin antibody followed by incubation with an Alexa 594-conjugated secondary antibody. (C) Co-immunoprecipitation of Mortalin-Myc and p53. Immunoprecipitates by Myc antibody were examined for the presence of p53. Mortalin-Myc was co-immunoprecipitated with p53 in whole cell lysates of MCF7-Mot F and MCF7-Mot B. The histogram shows relative band intensities of the immunocomplexes containing Mortalin and p53.

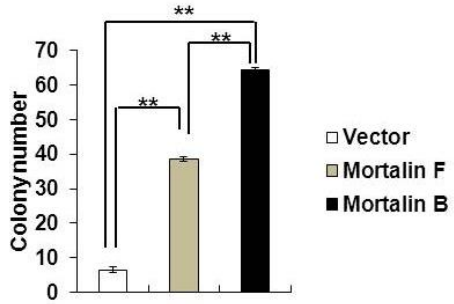
2. The MCF7 cells overexpressing Mortalin B (Non-mitochondrial) aggressively form tumors and show strong lung metastasis.

Cytoplasmic accumulation or sequestration of p53 in tumor is related with malignant phenotype of tumors.[45-50] I investigated whether ectopic Mortalin expression in human breast cancer cell (MCF7) can induce malignant transformation by an anchorage-independent growth assay in soft agar. The MCF7 cells with ectopic Mortalin B show higher colony forming capability compared to the cells with ectopic Mortalin F (Fig. 2A). Furthermore, because ectopic Mortalin B drive strong transformation into malignant cells, I tested whether the MCF7-Mot B cells can form tumor by xenograft assays using Balb/c nude mice. The MCF7-Mot B cells xenografted form aggressive tumors (Fig. 2B-3). The mouse xenografted with the MCF7-Mot B cells show large tumors compared with the mouse xenografted with the MCF7-Mot F cells (Fig. 2B-2 and 3). The mice with systematic injection of the MCF7–Mot B cells via tail vein show multiple tumors all over the mice body (Fig. 2B-4).

I also tested whether Mortalin can induce metastasis. First I tested whether MCF7-Mot B cells has infiltration capability by *in vitro* Boyden chamber assays. Mortalin F and B increase the number of the MCF7 cells infiltrated and Mortalin B appears to have more effect on the infiltration capability of the MCF7 cells. (Fig. 2C) Furthermore, I also examined whether injected via tail vein the MCF cells with ectopic Mortalin

expression can form tumor in the lung tissues through metastasis (Fig. 2D). Although Mortalin F caused metastatic about 30 lung tumors, Mortalin B shows 2-3 folds more potent effect on metastasis and cause as many as 80 lung tumors. The results suggest that Mot B causes more aggressive malignant transformation of the MCF7 cells, probably by cytoplasmic trapping or exclusion of p53 in nucleus via protein interaction with Mortalin B.

A


**
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70
60
50
40
30
20
10
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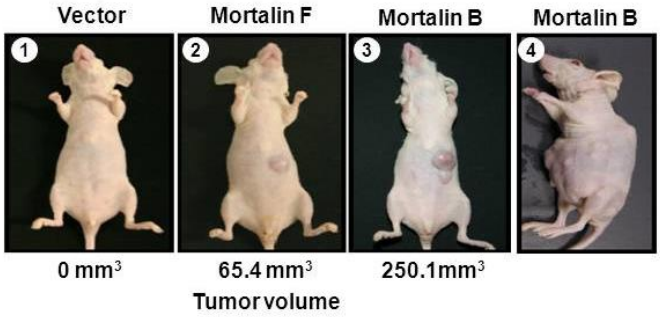
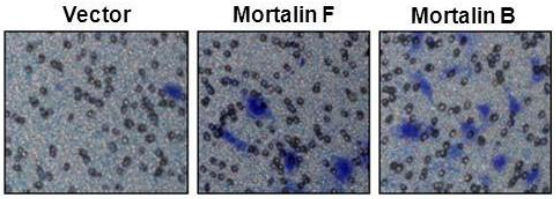
Colony number
Vector
Mortalin F
Mortalin B

B


Vector
Mortalin F
Mortalin B
Mortalin B
1
2
3
4
0 mm3
65.4 mm3
250.1mm3
Tumor volume

C


Vector
Mortalin F
Mortalin B

D

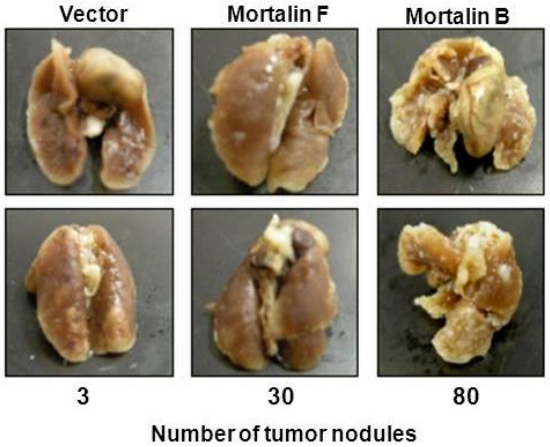

Vector
Mortalin F
Mortalin B
3
30
80
Number of tumor nodules

Figure 2. Mortalin F (full length) and Mortalin B lacking the mitochondria signal peptide (Mot B) increase malignant and metastatic characteristics of the breast carcinoma MCF7 cells. (A) Cell transformation assay. The MCF7-Mot B cells shows increase in number of anchorage independent cell growth colonies compared with the control and MCF7-Mot F cells. **, *P<0.01*. (B) Nude mice tumor formation assays of MCF7, MCF7-Mot F, or MCF7-Mot B cells. Both MCF-Mot F and B cells form tumor and Mortalin B increases tumor volume more strongly. (C) Cell invasion assay. MCF7-Mot B shows increased invasion as compared with MCF7-Mot F cells. (D) *In vivo* metastasis assays. Mice lung tissues were isolated after tail vein injections of MCF7, MCF7-Mot F, and MCF7-Mot B cells. MCF-Mot B cells showed significantly higher number of lung tumors.

3. Mortalin is expressed primary in cytoplasm, but Mortalin is also detected in nucleus, particularly high in the nucleus of malignant cells.

To investigate the subcellular localization of Mortalin F and Mortalin B, subcellular fractions of the MCF7, MCF7-Mot F, or MCF7-Mot B cells were prepared by Qproteome mitochondria isolation kit and Qproteome nuclear protein kit and were subjected to western blot analysis. As shown in Fig. 3A, Mortalin F is localized to the cytosol, microsomal, and mitochondrial fractions. However, Mortalin B is detected only in the cytosolic fractions and amount of Mortalin B detected in the cytosol is far less than the amount detected in the whole cell lysates (Fig. 3A). The result suggested that Mortalin may be localized in the nucleus. Indeed, both Mortalin B and Mortalin F are detected in the nuclear fractions (Fig. 3B).

Additionally, I examined whether Mortalin is enriched in the nucleus of cancer cells. Cytoplasmic and nuclear fractions of several human cancer cell lines were subjected to western blotting. As shown in Fig. 3C, Mortalin is detected in the nuclear fractions of several cell lines tested and is significantly enriched in the malignant cells that formed tumors in nude mice assays. Expression level of nuclear Mortalin is higher in malignant T47D cells compared to those of Saos-2, YKG-1, and A172 cells (Fig. 3C). I examined whether Mortalin is located in nucleus by immunocytochemical staining of the MCF7 overexpressing Mortalin F or B. Fluorescent microscopic image

analysis shows that Mortalin F is mainly at cytoplasm (probably mitochondrial locations) and Mortalin B is localized in cytoplasm and nucleus (Figs. 3D and E).

