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The centrosomal localization of KM-HN-1 (MGC33607) depends on the leucine zipper motif and the C-terminal coiled-coil domain

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Abbreviations: γ-TuRC, gamma-tubulin ring complex; NES, nuclear export signal

Abstract

KM-HN-1 is a C-terminal coiled-coil domain containing protein previously referred to as image clone MGC33607. This protein has been previously identified as a cancer/testis antigen and reported as nuclear and chromatin localizing protein. We raised polyclonal antisera with the GST fusion protein and identified them as a 105 kDa protein. Motif analysis showed that this protein harbors the leucine zipper motif in internal 1/3 region and the coiled-coil domain in the C-terminal region. Using the full length and various deletion mutants, we determined the motif that governs the subcellular localization of KM-HN-1. Immunofluorescence staining of the endogenous KM-HN-1 and various kinds of GFP-tagged KM-HN-1 revealed that KM-HN-1 localizes to the centrosomes as well as nucleus. The centrosomal localization-determining region of this protein is C-terminal coiled-coil domain in which the leucine zipper motif and the nuclear export signal (NES) harbor.

Keywords: CCDC110 protein, human; cell nucleus; centrosome; protein structure, tertiary; protein transport

Introduction

KM-HN-1 (MGC33607) was identified as a novel human cancer/testis antigen, in an attempts to discover cancer-specific target genes for cancer therapeutics development. From the RT-PCR analysis of cancer tissues and cancer cell lines, the mRNA expression level of KM-HN-1 was shown to be increased in a variety of cancer types, including tongue cancer, melanoma, hepatocellullar carcinoma (Monji *et al.,* 2004; Condomines *et al.,* 2007). We have obtained a KM-HN-1 clone from a yeast two-hybrid screening, using hCdc14A as bait. hCdc14 is one of the human orthologues of budding yeast cdc14p. Cdc14 is a dual-specificity protein phosphatase, which counteracts the cdc2/cyclin B1. Cdc14 plays a key role in the mitotic exit network and the centrosome cycle, from budding yeasts to metazoans (Pereira and Schiebel, 2001; Trautmann and McCollum, 2002; Stegmeier and Amon, 2004). hCdc14A also performs a crucial function in centrosome duplication and the completion of mitosis in human cells (Kaiser *et al.,* 2002; Mailand *et al.,* 2002). The KM-HN-1 protein was recognized by cellular and humoral immune responses from a number of cancer patients suffering from a variety of cancer types. KM-HN-1 has another splicing variant in which 80-116 amino acids of KM-HN-1 are missing (Figure 1). KM-HN-1 harbors a C-terminal coiled-coil domain in which the C-terminal half of the SMC (structure maintenance of chromatin) motif resides. The SMC motif originates from SMC protein, which performs pivotal function during chromosome segregation (Cobbe and Heck, 2000). In addition to the coiled-coil domain KM-HN-1 harbors a bipartite NLS (nuclear localization signal) (Boulikas, 1993) followed by a leucine zipper region, in the internal 1/3 region (Figure 1). The leucine zipper motif is generally comprised of five repeats of leucine (L1, L2, L3, L4, L5) in seven amino acid intervals, and this motif is preceded by basic amino acids. The leucine zipper motif functions as a protein dimerization domain of a variety of DNA binding proteins (Landschulz *et al.,* 1988). We identified KM-HN-1 as a 105 kDa protein via immunoblotting followed by immunoprecipitation. The clones initially obtained evidenced centrosomal localization and colocalization with hCdc14A in the centrosome. However Monji *et al.* (2004) previously reported that KM-HN-1 is a nuclear protein which evidences colocalization with chromatin. In this study, we found that KM-HN-1 localize to centrosomes as

