Identification and Functional Characterization of Nuclear Mortalin in Human Carcinogenesis

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Running title: Nuclear mortalin promotes tumorigenesis

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Background: Mortalin/mtHsp70 is an essential stress chaperone frequently enriched in cancers. Mortalin is present in the nucleus of cancer cells where it causes (i) strong inactivation of tumor suppressor protein p53 and (ii) activation of telomerase and heterogeneous ribonucleoprotein-K (hnRNP-K) proteins.

Results: Mortalin is an essential stress chaperone that is frequently enriched in cancer cells and exists in various subcellular sites including mitochondria, plasma membrane, endoplasmic reticulum (ER) and cytosol. Although, molecular mechanisms underlying its multiple subcellular localizations are not yet clear, their functional significance has been revealed by several studies. In the present study, we examined the nuclear fractions of human cells and found that the malignantly transformed cells have more mortalin than the normal cells. We then generated mortalin mutant that lacked mitochondrial targeting signal peptide. It was largely localized in the nucleus and hence called nuclear mortalin (mot-N). Functional characterization of mot-N revealed that it efficiently protects cancer cells against endogenous and exogenous oxidative stress. Furthermore, as compared to the full-length mortalin (mot-F) overexpressing cancer cells, mot-N derivatives showed increased malignant properties including higher proliferation rate, colony forming efficacy, motility and tumor forming capacity both in vitro and in vivo assays. We demonstrate that mot-N promotes carcinogenesis and cancer cell metastasis by (i) inactivation of tumor suppressor protein p53 functions and (ii) interaction and functional activation of telomerase and heterogeneous ribonucleoprotein-K (hnRNP-K) proteins.

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Mortalin/mtHsp70/Grp75 is a member of the Hsp70 family of proteins (1) that plays essential role in the mitochondrial import, oxidative stress response, regulation of mitochondrial membrane potential, energy generation, intracellular transport, chaperonization, p53 functions, immune response, protection against apoptosis and tumorigenesis (2-8). Mortalin and p53 co-localize and interact in transformed, but not in normal, human cells resulting in the nuclear exclusion and transcriptional inactivation of p53 (7, 9-11). Malignant transformation of NIH 3T3, life span extension of MRC-5 and attenuation of differentiation of HL-60 cells by overexpression of mortalin (mot-2) was attributed, at least in part, to transcriptional inactivation of p53 (12-14). Furthermore, it was shown to inhibit the p53-dependent suppression of centrosome duplication leading to aneuploidy (an established hallmark of cancer cells) (6, 15). Walker et al have reported that whereas mortalin and p53 proteins formed complex in the cytoplasm of leukemic clam hemocytes, normal hemocytes lacked this interaction. Treatment of leukemic clam hemocytes with MKT-077, a cationic mitochondriotropic dye that was shown to target mortalin-p53 interaction (16, 17), resulted in the translocation and reactivation of p53 in clam cells (11). These data implied that mortalin-mediated inactivation of p53 is an evolutionarily conserved feature of cancers.

Expression profile of mortalin in normal and a variety of immortal and tumorigenic cell lines revealed its bi-phasic behavior: an initial elevation during immortalization (relative to a down-regulation during replicative senescence of human fibroblasts) followed by an up-regulation at a later stage that coincides with the acquisition of an invasive phenotype (18-20). In proteomic analysis of cancer tissue arrays, mortalin was identified as a prognostic marker of colorectal cancers (21, 22). Associated with its phosphorylation, mortalin is known to show enhanced binding with FGF-1 and involved in regulation of its mitogenic activity (23). It has been shown that whereas cancers are frequently associated with higher level of mortalin expression, Alzheimer’s and Parkinson’s pathologies involve loss of mortalin and imbalance in mitochondrial homeostasis (3, 24-27). Overexpression of mortalin in experimental models of these diseases resulted in improvement of disease phenotypes and protection against the oxidative stress, a hallmark of these dementias (24-26, 28, 29).

In line with the role of mortalin in carcinogenesis, anti-mortalin molecules such as, antisense, ribozyme, siRNA, p53-antagonist polypeptides and chemicals that abrogated mortalin-p53 interaction and caused relocation of p53 to cell nucleus resulted in growth arrest/apoptosis of cancer cells (2, 4, 6, 30). Mortalin targeting adenovoncolytic virus caused tumor suppression in vivo by activation of p53, induction of apoptosis and inhibition of angiogenesis (31). Furthermore, up-regulation of mortalin was correlated with an early recurrence of hepatocarcinoma in post-operative patients and liver cancer metastasis (32) suggesting that anti-mortalin molecules, not only serve as anticancer agents, but could also be potentially very important in prevention of cancer recurrence. Altogether, these reports have necessitated the investigations on the molecular mechanisms of the roles of mortalin in human tumorigenesis.