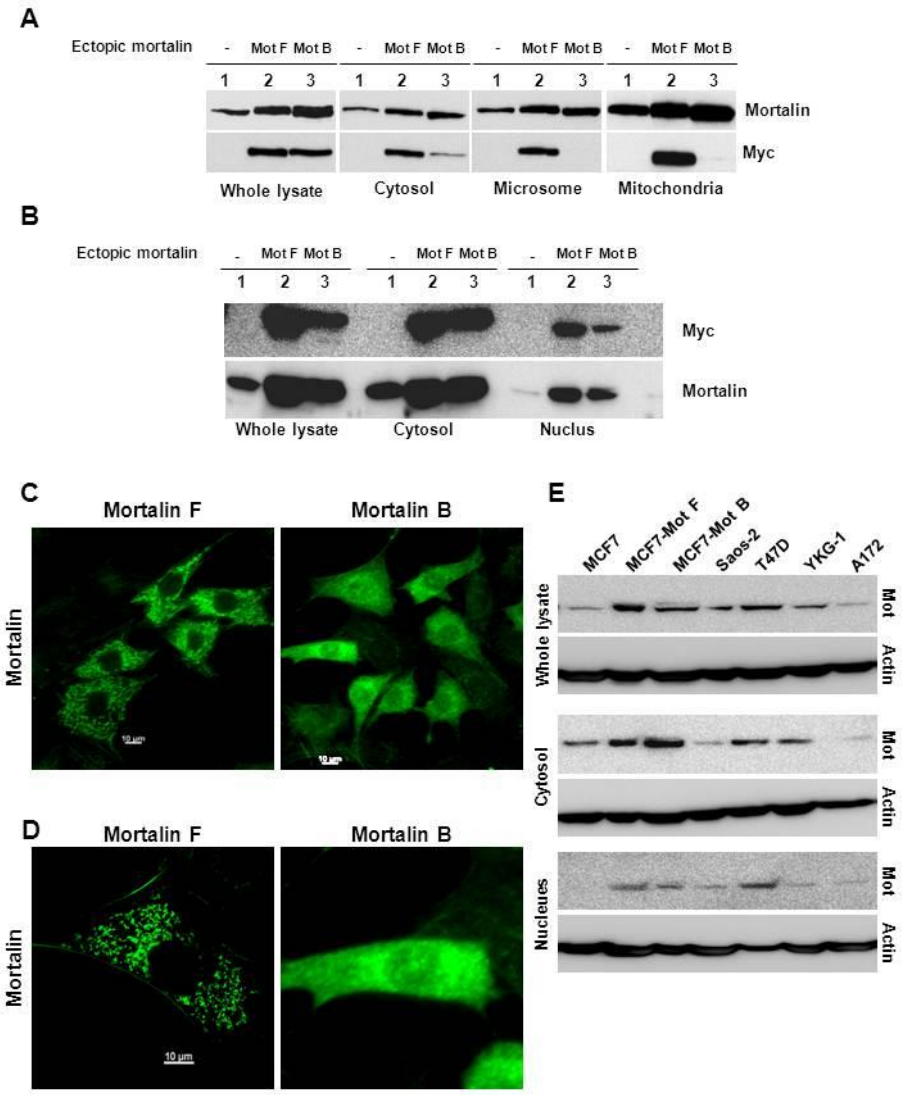

A
Ectopic mortalin
- Mot F Mot B
1 2 3
Mortalin
Myc
Whole lysate
Cytosol
Microsome
Mitochondria
B
Ectopic mortalin
Myc
Mortalin
Whole lysate
Cytosol
Nuclus
C
Mortalin F
Mortalin B
Mortalin
D
E
MCF7
MCF7-Mot F
MCF7-Mot B
Saos-2
T47D
YKG-1
A172
Whole lysate
Cytosol
Nucleues
Mot
Actin

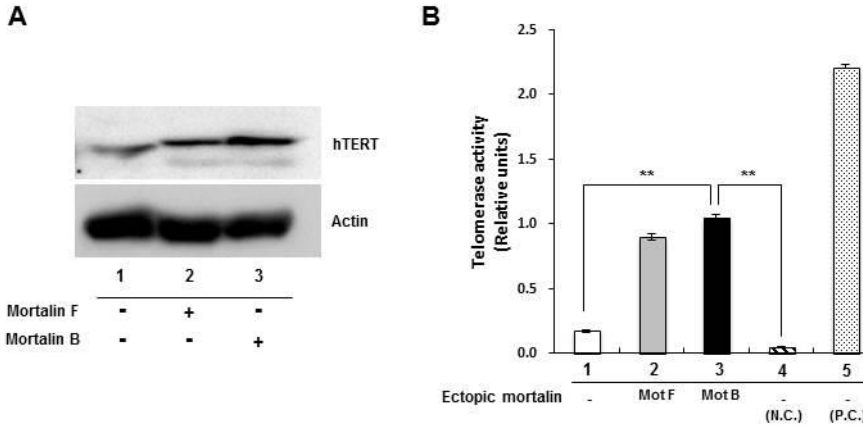
Figure 3. Subcellular localization of Mortalin F and Mortalin B (A) Western blotting assay. The subcellular fractions of the MCF7 cells over expressing Mortalin F or Mortalin B were separated by SDS-PAGE and analyzed by western blot assays using by anti-Myc and anti-Mortalin antibodies. Mortalin F (lane 2), but not Mortalin B (lane 3) is localized in the microsomes and mitochondria. (B) Western blotting assay. The subcellular fractions of the MCF7 cells over expressing Mortalin F or Mortalin B were analyzed as above. Mortalin F (lane 2) and Mortalin B (lane 3) are localized in the cytoplasm and nucleus. (C, D) Immunocytochemical analysis of Mortalin expression. Mortalin F is mainly expressed in cytoplasm and Mortalin B is located both in cytoplasm and nucleus. (E) Western blotting assay. The subcellular fractions of the MCF7 cells over expressing Mortalin F or Mortalin B, and various cancer cells were separated by SDS-PAGE and analyzed by western blot assays using by anti-Mortalin antibody. Actin, loading control.

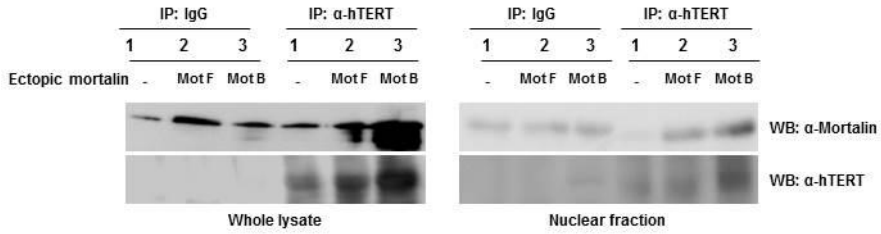
4. Nuclear Mortalin interacts with hTERT in malignant cancer cells.