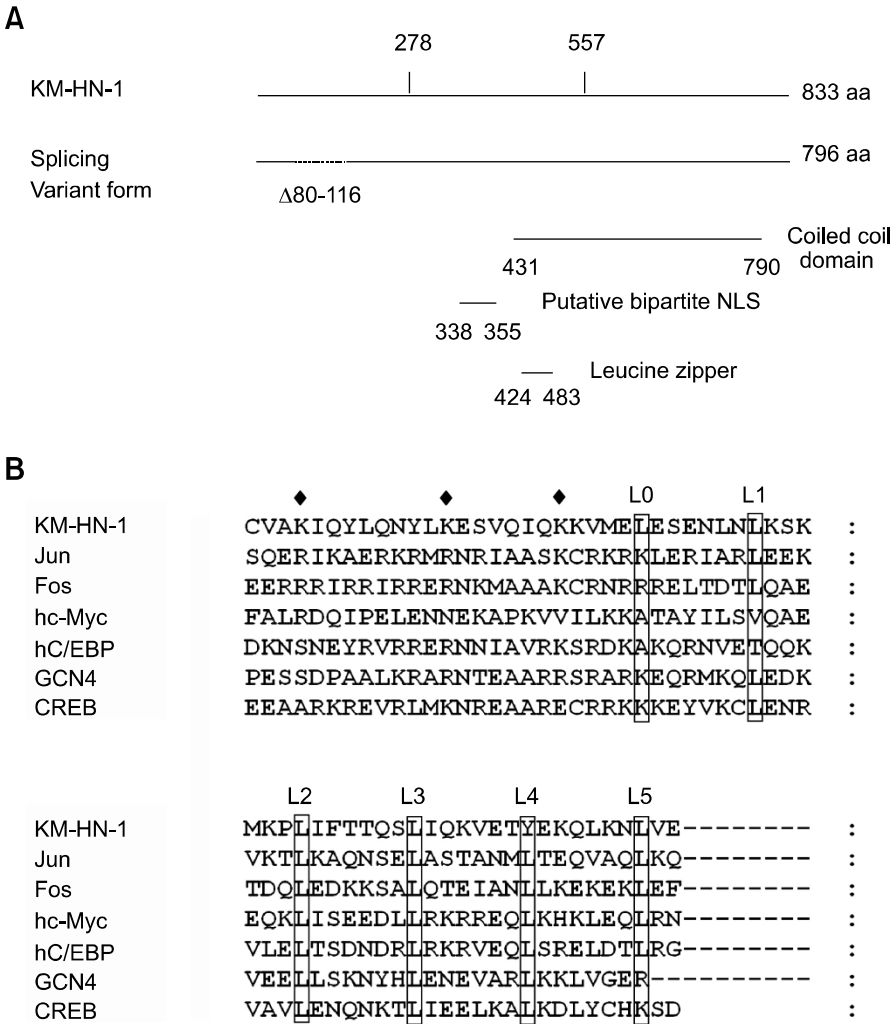

Figure 1. Schematic illustrations of KM-HN-1 and its splice variant and conserved motifs. (A) KM-HN-1 consists of 833 amino acid residues; the splicing variant (796 amino acid residues) has missing 80-116 of KM-HN-1. Both harbor a C-terminal coiled- coil region in which the SMC motif resides. The internal 1/3 region harbors leucine zipper (LZ) region preceded by a putative bipartite NLS. (B) Amino acid alignment of leucine zipper motif. KM-HN-1 has at least five leucine zipper repeats. Key leucine zipper domain molecule (L0-L5) region of KM-HN-1 and their aligned replacements are shaded. The closed diamond (◆) represents conserved basic amino acid residues.

well as nucleus. We also determined the region of KM-HN-1 critical to centrosomal localization, and other features of the intracellular localizations using KM-HN-1 deletion mutants.

Materials and Methods

Cell culture

A U-2 OS human osteosarcoma cell line (American Type Culture Collection HTB 96) and a 293 human embryonic kidney cell line (American Type Culture Collection, Rochville, MD) were maintained in DMEM supplemented with 10% FBS (HyClone Laboratories Inc., Logan, UT) and 100 U/ml of penicillin G plus 100 μg/ml of streptomycin (HyClone Laboratories Inc.).