Mortalin has been reported to exist in multiple subcellular localizations including mitochondria, ER, plasma membrane, cytosol and centrosomes (6, 15, 27, 33, 34). Recently, Rozenberg et al have reported circulating mortalin in serum of colorectal cancer patients and its elevated levels (>60 ng/ml) were assigned as risk factor for shorter survival (22). On the other hand, Shih et al reported that the nuclear translocation of mortalin is critically involved in neuronal cell differentiation (35). In light of these reports, we examined whether mortalin exists in the nucleus of human normal and transformed cells. We demonstrate that mortalin is present in the nucleus of cancer cells where it promotes tumor aggressiveness by mechanisms involving inactivation of p53 functions, activation of telomerase, hnRNPK and MMPs.

**EXPERIMENTAL PROCEDURES**

**Cell Culture And Fractionation**

Normal human fibroblasts (MRC5, TIG-1...
and WI-38), breast carcinoma (MCF7, MDA-MB-231, T47D), osteosarcoma (U2OS and Saos-2), fibrosarcoma (HT1080), cervical carcinoma (HeLa), lung adenocarcinoma (A549), colon carcinoma (HCT116) and prostate carcinoma (DU145) were maintained in Dulbecco’s Modified Eagle’s Medium (DMEM; Gibco BRL, Grand Island, NY, USA) as described earlier (4). Cells were procured from Japanese Collection of Research Bioresources, Cell Bank, National Institute of Biomedical Innovation, Japan (MRC5, TIG-1, WI-38, HT1080, HeLa and A549), DS Pharma Biomedical Co. Ltd, Japan (MCF7, T47D, U2OS), Cell Resource Center for Biomedical Research, Institute of Development Aging and Cancer, Tohoku University, Japan (DU145). Nuclear and mitochondrial fractions were prepared using the Qproteome Cell Compartment kit and Mitochondria Isolation kit, respectively (Qiagen, Hilden, Germany).

Cell Proliferation Rate And Oxidative Stress Response - Cell proliferation was measured by MTT assay (Invitrogen, Carlsbad, CA) using 96-well plate (10^3 cells/well). For oxidative stress, cells were treated with 300 µM (MCF7) or 1.0 mM (U2OS) hydrogen peroxide (2 h), followed by recovery in the fresh media (48-72 h). All the assays were performed independently and at least three times.

Detection of Reactive Oxygen Species - Cells were cultured on glass coverslips placed in 12-well plates and stained for ROS by fluorescent staining using the Image-iTTM LIVE Green Reactive Oxygen Species (ROS) Detection Kit (Molecular Probes, Eugene, OR). Images were captured on a Zeiss Axiovert 200M microscope and analyzed by AxioVision 4.6 software (Carl Zeiss). To examine the presence of mortalin in the nucleus, images were acquired with a Zeiss LSM 700 confocal microscope. The files were transferred to a graphic workstation and analyzed with the ‘Imaris’ software (Bitplane, Zurich, Switzerland).

Immunohistochemistry - Paraffin-embedded cancer and normal ovarian tissue slides were purchased from BioChain Institute, Inc. (Newark, CA, USA). Slides were deparaffinized in xylene and incubated with anti-mortalin antibody. For detection of antibody binding, the REAL-HRP system (Dako REAL™ EnVision™ Detection System, Peroxidase/DAB+, Rabbit/Mouse (DAKO, Carpinteria, CA) were used. The color was developed using Liquid DAB+ Substrate Chromogen System (DAKO) followed by counterstaining with masto’s hematoxylin (WAKO, Tokyo, Japan). Slides were examined using a fluorescence microscope (BZ-9000, Keyence, Tokyo, Japan), and images were obtained using BZ-II Analyzer software (Keyence).
Construction of Mortalin Mutants and Cell Lines - Full-length mortalin and its three deletion mutants (mot-F, full-length mortalin; mot-N, amino acid residues 42-679; mot-A, N-terminal amino acid residues 1-180; mot-B, amino acid residues 180-300) were generated by PCR and cloning into the Hind III site of the vector pCX4neo. Retroviruses were produced in Plat-E cells as described previously (18). The stably infected cells were maintained in 100 µg/ml G418-supplemented medium. V5-tagged full length and signal peptide lacking deletion mutant were generated using pcDNA3.1 plasmid vector. Transfections were performed using X-tremeGENE 9 DNA Transfection Reagent (Roche, Indianapolis, IN) following manufacturer’s instructions.