The high level of telomerase expression and activity has been founded in almost all cancer cells.[51] Several reports suggested that telomerase activation is related with the acquisition of malignant phenotype.[52-54] Accordingly, I investigated whether ectopic Mortalin F or Mortalin B expression affects telomerase activity or expression by western blotting and TRAP assay. As shown in Fig 4A, MCF7-Mot B cells show higher telomerase protein expression than MCF7 and MCF7-Mot F. Furthermore, MCF7-Mot B cells show higher telomerase activity compared with MCF7 and MCF7-Mot F (Fig. 4B). I further examined whether Mot F- or Mot B stabilizes and activates hTERT by the molecular interaction between the two proteins. Co-immunoprecipitation of the whole cell lysates and nuclear fractions of MCF7, MCF7-Mot F, and MCF7-Mot B cells show that hTERT forms a protein complex with the Mortalins. Interestingly, hTERT forms a stronger complex with Mot B compared with Mortalin F, both in cytoplasm and nucleus (Fig. 4C).

Because nuclear molecular interaction between Mortalin and hTERT is present, although relatively weak compared to that of cytoplasm, I investigated the potential correlation with malignant properties of cancer cells. Interestingly, malignant cells such as MCF7-Mot B and T47D show nuclear Mortalin and hTERT complex, but nonmalignant cells Saos-2, and YKG-1 do

not show nuclear protein complex (Fig. 4D). These data suggest that the nuclear Mortalin interacts with hTERT, which may be important in the acquisition of cancer cell malignancy.


A
hTERT
Actin
1 2 3
Mortalin F - + -
Mortalin B - - +
B
Telomerase activity (Relative units)
2.5
2.0
1.5
1.0
0.5
0.0
**
**
1 2 3 4 5
Ectopic mortalin - Mot F Mot B - (N.C.) - (P.C.)


C
IP: IgG
IP: α-hTERT
IP: IgG
IP: α-hTERT
1 2 3 1 2 3 1 2 3 1 2 3
Ectopic mortalin - Mot F Mot B - Mot F Mot B - Mot F Mot B - Mot F Mot B
WB: α-Mortalin
WB: α-hTERT
Whole lysate
Nuclear fraction

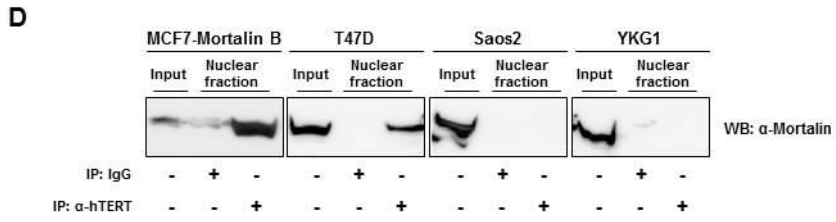

D
MCF7-Mortalin B
T47D
Saos2
YKG1
Input
Nuclear fraction
WB: α-Mortalin
IP: IgG - + - - + - - + - - + -
IP: α-hTERT - - + - - + - - + - - +

Figure 4. Mortalin (Mot F and Mot B) interacts with and activates telomerase in the cells overexpressing Mortalin and in malignant cancer cells. (A) Western blotting assay of hTERT in the presence of ectopic Mot F and Mot B. Cell lysates of the MCF7 cells overexpressing Mortalin F or Mortalin B were separated by SDS-PAGE and analyzed by western blot assays using by anti-hTERT antibody. Actin, loading control. (B) TRAP assays of the MCF7 cells expressing Mortalin F or Mortalin B. U2OS cells heated at 85℃ for 10 min and MRC5 cells transfected with hTERT were used as negative (N.C.) and positive controls (P.C.), respectively. **, $P<0.01$. (C) Co-immunoprecipitation of Mortalin and hTERT. Immunoprecipitates by hTERT antibody were examined for the presence of Mortalin. Both in the whole cell lysates and the nuclear fractions, Mortalin was co-immunoprecipitated with hTERT. The histograms of the western bands intensities are shown below. (D) Mortalin and hTERT interact with each other in the nucleus of malignant cancer T47D cells. Nuclear fractions of various cancer cells lysates were immunoprecipitated with anti-hTERT antibody and analyzed as described in (C).

5. Mortalin-specific shRNA induces cytotoxicity and suppresses colony forming potential of cancer cells.

The results described above suggest that Mortalin may be related with the increased malignant property of cancer cells by the molecular interaction with p53. I investigated whether knocking-down of Mortalin expression can affect the cell colony formation ability. I prepared Mortalin-specific shRNA-expressing vectors and tested how knock-down affects human cancer cells colony formation. Two kinds of shRNA expression plasmid constructs, (i) U6 promoter driven, constitutive (#7 and #8) and tetracycline-inducible (#596, #696, and #906) plasmids and (ii) oncolytic adenoviruses (Ad-ΔB7-shMot3, Ad-ΔB7-shMot4, Ad-ΔB7-shMot7, Ad-ΔB7-shMot8, and Ad-ΔB7-shMot9), were prepared as described in Materials and Methods. Cells transfected with Mot-shRNA plasmids were selected in puromycin-supplemented medium and were examined for Mortalin expression by immunocytochemistry. As shown in Fig. 5A and C, many cells demonstrated negligible staining of Mortalin; Bcl-xL staining was used as an internal control. The cells transfected with Mortalin shRNA show normal Bcl-xL staining, suggesting that knock-down does not affect Bcl-xL expression (Fig. 5C). Knocking-down Mortalin causes cytotoxicity in the cells transfected with shRNA targeting Mortalin (Fig. 5B), which is also confirmed by colony-forming assays. All the shRNA expression constructs suppress the colony-forming potential of U2OS cells; #007 and

#008 shRNA show more strong inhibitory activity than #596, #696 and #906 (Fig. 5D).