Colonies Forming Assay - Cell transformation assay was performed using Cell Transformation Detection Kit (CHEMICON, CA). Colony forming efficacy is expressed as the mean number of colonies from three independent experiments. For colony forming assays, 1x10^5 cells were seeded on 6-well plate. Cells were maintained until the appearance of colonies with regular change of medium. Colonies were fixed in methanol, stained with 0.5% crystal violet, photographed and counted.

Chemotaxis and Cell Invasion Assay - Cells at 60-70% confluency were trypsinized and re-suspended in DMEM. 2x10^5 cells were plated in 12 mm-pored Transwell inserts for Chemotaxis assay (Costar, MA). Fibronectin from human plasma (Sigma, MO) was used as a chemo-attractant. Cell invasion assay was performed using QCM™ 24-Well Cell Invasion Assay kit (Millipore). The fluorescence of invaded cells was read using a microplate reader (Infinity 200 PRO; Tecan Group Ltd.) with 480/520 nm filter set.

Nude Mice Tumor Assay - Balb/c nude mice (4 weeks old, female; Nihon Clea, Japan) were given subcutaneous injections of cells (1x10^6 suspended in 0.2 ml of growth medium). Tumor formation was monitored for a month. The assay was regarded as positive if tumors appeared and grew progressively. For metastasis assay, cells were injected intravenously into the lateral tail vein of Balb/c nude mice. Two weeks after injection, lungs were harvested and screened for tumor nodules. Five mice were used for each group and the experiment was repeated twice.

p53 Dependent Reporter Assays - Control, mot-F and mot-N cells (1x10^5/well) were plated in 6-well plate and transfected with 1 µg of pGL13-luc plasmid vector containing firefly luciferase gene and p53 responsive elements. pRL-TK (Promega, Madison, WI) vector was used as transfection efficiency control. Cells were lysed and luciferase activity was measured using the dual-luciferase reporter assay system (Promega) and microplate reader (Infinity 200 PRO; Tecan Group Ltd.).

Telomere Repeat Amplification Protocol (TRAP) Assay - TRAP assay (for semi-quantitative detection of telomerase activity) was performed using the TeloTAGGG Telomerase PCR ELISA kit (Roche Applied Science, Mannheim, Germany). Cell lysates were prepared in lysis reagent from cultured (2x10^5) cells. The supernatant collected after centrifugation at 16,000 x g for 20 min at 4°C was used for TRAP assay. Telomerase activity in the sample was calculated as units of activity in relative to the positive control (MRC5 cells transfected with hTERT). U2OS cells that lack telomerase activity were used as negative control. All the assays were performed in triplicate.

Co-immunoprecipitation and Immunodepletion Assays - For co-immunoprecipitation, cell lysates (300 µg protein) in 300 µl RIPA buffer were incubated at 4°C for 1-2 h with anti-myc or anti-hTERT or anti-mortalin antibody (as indicated). Immunocomplexes were separated by incubation with Protein-A/G Sepharose (Santa Cruz Biotechnology), and Western blotting was performed with the indicated antibodies using procedure described above. For immunodepletion of hTERT, cell lysates were incubated with anti-telomerase antibody (a kind gift from Dr. Reddel, CMRI, Australia). Immunoprecipitated hTERT was separated by binding of IgG with the Protein-A/G Sepharose beads. The supernatant was incubated with the anti-hTERT antibody again and the process was repeated four times.
times. An aliquot of the supernatant after each round of immunoprecipitation was resolved on a SDS gel and was processed for Western blotting with anti-mortalin antibody. Control immunodepletion was performed with isotype matched irrelevant pre-immune IgG.

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

**Results and Discussion**

Mortalin is Present in The Nucleus of Cancer Cells - Cytoplasmic and nuclear fractions of several human cancer cell lines were Western blotted with anti-mortalin antibody. As shown in Fig. 1A, mortalin was enriched in cancer cells and was detected in their nuclear fractions. In order to rule out the cross contamination of subcellular fractions, the blots were probed with antibodies to α-tubulin (for cytoplasmic fractions) and lamin A/C (for nuclear fractions) (Figs. 1A and 1B). Furthermore, we performed high-resolution single cell image analysis that confirmed the presence of mortalin in the nuclei of cancer cells (Figs. 1C and 1D), and raised the proposition that nuclear mortalin may functionally contribute to the cancer properties.

Nuclear Mortalin Possesses Pro-Proliferative Function and Protects The Cells Against Oxidative Stress - In order to delineate the functional significance of nuclear mortalin in cancer cells, we generated its mutant that lacked the N-terminal 41 amino acids, mitochondrial targeting signal peptide sequence. Retrospliac expression constructs encoding mot-F (full-length protein 1-679 residues) and mot-N (amino acid residues 42-679) tagged with myc epitope were transduced into the non-malignant MCF7 breast cancer cells. Expression of mot-F and mot-N proteins was detected by Western blotting with anti-myc and anti-mortalin antibodies. As shown in Fig. 2A-C, Western blotting and immunostaining of the control and infected cells revealed that (i) the mot-F and mot-N proteins were equally expressed, and could be distinguished from the endogenous mortalin (Fig. 2B) and (ii) mot-N was efficiently translocated to the nucleus (Fig. 2C). Similar results were obtained when V5-tagged mot-F and mot-N proteins were expressed in MCF7 and U2OS cells (Figs. 2D and 2E). High-resolution 3D imaging confirmed the presence of mot-F and mot-N in the nucleus; mot-N was more than mot-F. We next performed the fractionation of the transduced cells. Western blotting of cell fractions with anti-myc and anti-mortalin antibodies also revealed that the mot-F localized in the cytosol, microsomal and mitochondrial fractions (Fig. 2F, lanes 2). On the other hand, only a fraction of the total mot-N protein in the cytosol fractions and none in the microsomal and mitochondrial fractions was detected (Fig. 2F, lanes 3 and data not shown). Examination of the nuclear fractions confirmed the nuclear localization of mot-N (Fig. 2G). Similar results were obtained using different cell lines and in independent experiments. Of note, we found that exogenously expressed full-length mortalin, mot-F, was also detected in the nuclear fractions endorsing that the nuclear translocation of mortalin is an innate phenomenon in cancer cells as also supported by data in Fig. 1.

We next examined the effect of mot-F (mimics the endogenous mortalin localized at multiple subcellular sites) and mot-N (nuclear enriched) on cancer cell properties. Multiple clones with stable expression of mot-F and mot-N were isolated, and first examined the level of mortalin expression for 2-3 passages (Fig. 3A). The clones were then subjected to cell proliferation, oxidative stress and malignant transformation assays. As shown in Fig. 3B, mortalin overexpressing cells showed higher proliferation rate as compared to the untransfected/vector transfected (control) cells. Furthermore, mot-N derivatives showed higher proliferation as compared to the mot-F derivatives (Figs. 3B and 3C and data not shown). In order to further resolve the effect of mot-N on cell proliferation, we isolated low and high mot-N expression clones. As shown in Figs. 3C and 3D, dose dependent increase in cell proliferation was detected. Cells were examined for their endogenous and exogenous oxidative stress.
tolerance by quantitation of Reactive Oxygen Species (ROS). As shown in Fig. 3E, mot-F and mot-N overexpressing cells showed significantly lower ROS level (3 fold) suggesting decrease in the level of oxidative stress in these cells as compared to the untransfected as well as vector transfected (control) cells. Furthermore, mot-F and mot-N overexpressing cells when exposed to oxidative stress by incubating in H2O2-supplemented medium showed better survival than the control cells suggesting their increased tolerance to exogenous oxidative stress (Fig. 3F). In both the assays, mot-N cells were more tolerant than the mot-F cells. Similar data were obtained in U2OS and MCF7 cells (Figs. 3E and 3F).

In vitro and in vivo cell transformation assays revealed the malignant transformation capacity of mot-F and mot-N overexpressing cells (Fig. 4, A-C). Overexpression of mot-F caused malignant transformation of cancer cells as detected by subcutaneous, bone and breast xenograft models of nude mice tumor formation assay (12, 18 and data not shown). Furthermore, we found that the mot-N derivatives were more aggressively transformed than mot-F cells (Fig. 4). The mot-N derivatives showed rounded morphology (Fig. 4A-a), higher anchorage-independent growth capability (Fig. 4A, b) and higher colony forming efficiency (Fig. 4A, c and d), as compared to the mot-F derivatives. Cell migration assays revealed higher migration (Fig. 4B, a) and invasion capacity (Fig. 4B, b and c) of mot-N cells. In vivo tumor forming capacity assay in nude mice revealed that mot-N derivatives were aggressively transformed. They formed tumors rapidly and aggressively as compared to the mot-F derivatives (Figs. 4C, a and 4D). Furthermore, when MCF7/mot-F and MCF7/mot-N cells were injected in the opposite flanks of the same mouse, MCF7/mot-N tumors appeared earlier and rapidly increased in size as compared to the MCF7/mot-F tumors (Fig. 4C, b). Some mice injected with mot-N derivatives showed multiple tumors all over the injected side of the body (Fig. 4C, c). Furthermore, consistent with the in vitro migration assay data, MCF7/mot-N cells showed aggressive lung metastasis in vivo (tail vein injection assays) models (Figs. 4E). Taken together, the data demonstrated that mot-N has pro-proliferative function and it contributed to the malignant transformation of cancer cells.