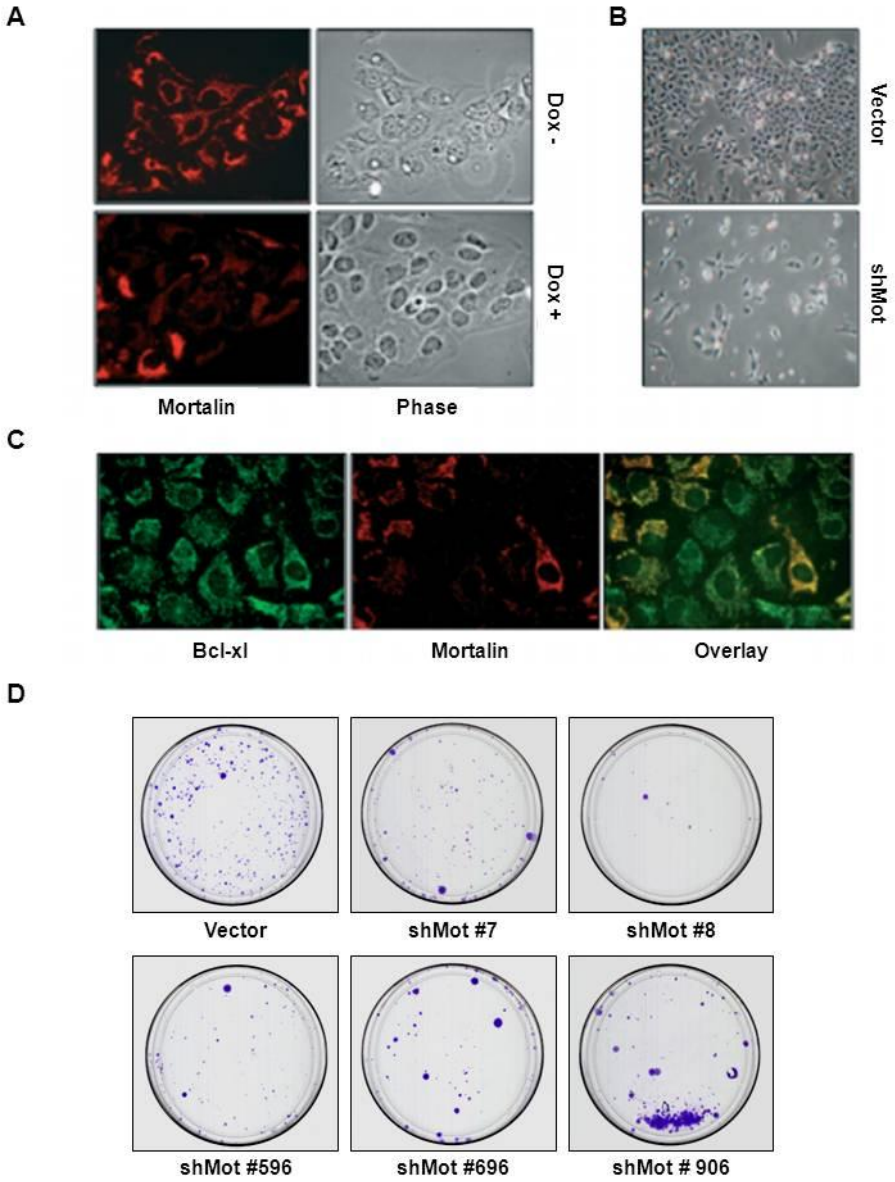


Figure 5. Mortalin-specific shRNA inhibits cell proliferation and suppresses colony forming potential of cancer cells. The transfected cells were selected in medium supplemented with puromycin (2 g/µl) for 48-96 h. Tetracycline inducible constructs were induced by addition of doxycycline (1 g/ml in the medium). (A) Immunocytochemistry of U2OS cells transfected with control or shRNA Mortalin expression vector. shRNA Mortalin transfected cells showed weak staining. (B) While the U2OS cells transfected with control expression vector grow healthy, the cells transfected with shRNA show poor growth. (C) Double immunostaining of Mortalin and Bcl-xL expression in the U2OS cells transfected with shRNA Mortalin. Knock-down of Mortalin expression do not affect Bcl-xL expression. (D) Colony forming assays of the stable U2OS cells stably overexpressing sh RNA against Mortalin. The stable U2OS cells were selected with Puromycin and the cells were plated at low density (2000 cells per 10 cm dish) and were allowed to form colonies for 15 days. Significant decrease in the colony forming efficiency is noted in the cells stably overexpressing shRNA against Mortalin.

6. Cancer cell specific killing effect of recombinant oncolytic adenovirus expressing shRNA Mortalin *in vitro*

To examine the oncolytic abilities of the six recombinant oncolytic adenoviruses expressing shRNA Mortalin (Fig. 6A), cytopathic effect (CPE) assay was done using human normal (HDF, TIG, WI38) and cancer (U2OS) cells. Replication-incompetent adenovirus, Ad-ΔE1, was used as negative control. The adenoviruses do not shown any effect on normal cells, but they show strong cytotoxicity in the U2OS cancer cells (Fig. 6B). To quantitate the cytopathic effect of the oncolytic adenoviruses, the cell viability was examined by MTT assay. As shown in Fig. 6C, the oncolytic adenoviruses expressing shRNA Mortalin show significant cytotoxic effect in U2OS, whereas they show only a minor cytotoxic effect in normal fibroblasts. Also I tested the cell type specific cytotoxic effect of the oncolytic adenoviruses expressing sh Mortalin (MOI of 50, 100, and 300) by infection of normal TIG and U2OS cells. Crystal violet staining of viable cells showed that the oncolytic adenovirus penetrated into TIG cells does not show any cytotoxic effect even at 300 MOI, but the virus show potent cytotoxic effect in U2OS cancer cells at 100 and 300 MOI. The results suggest that oncolytic adenoviruses expressing shMot shows cancer cell specific cytotoxic activity (Fig. 6D).

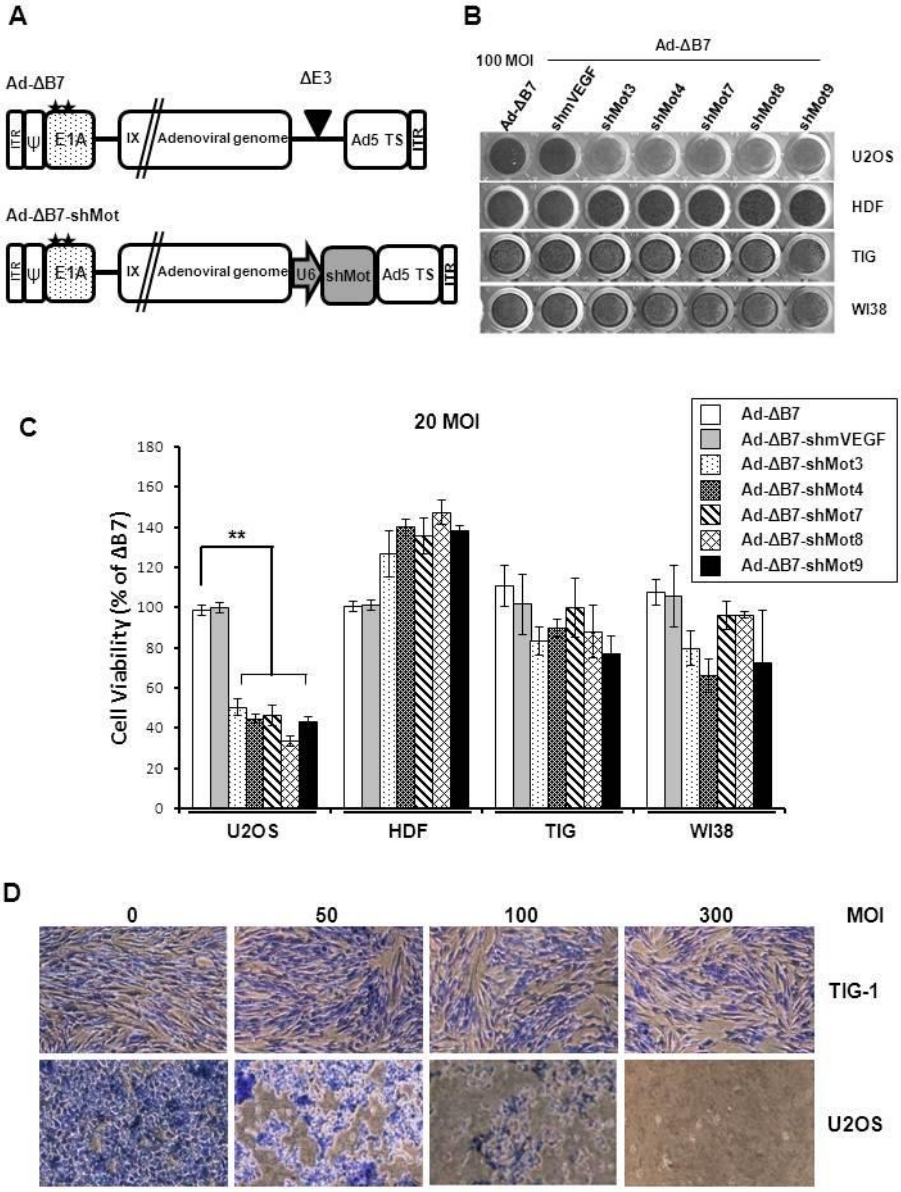

A
Ad-ΔB7
ΔE3
ITR
Ψ
E1A
IX
Adenoviral genome
Ad5 TS
ITR
Ad-ΔB7-shMot
U6
shMot
B
100 MOI
Ad-ΔB7
Ad-ΔB7
shmVEGF
shMot3
shMot4
shMot7
shMot8
shMot9
U2OS
HDF
TIG
WI38
C
20 MOI
Ad-ΔB7
Ad-ΔB7-shmVEGF
Ad-ΔB7-shMot3
Ad-ΔB7-shMot4
Ad-ΔB7-shMot7
Ad-ΔB7-shMot8
Ad-ΔB7-shMot9
Cell Viability (% of ΔB7)
**
U2OS
HDF
TIG
WI38
D
0
50
100
300
MOI
TIG-1
U2OS