Nuclear Mortalin Contributes to Malignant Transformation by Strong Inactivation of p53 - Mortalin has been shown to inactivate p53 by sequestering it in the cytoplasm, and also by binding it in the nucleus at G1 stage of the cell cycle where it inactivates its control of centrosome duplication function (4, 6, 9, 10, 15). In light of the present finding that mortalin exists in the nucleus of malignantly transformed cancer cells, we investigated the impact of mot-F and mot-N on p53 function by binding and activity assays. For this study, two more deletion mutants (i) mot-A, N-terminal amino acid residues 1-180 that lack p53 binding region, and (ii) mot-B, amino acid residues 180-300, containing the p53 binding region were generated. Similar to mot-F and mot-N, these constructs were designed to have myc tag at the carboxy-terminus (Fig. 5A). The cells infected with mot-F, mot-N, mot-A and mot-B were examined for nuclear translocation of p53 in response to DNA damage stress in U2OS cells that harbor wild type p53 function. As shown in Fig. 5B, control cells showed that the etoposide caused nuclear translocation of p53 (green) as seen by its intense nuclear staining and absence of p53 (green) in cytoplasm (Fig. 5B, compare a with b). Cells infected with mot-F, mot-N and mot-B strongly inhibited the nuclear translocation of p53 (green) as seen by its intense nuclear staining and absence of p53 (green) in cytoplasm (Fig. 5B, compare a with b). Mutant mot-A (that lacked p53 binding region of mortalin) -infected cells did not show any difference from the control etoposide-treated cells, and there was no co-localization of mortalin and p53 in the cytoplasm (Fig. 5B, b and e). In order to quantitate this effect, we counted the cells with very intense p53 staining (Fig. 5C) and found that mot-F, mot-N and mot-B strongly inhibited the nuclear translocation of p53. Of note, we found that the inactivation of p53 by mot-N was significantly higher than mot-F. Similar results were obtained in etoposide-treated MCF7 cells overexpressing either mot-F or mot-N (data not shown).
We anticipated that the stronger inactivation of p53 by mot-N may explain increased malignant characteristics of cancer cells. To investigate this proposition, we examined the binding of p53 to mot-F and mot-N derivatives by co-immunoprecipitation assays. As shown in Fig. 5D, the amount of p53 precipitated with mot-N was more than mot-F, suggesting higher complex formation between p53 and mot-N (Fig. 5D). Furthermore, p53 dependent reporter assays on mot-F and mot-N derivatives of U2OS cells revealed stronger inactivation in the latter (Fig. 5E). Control of centrosome duplication by p53 has been shown to be regulated by p53-mortalin interaction at the centrosome and mitotic kinase, Aurora A (6, 15). Hence, we examined the localization of mot-F and mot-N at the centrosome by co-immunostaining of mortalin and Aurora A. As shown in Fig. 5F, mot-N cells showed enhanced co-localization with Aurora A. Furthermore, inactivation of the control of centrosome duplication function of p53 resulting into the aneuploid chromosomes as examined by γ-tubulin staining revealed higher aneuploidy in mot-F and mot-N derivatives as compared to the control MCF7; the aneuploidy score was higher in cells expressing mot-N as compared to the ones expressing mot-F protein (Fig. 5G and data not shown). These data suggested that the nuclear mortalin contributes to malignant properties of cancer cells through strong inactivation of p53 functions including transcriptional activation and control of centrosome duplication.

Nuclear Mortalin Contributes to Malignant Transformation by Activation of Telomerase and hnRNP-K - In view of nuclear localization of mortalin and its impact on cell proliferation, transformation and metastasis of cancer cells, we next examined its impact on two oncogenes, telomerase and hnRNP-K that have been shown to act as key regulators of tumorigenesis and metastasis, respectively. As shown in Figs. 6A and 6B, mot-F and mot-N cells showed higher level of telomerase protein (Fig. 6A) and activity (Fig. 6B) as compared to the control. Furthermore, telomerase activity was higher in mot-N derivatives than in mot-F cells. We next investigated whether the increase in telomerase activity was due to direct interaction with mortalin in the nucleus. Co-immunoprecipitation of hTERT and mortalin from the nuclear fractions of highly malignant cells, such as MCF7/mot-N and T47D, showed the presence of mortalin-hTERT complexes. Non-malignant cells, MCF7, Saos-2 and YKG-1, on the other hand, lacked mortalin-telomerase interaction (Fig. 6C and data not shown). In order to quantitate these interactions, we performed serial immunodepletion assays on mot-F and mot-N cell lysates. Serial immunodepletion of hTERT using a hTERT specific antibody caused parallel immunodepletion of mortalin (detected by anti-myc and anti-mortalin antibodies) in mot-F and mot-N cells (Fig. 6D) suggesting the direct interaction of telomerase and mortalin. These data demonstrated, for the first time, that mortalin interacts with telomerase in the nucleus, causes its stabilization and activation contributing to malignant transformation of cancer cells.

We next investigated the mechanism of increased metastatic properties of mot-F and mot-N cells (Figs. 7, A-D). Analyses of matrix metalloproteases (well established markers of cancer cell metastasis) in MCF7, MCF7/mot-F and MCF7/mot-N cells revealed statistically significant increase in the level of MMP-2, -3 and -9 expression (Fig. 7A). Furthermore, we investigated whether the increase in MMPs was due to activation of their upstream regulator, heterogeneous nuclear ribonucleoprotein K (hnRNP-K) (36-38) by nuclear mortalin. As shown in Fig. 7A, MCF7/mot-F and mot-N cells showed higher level of expression of hnRNP-K that matched well with their high migratory characteristics in vitro and in vivo (Fig. 4). In order to investigate the crosstalk between mortalin and hnRNP-K, we also generated hnRNP-K overexpressing cells and examined the level of mortalin expression. We found that the hnRNP-K overexpressing cells possessed highly malignant characteristics (39). However, as shown in Fig. 7B, the level of mortalin expression in these cells remained unchanged as compared to the parent cells suggesting that mortalin may work upstream of hnRNP-K in promoting the cancer metastasis. Co-immunoprecipitation of mortalin and hnRNP-K showed that the two proteins form a complex (Fig. 7C), and an
increased level of expression of hnRNP-K in mortalin overexpressing cells might be the result of its stabilization and protection from HDM2-mediated degradation (40). In support, immunostaining revealed that the significantly high proportion of MCF7/mot-F and MCF7/mot-N cells have nuclear hnRNP-K more than 75% of either the MCF7/mot-F or MCF7/mot-N cells exhibited strong nuclear staining (Fig. 7D).

Telomerase is a ribonucleoprotein complex composed of the catalytic protein subunit hTERT (human telomerase reverse transcriptase) and RNA subunit hTR (human telomerase RNA) that serves as the template for the synthesis of telomeric DNA and is constitutively expressed in cells. Telomerase activity is closely associated with hTERT expression. In contrast to the normal somatic cells, most human tumor cells possess high level of hTERT expression. Several studies have demonstrated that the telomerase activity is required for the malignant properties of cancer cells and hence may serve as a good target for the development of anti-cancer drugs (41). Studies on the regulation of hTERT expression have revealed that it is regulated by trans-regulatory factors including Sp1/Sp3, AP-2β, Myc/Max heterodimer, HIF-2α, TAK1, MSH2, GRHL2, p53, HER2, hnRNP-K and hnRNP-D (42-44) that show physical interactions with the hTERT promoter. It was earlier shown that mortalin overexpression cooperates with telomerase to extend the in vitro lifespan of normal human fibroblasts (13). Taken together with the present data, it is concluded that nuclear mortalin interacts with telomerase and activates its function contributing to proliferation and malignant characteristics of cancer cells.

hnRNP-K is a member of the family of about 20 hnRNP proteins that are ubiquitously expressed, complex with heterogeneous nuclear RNA (hnRNA) and influence pre-mRNA processing and transport involved in transcription and post-transcriptional messenger RNA metabolism. hnRNP-K is known to regulate the multitude of gene expression, including p53 (40), as a transcription factor or by alteration of mRNA stability and translation (45). Kang et al have shown that hnRNP-K physically associates with hTERT promoter in vitro and in vivo (44). Although the knockdown of hnRNP-K did not affect the telomerase activity, its knockdown along with GRHL2 resulted in an inhibition of telomerase function in oral squamous cell carcinoma (44). Consistent with its telomerase activity enhancing function, hnRNP-K was enriched in human oral squamous cell carcinoma (OSCC) cells than in the normal cells (lack telomerase activity). hnRNP proteins are known to shuttle between nucleus and cytoplasm and have a role during cell cycle progression. Cytoplasmic accumulation that is required for the ability of hnRNP-K to silence mRNA translation is dependent on phosphorylation mediated by MAPK/ERK (46). Several studies have reported up-regulation of hnRNP-K and its altered subcellular localization in tumors (47). Higher level of nuclear hnRNP-K has been reported in proliferating compared to resting cells. In contrast, the level of cytoplasmic hnRNP-K protein was either the same or lower in dividing as compared to the quiescent cells. States of enhanced proliferation were also associated with increased phosphorylation of hnRNP-K. Based on these findings, it was suggested that the nuclear hnRNP-K is involved cell proliferation-regulatory signaling (48). It is modified in response to changes in intracellular and extracellular environment including cytokines, growth factors and oxidative stress (49) and bridges signal transduction pathways to chromatin remodeling and nucleic acid directed processes. hnRNP-K has also been shown to bind to the 3’ UTR of VEGF mRNA and regulate its translation (50) and in turn would affect the expression of matrix metalloproteinases (MMPs). Mot-F and Mot-N cells showed increase in the level of expression of MMP-2, -3 and -9. MMPs are up-regulated in many tumors. They cause epithelial-mesenchymal transition, malignant transformation and genomic instability by mechanisms involving articulation of tumor environment, oxidative stress and induction of the transcription factors.