Figure 6. Recombinant oncolytic adenovirus expressing shRNA Mortalin showed cancer cell specific killing effect. (A) Schematic representation of replication incompetent Ad-ΔE1, Ad-ΔB7, and Ad-ΔB7 derived oncolytic adenoviruses expressing shRNA targeting Mortalin. Ad-ΔE1 has the whole E1 region deleted; Ad-ΔB7 has mutation in retinoblastoma (Rb) binding site of E1A and also has deletion in E1B. Ad-ΔB7-shMot3, Ad-ΔB7-shMot4, Ad-ΔB7-shMot7, Ad-ΔB7-shMot8, and Ad-ΔB7-shMot9 carry the Mortalin-specific shRNA sequences driven by the U6 promoter cloned into ΔE3 region. ΔE1A, ΔE1B, and ΔE3 denote the deletion of E1A, E1B, and E3 gene, respectively. ITR= inverted terminal repeat; Ψ=packaging signal; IX=protein IX. The asterisks indicate the mutations of the Rb protein binding sites on the E1A. Mortalin-shRNA expressing oncolytic adenoviruses were prepared as described elsewhere.[41] (B) Crystal violet staining of cells infected with indicated Mortalin targeting oncolytic adenoviruses. Normal (HDF, TIG-1 and WI38) and cancer (U2OS) cells were infected with viruses at 100 MOI. Vector backbone Ad-ΔB7 and Ad-ΔB7/shmVEGF (identical backbone containing mouse VEGF-specific shRNA) were used as controls. (C) MTT assays. Viability of the normal (HDF, TIG, and WI38) and cancer cells (U2OS) was examined at 48 h post infection. Oncolytic viruses significant decrease in cancer cell viability but did not show any significant effect in normal cells. **, $p<0.01$ (D) Crystal violet staining of cells infected with

indicated Mortalin targeting oncolytic adenoviruses. Normal TIG-1 and cancer (U2OS) cells were infected with viruses at 100-300 MOI.

7. Inhibition of Mortalin expression by Mortalin targeting oncolytic adenovirus *in virto*

In order to further confirm that Mortalin silencing induces cytotoxicity of cancer cells, U2OS and U2OS-Mot F cells was infected by Ad-ΔB7, Ad-ΔB7-shMot7, Ad-ΔB7-shMot8, or Ad-ΔB7-shMot9. As shown in Fig. 7A, oncolytic adenoviruses expressing shRNA Mortalin show cytotoxic effect in both U2OS and U2OS-Mot F. The cytotoxic effect is stronger in U2OS than U2OS-Mot F. Moreover, the cytotoxicity of Ad-ΔB7-shMot7, Ad-ΔB7-shMot8, and Ad-ΔB7-shMot9 in U2OS-motF cells are much stronger than the control oncolytic adenovirus, Ad-ΔB7 (Fig. 7B). The data suggested that knocking-down of Mortalin can induce the killing of cancer cells. (Fig. 7A and C).

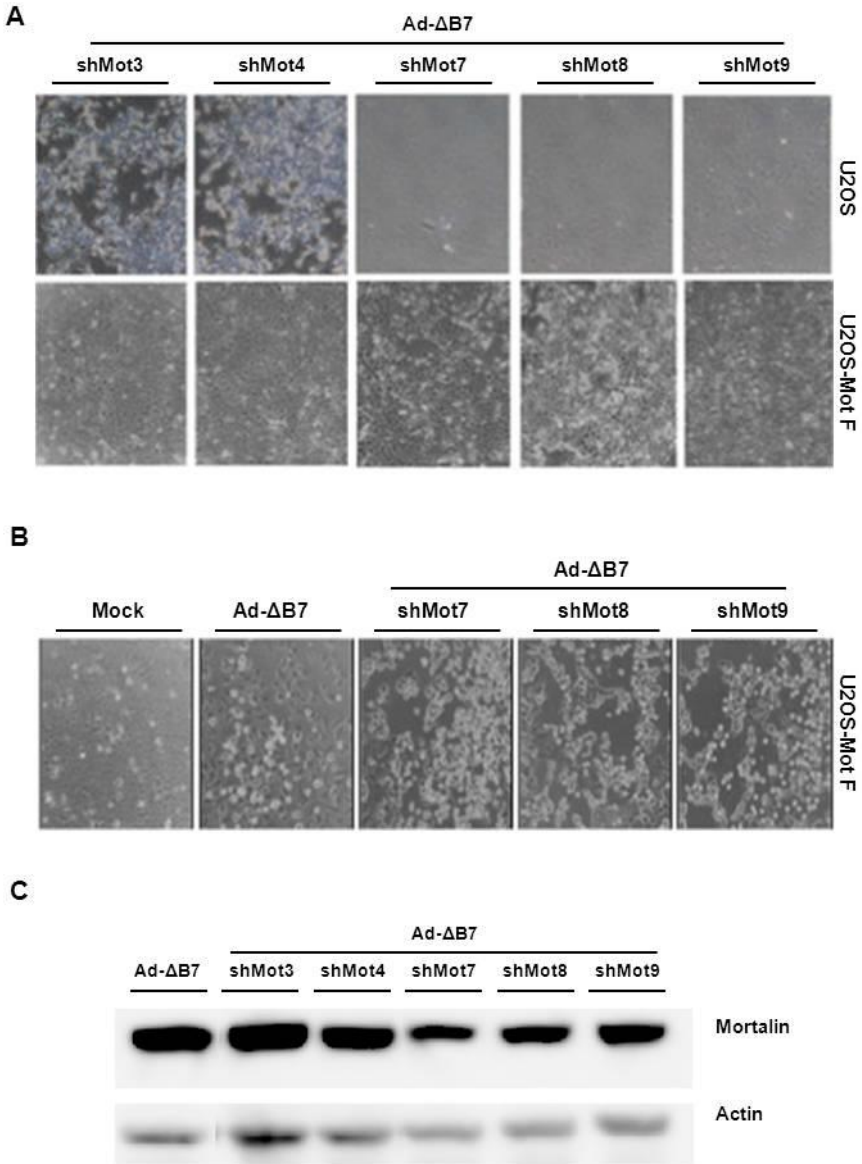

A
Ad-ΔB7
shMot3
shMot4
shMot7
shMot8
shMot9
U2OS
U2OS-Mot F
B
Ad-ΔB7
Mock
Ad-ΔB7
shMot7
shMot8
shMot9
U2OS-Mot F
C
Ad-ΔB7
Ad-ΔB7
shMot3
shMot4
shMot7
shMot8
shMot9
Mortalin
Actin

Figure 7. Ectopic Mortalin protected the cells from oncolytic adenoviruses targeting Mortalin expression. (A) U2OS cells expressing Mortalin F were generated as described.[26] Control and Mortalin-overexpressing U2OS cells were then infected with each of the six oncolytic adenoviruses expressing shRNA Mortalin along with their parental U2OS cells. Oncolytic adenoviruses showed effective killing effect in U2OS cells, whereas they showed less cytotoxicity in U2OS-Mot F cells. (B) U2OS cells were infected by shRNA Mortalin-expressing oncolytic adenoviruses, Ad-ΔB7-shMot7, Ad-ΔB7-shMot8, and Ad-ΔB7-shMot9 showed higher cytotoxicity than the backbone adenovirus, Ad-ΔB7. (C) The level of Mortalin expression subsequent to the oncolytic adenoviruses expressing shRNA Mortalin infection was determined by western blotting with anti-Mortalin antibody.