In order to further get insights into clinical relevance of nuclear mortalin in cancer, we examined the normal and tumor (ovary, kidney, lung, liver and brain) tissues
by mortalin immunohistochemistry. Mortalin was found to be highly expressed and enriched in the nuclei of all the tumor tissues examined as compared to the controls (Fig. 7E and data not shown). Similar increase in mortalin has also been reported in liver cancers and melanoma biopsies (2, 10, 51). In agreement with the role of nuclear mortalin in tumors, human normal lung fibroblasts lacked endogenous nuclear mortalin staining (data not shown). Furthermore, although the exogenous mot-F and mot-N were localized in the nuclei, the cells neither showed increase in proliferation, transformation nor malignant properties (data not shown). The unaltered phenotype of normal cells could be due to the lack of mortalin-p53 interaction (9), telomerase and hnRNP-K proteins (39-41). Taken together, the present study demonstrates, for the first time, a new line of action of stress chaperone mortalin in human carcinogenesis. In addition to its previously described cytoplasmic sequestration and inactivation of p53, it localizes in the nucleus, inactivates p53-mediated control of centrosome duplication causing genomic instability, activate telomere maintaining enzyme hTERT (a hallmark of most cancers) and a multifunctional chromatin-remodeling protein hnRNP-K involved in regulation cell migration, and thus promotes carcinogenesis.

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LEGENDS TO THE FIGURES

FIGURE 1. Mortalin is expressed in the nucleus of cancer cells. (A) Western blotting of the cytoplasmic and nuclear cell fractions with anti-mortalin antibody showed its presence in the nuclear fractions. Cytoplasmic protein, α-tubulin and nuclear protein, lamin A/C were used as respective controls for the cytoplasmic and nuclear fractions. (B) Quantitation of the signals, SD and statistical significance from three independent experiments. (C) High-resolution spectral imaging showing mortalin in the cross-section of the nucleus (right bottom) of T47D cells. Left top, immunostaining of mortalin (green); nuclei were stained with Hoechst (blue). White lines in the left-bottom, right-top and -bottom panels show the cross section points. (D) Western blotting analysis of cytoplasmic and nuclear cell fractions of T47D cells with anti-mortalin antibody showing the presence of mortalin predominantly in the nuclear fraction of cancer cells.

FIGURE 2. Subcellular localization of native and exogenously expressed, normal and mutant, mortalin. (A) Schematic representation of mortalin mutants. (B) Expression of exogenously expressed full-length mortalin (mot-F) and its mutant lacking the N-terminal signal peptide (mot-N) as detected by anti-myc antibody. (C) Immunostaining of mortalin with anti-mortalin antibody (green) in mot-F and mot-N transduced cells. Actin was used as a control. Enlarged images, on the right, showing predominant localization of mot-N in the cell nuclei. Scale bar; 10 µm. (D and E) Immunostaining of V5 tagged mot-F and mot-N transfected cells with anti-V5 antibody (green) in MCF7 (D) and U2OS (E) cells. Nuclei were stained blue with Hoechst 33342. White and red arrows point to the weak and strong nuclear staining in mot-F/V5 and mot-N/V5 transfected cells, respectively. (E) 3D image and cut section of the V5 stained U2OS cell showing the presence of V5-tagged mot-N in the nucleus. (F and G) Western blots showing mortalin in the subcellular fractions detected by anti-myc and anti-mortalin antibodies. Mot-F (lane 2), but not Mot-N (lane 3), was localized in the microsomal and mitochondrial fractions (F). Mot-N (lane 3) was detected in the cytosol (F) only. (G) Western blotting of cytoplasmic and nuclear fractions of mot-F and mot-N cells revealed that the transfected mot-F protein also localized in the nucleus; although less than mot-N protein. α-tubulin and lamin A/C were used as internal controls for loading and purity of cytoplasmic and nuclear fractions, respectively.