8. Oncolytic adenovirus expressing shRNA targeting Mortalin decreases tumor growth and increases survival of the mice xenografted with MCF7 Mot F cells

I next investigated the *in vivo* potency of oncolytic adenovirus expressing Mortalin-specific shRNA. Since U2OS cells do not form tumors, the human breast cancer MCF7 (MCF7-Mot F) cells that overexpresses Mortalin and forms aggressive tumors in nude mice were used. *In vitro* assays using MCF7-Mot F cells revealed that the Mortalin targeting oncolytic adenoviruses are cytotoxic at MOI in the range 100–1000 (Fig. 8A). On the basis of in vitro efficacy data, I selected Ad-ΔB7-shMot7 and Ad-ΔB7-shMot8 for *in vivo* studies and evaluated the antitumor effect by injecting adenoviruses into tumors established with MCF7-Mot F cells. The tumor volume analysis reveals a significant difference in tumor volume between control and all the treatment groups. (Fig. 8B) By 25th day post-treatment, control tumors that received PBS show robust growth with an average volume of 1909.3 ± 379 mm^3. In contrast, Ad-ΔB7-, Ad-ΔB7-shMot7-, or Ad-ΔB7-shMot8-treated tumors that reach to an average volume of 1101.04 ± 123.4 mm^3, 762.8 ± 108.3 mm^3, and 669.8 ± 56.4 mm^3, respectively, show 43%, 60%, and 65% growth inhibition ($P^* < 0.05$). Ad-ΔB7-shMot7 and Ad-ΔB7-shMot8 elicit more potent antitumor effect than control oncolytic Ad, Ad-ΔB7, in xenograft model. Of note, the survival advantage is also significantly enhanced in

animals treated with Ad-ΔB7-shMot7 or Ad-ΔB7-shMot8 compared with Ad-ΔB7-treated mice ($p<0.05$) (Fig. 8C). 100% and 60% of the animals that received Ad-ΔB7-shMot8 and Ad-ΔB7-shMot7, respectively, are still viable 35 days after the initial treatment (Fig. 8C), whereas only 20% of control oncolytic Ad, Ad-ΔB7-treated mice are viable and no animal in PBS-treated control group are viable in the same time period. Throughout the course of the study, no systemic toxicity, such as diarrhea, loss of weight, or cachexia was observed.

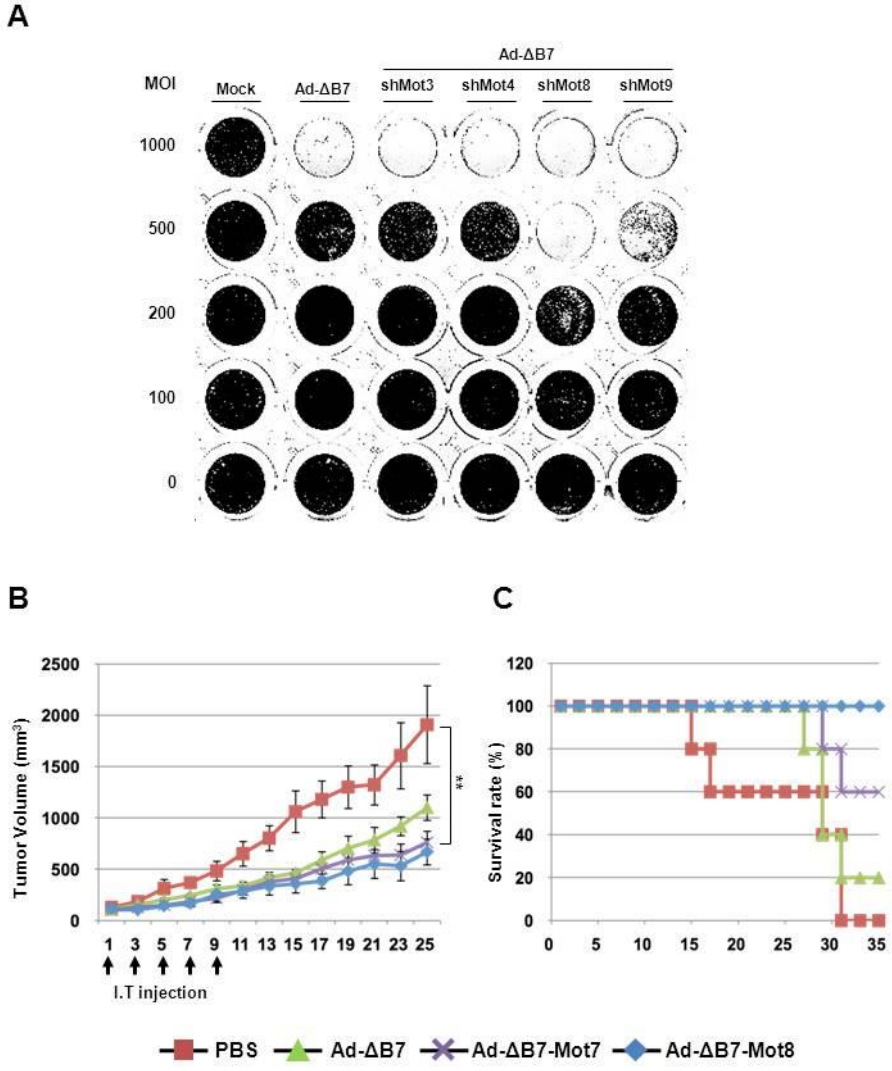

A
Ad-ΔB7
MOI
Mock
Ad-ΔB7
shMot3
shMot4
shMot8
shMot9
1000
500
200
100
0
B
C
Tumor Volume (mm3)
2500
2000
1500
1000
500
0
1 3 5 7 9 11 13 15 17 19 21 23 25
I.T injection
**
Survival rate (%)
120
100
80
60
40
20
0
0 5 10 15 20 25 30 35
PBS
Ad-ΔB7
Ad-ΔB7-Mot7
Ad-ΔB7-Mot8

Figure 8. ***In vivo*** **anti-tumor effects of adenoviruses targeting Mortalin.**

(A) Mortalin overexpressing MCF7 cells (MCF7-Mot F) that form aggressive tumors in nude mice were infected with viruses at 100-1000 MOI. Crystal violet staining of cells infected with indicated Mortalin targeting oncolytic adenoviruses is shown. (B) Mortalin targeting oncolytic adenovirus suppressed the growth of a Mortalin overexpressing MCF7 (MCF7-Mot F) human breast tumor xenograft model. MCF7-Mot F cells were injected into the flanks of 6- to 8-week-old male nude mice. When the volumes of tumors reached 90-120 mm^3, tumors were treated with Ad-ΔB7, Ad-ΔB7-shMot7, or Ad-ΔB7-shMot8, along with PBS as a negative control. Tumor volume was monitored over time (days) after treatment with adenoviruses. The arrow indicates when treatment was given ($1x10^8$ PFU per mouse). Values represent the means ± SE for five animals per group. (C) The survival analyses of adenovirus-treated mice were performed over a period of 35 days. In comparison, whereas all PBS treated mice died at 35 days, 20% of the Ad-ΔB7, 60% of Ad-ΔB7-shMot7, and 100% of Ad-ΔB7-shMot8 were viable after 35 days.

9. Histological and immunohistochemical analyses of tumor tissues

The enhanced anti-tumor effect and survival benefit of Mortalin-specific shRNA-expressing oncolytic adenoviruse, Ad-ΔB7-shMot8 was further investigated by histological examination. Tumors were harvested from each treatment group on the day 3 after the three sequential viral infections with days interval. Hematoxylin and eosin (H & E) staining revealed that the most of tumor mass treated with PBS or control oncolytic adenovirus (Ad-ΔB7) display a large area of proliferating tumor cells, whereas tumor tissues treated with Ad-ΔB7-shMot8 displayed a large area of necrosis (Fig 9). As expected, tumor tissue injected with the control and Mortalin targeting oncolytic adenovirus, but not tumors injected with PBS, showed E1A staining. To determine whether the enhanced anti-tumor effect of Ad-ΔB7-shMot8-treated tumors coincides with reduced expression of Mortalin, tumor tissue section slides were immunostained. A marked decrease in Mortalin expression was detected in the Ad-ΔB7-shMot8-treated tumors compared with PBS- or Ad-ΔB7-treated tumors (Fig. 9). To determine whether reduced Mortalin expression induced apoptosis *in vivo*, TUNEL assay was carried out using the same tumor sections. As seen in Fig. 9, only a few TUNEL-positive cells were detected in the PBS treated group, but tumors treated with Ad-ΔB7-shMot8 exhibited a significant increase in apoptosis. To determine whether the reduced size of Ad-ΔB7-shMot8-treated tumors coincided with reduced

neovascularization, microvessel density was assessed. Immunostaining with anti-PECAM antibody showed a marked decrease in endothelial cells and vessel structures in Ad-ΔB7-shMot8-treated tumors compared with PBS- or Ad-ΔB7-treated tumors (Fig. 9).

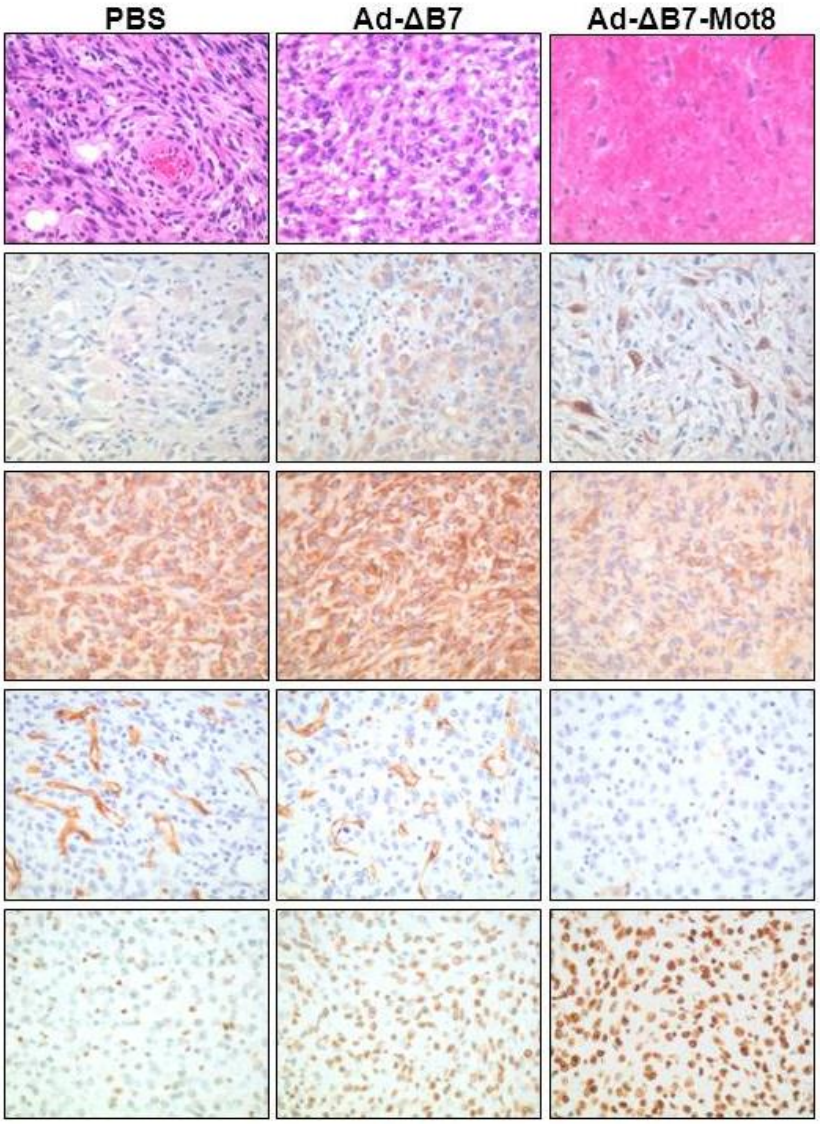

PBS
Ad-ΔB7
Ad-ΔB7-Mot8
H&E
E1A
Mortalin
CD31
TUNEL

Figure 9. Histological analyses of xenogafted tumor tissues. Three days following final oncolytic adenovirus infection, tumors were fixed with zinc fixative, stained with H&E, anti-E1A, anti-Mortalin antibody, and anti-CD31 antibody as described under Materials and Methods. Ad-ΔB7-shMot8-treated tumors showed decreases in Mortalin, necrosis, decrease in vessel formation (CD31 staining) and marked increase in apoptosis (Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling, TUNEL staining). Original magnification: x 400.

IV. DISCUSSION

Cancer cells display several hallmarks that distinguish them from normal cells: limitless replicative potential, self-sufficiency in growth signals, insensitivity to growth inhibitory signals, evasion of programmed cell death, sustained angiogenesis, tissue invasion and metastasis. Several recent studies have shown that heat shock proteins play essential functions for tumor cell survival including protection from apoptosis and resistance to cytotoxins.[18, 23, 55-60] Mortalin, mitochondrial heat shock protein 70, is upregulated during carcinogenesis. And Mortalin-overexpressing cells show malignant properties.[13, 26, 27, 35, 61, 62] Because of mitochondrial targeting signal peptide at N-terminus, Mortalin was initially considered as a mitochondrial stress chaperone. However, many later studies demonstrated that Mortalin is also expressed in other subcellular compartments including ER, plasma membrane and cytoplasm.[13, 63] Mortalin was shown to perform multiple functions by interacting with other proteins which include GRP94 (ER), Interleukin-1 receptor alpha (cell surface), MPD (vacuoles) HSP60 (mitochondria), FGF-1 (cytoplasm) and RAR/RXR.[14, 15, 43, 44, 64-66] Intriguingly, several report demonstrated that Mortalin also interacts with p53 in human cancer cells and inhibits functions of p53 including the transcriptional activation and apoptosis.[9, 16, 23, 31, 67, 68]

I hypothesized that Mortalin located outside of mitochondria might play

some role in malignant property of cancer cells. To prove the hypothesis, I generated Mortalin mutant without mitochondrial targeting signal peptide (Mortalin B) and investigated whether the Mortalin mutant can inhibit nuclear translocation of p53. Mortalin B inhibited nuclear translocation of p53 in the U2OS cells treated with etoposide. Mortalin B appears to form a more stable complex with p53 compare to Mortalin F, possibly suggesting that Mortalin localized outside mitochondria may form a relatively more stable complex with p53 in cytoplasm and inhibits nuclear translocation of p53. Because cytoplasmic sequestration of p53 is often found in malignant cancer cells, I investigated whether cytoplasmic trapping of p53 by Mortalin could contribute to malignant property of cancer cells. The MCF7 cells expressing Mortalin B showed increased colony forming capability *in vitro* and developed into more aggressive tumor upon xenograft. I found that MCF7 cells expressing Mortalin B show more metastatic tumor nodules in the lung than MCF7 cells expressing Mortalin F. Mortalin B may cause malignant transformation of MCF7 cells and inactivation of p53 activity by cytoplasmic sequestration may be responsible for the malignant transformation.