FIGURE 3. Nuclear mortalin contributes to pro-proliferative and oxidative stress tolerance in cancer cells. (A) Expression level of transduced mot-F and mot-N protein in several independent clones as examined by Western blotting. All isolated clones (5 each) showed comparable level of expression. (B) Comparison of the growth rate of control, mot-F and mot-N derivatives, as determined by time-dependent increase in cell number (48 h post-plating) and (C) Average proliferation capacity of control, mot-F and mot-N derivatives showed increased proliferation capacity of mot-N cells as compared to the control and mot-F cells. (D) Proliferation rate of cells corresponding to low and high level of mot-N expression. (E) Endogenous level of oxidative stress as detected by ROS levels showed low level of oxidative stress in mot-N cells; quantitation of the data from three independent experiments in U2OS and MCF7 is shown. Scale bar, 20 µm. (F) Response to the exogenous oxidative stress as evaluated by exposure of cells to hydrogen peroxide showed better stress tolerance in mot-N cells as compared to the control and mot-F cells.

FIGURE 4. Nuclear mortalin contributes to pro-proliferative and malignant characteristics of cancer cells. (A) Effect of exogenous expression of mot-F and mot-N on cell morphology (a), anchorage independent growth of cells (b), colony-forming efficacy (c and d). (B) Cell migration assay (a) and cell invasion assay (b-c) showing increased migration of MCF7/mot-N as compared to MCF7/mot-F cells. (C) Nude mice tumor formation for MCF7, MCF7/mot-F and MCF7/mot-N cells (a) and aggressiveness of mot-N as compared to the mot-F tumors (b and c). (D) Average tumor volume of mot-F and mot-N
derivatives of MCF7 is shown; MCF7 untransduced cells did not form tumors. (E) In vivo lung metastasis due to MCF7, MCF7/mot-F and MCF7/mot-B cells showing significantly higher lung metastasis in MCF/mot-N cells.

FIGURE 5. **Nuclear mortalin causes strong inactivation of p53.** (A) Schematic representation of mortalin mutants (B) Overexpression of mortalin and its p53-binding mutants inhibited etoposide-induced nuclear translocation of p53. Double immunostaining with anti-p53 (green) and anti-mortalin (red) antibodies showing nuclear translocation of p53 in control, mot-A mutant transected etoposide-treated cells. Mot-F, -N and -B transduced cells showed cytoplasmic retention of p53 as seen by cytoplasmic staining that is co-localized with mortalin and hence seen as yellow. (C) Quantitation of cells with etoposide-induced nuclear translocation of p53. (D) Co-immunoprecipitation of mot-F and mot-N with p53. Immunoprecipitated mortalin-myc by anti-myc antibody (myc-IP) was examined for the presence of p53. Control immunoprecipitation was performed with isotype matched IgG. Quantitation of p53 precipitated with mortalin-myc is shown (E) p53 dependent reporter assay showed stronger inactivation of p53 activity in mot-N transduced cells. (F) Co-immunostaining of mortalin and Aurora A revealed stronger intensity of co-immunostaining (yellow signal) of the two proteins in MCF7/mot-N than MCF/mot-F cells. (G) Co-immunostaining of mortalin-myc and gamma-tubulin showing the presence of aneuploid cells in mot-F and mot-N cells; cell aneuploidy and anomalies score was higher in mot-N cells.

FIGURE 6. **Mortalin interacts with telomerase leading to activation of its function.** (A) Western blotting of hTERT showing higher level of hTERT expression in mot-N cells. (B) TRAP assay showing activation of telomerase in mot-F and mot-N cells. U2OS (an ALT cell line) and MRC5 cells transfected with hTERT were used as negative and positive controls, respectively. (C) Co-immunoprecipitation of mortalin and hTERT in which hTERT complexes were examined for the presence of mortalin. The latter was seen to co-immunoprecipitate with hTERT both in the whole cell lysates and the nuclear fractions of malignantly transformed cells. (D) Immunodepletion of hTERT by repeated immunoprecipitation (lower panel, lanes 1 to 4) of hTERT from cell lysates revealed decrease in mortalin both in mot-F and mot-N cells. Irrelevant IgG immunoprecipitation used as control did not show decrease in mortalin in serial immunodepletion rounds (upper panel, lanes 1 to 4).

FIGURE 7. **Mortalin interacts with hnRNPK and leads to activation of its downstream effectors MMP-2, -3 and -9.** (A) MCF7/mot-F and mot-N cells showed increase in MMP-2, MMP-3, MMP-9 and hnRNP-K. (B) hnRNP-K overexpression did not affect the level of mortalin. (C) Co-immunoprecipitation assay showing the presence of hnRNP-K in mortalin immunocomplexes. (D) Immunostaining of hnRNP-K revealed its enhanced nuclear localization in mot-F and mot-N cells. (E) Immunohistochemical detection of mortalin in human normal and cancer ovarian tissues. The increased level of mortalin expression and its enrichment in nuclei are seen in cancer tissue.
Identification and Functional Characterization of Nuclear Mortalin in Human Carcinogenesis
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