I found that Mortalin F was detected in various cell fractions. However, Mortalin B was only detected in cytoplasmic fractions but not in microsomal and mitochondrial fractions. Interestingly, both Mortalin F and Mortalin B were found in nuclear fractions. Expression of nuclear Mortalin is

significantly high in malignant cancer cells including MCF7-Mortalin F, MCF7-Mortalin B and T47D. Because a very small amount of Mortalin is detected in nuclear fractions of MCF7, compare to MCF7-Mortalin F and MCF7-Mortalin B, nuclear Mortalin may be related with in malignant property of cancer cells.

Cancer cells with enhanced telomerase activity showed progressive increase in the malignant properties.[52] Because cancer cells with non-mitochondrial Mortalin show malignant properties, I investigated whether Mortalin is related with expression of human telomerase reverse transcriptase (hTERT). MCF7-Mortalin B cells showed higher hTERT expression and telomerase activity than MCF7 or MCF7-Mortalin F, suggesting that non mitochondrial Mortalin may increase telomerase expression. I found that Mortalin and hTERT interact with each other in nucleus. The data suggested that nuclear Mortalin may increase hTERT activity by molecular interaction. Malignant cancer cells such as MCF7-Mortalin B and T47D showed nuclear Mortalin and hTERT complex, but not in nonmalignant cells. Nuclear Mortalin may contribute to development of aggressive malignant properties in cancer cells by interaction with hTERT.

Considering these unique features of Mortalin, Mortalin may be useful target for anti-cancer therapy. I investigated whether knocking down Mortalin expression can be useful for cancer therapy. To repress Mortalin expression, I

constructed plasmids expressing shRNA targeting Mortalin (shMot). Even if vector-based siRNA technology was helpful tool for inhibiting target gene expression, short duration of shRNAs expression was limitation. Yoo *et al*. showed that VEGF-specific shRNA-expressing oncolytic adenovirus could repress VEGF expression and long-term expression of shRNA resulting in potent anti-tumor effect.[69] Accordingly I prepared recombinant oncolytic adenoviruses expressing shMot (Ad-ΔB7-shMot). The viruses selectively killed cancer cells by knocking down Mortalin expression.

In xenograft studies, Ad-ΔB7-shMot demonstrated an enhanced anti-tumor effect compare to the control oncolytic adenovirus, Ad-ΔB7. The mouse infected with Ad-ΔB7-shMot showed higher survival rate than the mouse infected with Ad-ΔB7. H&E staining of tumor infected with Ad-ΔB7-shMot showed broad necrotic lesion but PBS or Ad-ΔB7 treated tumor showed little necrotic lesion. TUNEL assay showed that Ad-ΔB7-shMot significantly increased apoptotic cells and in contrast, PBS or Ad-ΔB7 treated tumor showed only few apoptotic cells. We also examined for micro-vessel density of the tumors. Ad-ΔB7-shMot treated tumor showed marked decrease in vessel liked structures in Ad-ΔB7-shMot treated tumors compared with PBS- or Ad-ΔB7-treated tumors. Ad-ΔB7-shMot could suppress expression of Mortalin, formation of micro-vessel and induce apoptosis in xenograft tumor induced by MCF7-Mortalin F, which explain inhibition of tumor growth and

survival benefit.

Taken collectively, these findings suggested that non-mitochondrial Mortalin plays important role for malignancy of cancer cells. The oncolytic recombinant adenovirus expressing shRNA Mortalin potently knocked down Mortalin expression and inhibited growth of xenograft tumors derived from MCF7-Mortalin. The oncolytic recombinant adenovirus expressing shRNA against Mortalin may be useful as an anti-cancer gene therapeutic agent.

V. CONCLUSION

I found that Mortalin inhibits nuclear translocation of p53 and increases expression and activity of hTERT by molecular interaction. Moreover, the cancer cells overexpressing non-mitochondrial Mortalin form aggressive tumor and metastatic tumor nodules. The oncolytic recombinant adenovirus expressing shRNA Mortalin enhances the anti-tumor effect and survival rate through suppressing expression of Mortalin, inducing apoptosis and suppressing formation of micro-vessel. These results suggest that Mortalin may be useful target for anti-cancer therapy.

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ABSTRACT (IN KOREAN)

Mortalin 이 암세포의 악성화에 미치는 영향 및 작용기전 연구: Mortalin 특이적 shRNA 를 발현하는 아데노바이러스의 항종양 효과

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류 지 훈

Mortalin 은 heat shock protein 70 (hsp70) family 에 속하는 단백질이다. Mortali 은 다양한 암세포에서 과발현되고, 암의 발생과 악성화에 밀접한 연관이 있다고 보고되어지고 있다. 또한, Mortalin 은 미토콘드리아 뿐만 아니라 세포내 여러 위치에서 발견되며 많은 단백질들과 상호작용을 통해 다양한 기능을 수행한다. 본 저자는 시그널펩티드를 제거한 변형 Mortalin 이 미토콘드리아를 제외한 세포질과 핵에 존재하고, 상호작용을 통해 p53 이 핵으로 이동하는 것을 저해하며, hTERT 의 발현 및 활성을 증가시키는 것을 확인하였다. 변형 Mortalin 을 과발현하는

암세포는 빠른 종양형성과 성장을 보였고, 다발성 폐전이 또한 관찰할 수 있었다.

암세포에서 Mortalin 의 발현을 억제하기 위해 shRNA Mortalin 을 발현하는 종양 선택적 살상 아데노바이러스 (Ad-ΔB7-shMot)을 제작하였다. Ad-ΔB7-shMot 을 암세포에 감염시킨 결과 암세포 내 Mortalin 의 발현이 감소되었고, 암세포의 살상능이 증가하였다. 항종양 효과를 확인한 동물실험에서 Ad-ΔB7-shMot 을 처리한 실험군의 종양 성장이 억제되었고, 실험 동물이 높은 생존율을 보였다. 게다가, 종양 조직의 괴사 및 세포고사가 증가되었으며 신생혈관의 생성이 억제되었다.

본 저자의 연구결과는 미토콘드리아 밖에 발현되는 Mortalin 이 암세포의 악성도에 영향을 미칠 수 있음을 보여주었다. 또한, 종양 선택적 살상 아데노바이러스를 이용하여 암세포 내에서 Mortalin 의 발현을 억제하였을 때, 항종양 효과가 증가함을 관찰할 수 있었다. 따라서 Mortalin 은 암 치료에 사용될 수 있는 표적인자이다.

핵심 되는 말: Mortalin, 미토콘드리아, p53, siRNA, 종양선택적 살상 아데노바이러스, 세포고사, 암 치료

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