Cloning of KM-HN-1 and construction of expression vectors

KM-HN-1 was initially obtained via yeast two-hybrid screening using hCdc14A as bait. The obtained gene was a partial c-terminal fragment clone. In order to obtain the full length KM-HN-1, we employed the PCR method. In addition to the PCR cloning of KM-HN-1 we purchased a full-length of IMAGE clone and verified it by DNA sequencing. The ORF of KM-HN-1 was further subcloned into pGEX (Pharmacia, Piscataway, NJ), pCIneo (Promega, Madison, WI), pEGFP (Clontech, Palo Alto, CA) and pShuttle-EGFP adenoviral shuttle vectors.

Northern blot analysis

Human multiple tissue Northern blots (hMTN) were purchased from Clontech. The blots were prehybridized with MyracleHyb (Stratagen, La Jolla,

CA) hybridization solution at 68°C for 2 h, and then hybridized with the same prehybridization solution containing ^{32}P-labeled KM- HN-1 probe for 6 h. The hybridized hMTN blots were washed in 2 × SSC containing 0.5% SDS twice for 15 min each and with 0.5 × SSC containing 0.5% SDS twice for 15 min each, then exposed to X-ray film (Kodak, Rochester, NY) for 24 h for autoradiography.

RT-PCR analysis of KM-HN-1

0.5 g of colon cancer tissues and surrounding normal tissues from surgical resection samples were used for RNA purification. Total RNA purification was conducted out using Trizol reagents (Invitrogen, San Diego, CA). After the precipitation of RNA with isopropanol, the RNA was dissolved in DEPC- treated water. For cDNA synthesis, 1 μg of total RNA was incubated in 20 μl of reaction mixture containing 2 μM oligo-dT primer (Bioneer, Daejeon, Korea), MMLV reverse transcriptase (Enzynomics, Korea), RNasin (Promega) and 0.2 mM NTP for 30 min at 42°C. After cDNA synthesis, PCR was performed for 30 cycles for the quantitation of KM-HN-1 and for 25 cycles for human actin. The sequences of the primers used were as follows: KM-HN-1 (380 bp product): forward, 5'-TACAAGAATCTACTGCCAGAC-3'; reverse, 5'-ATCTGTTTTGGTTTCCATTACCAT-3'. Human actin (160bp product): forward, 5'-ATCATGAAGTGTGACGTGGACAT-3'; reverse, 5'-AGGAGGAGCAATGATCTTGATCT-3'. The PCR-products were electrophoresed on 2.5% NuSieve 3:1 agarose (FMC).

Generation of antisera and immunoprecipitation

To generate recombinant KM-HN-1, the N-terminal half fragment (1-417) and C-terminal half fragment (418-833) were PCR-amplified and subcloned into pGEX4T-1 vector. After confirmation via DNA sequencing the recombinant GST fusion proteins were purified and immunized to rabbits in order to raise anti-sera. To purify the anti-KM-HN-N antibody, the immunized sera were first precleared with GST-bound GST agarose. The precleared anti-KM-HN-N antisera were further purified using GST-KM-HN-N (1-417) immobilized with Affigel-10 (Bio-Rad Laboratories). For immunoprecipitation, cultured cells were lysed in Tris-buffered saline containing 0.5% NP-40 and 0.5% sodium deoxycholate. The cell lysates were centrifuged at 15,000 × *g* for 15 min at 4°C. The supernatant was precleared with normal rabbit IgG complexed with protein-A agarose (Santa Cruz). The anti KM-HN-1 antisera bound by protein-A agarose were directly used for the immunoprecipitation of KM-HN-1.

Plasmid construction and generation of GFP tagged KM-HN-1 adenovirus

Recombinant adenoviruses were constructed according to the procedure described previously (He *et al.,* 1998; Seong *et al.,* 2002). In brief, coding areas of the full ORF (1-833, KM-HN-full), N-terminal 2/3 region (1-557, KM-HN-N1), N-terminal 1/3 region (1-278, KM-HN-N2), C-terminal 2/3 region (278-833, KM-HN-C1), C-terminal 1/3 region (557-833, KM-HN-C2) and internal 1/3 region (278-557, KM-HN-M) were PCR-amplified with Turbo *pfu* (Stratagene) and subcloned into the pShuttle-EGFP adenoviral shuttle vector. DNA sequencing and immunoblotting with anti-GFP antibody confirmed the construction of recombinant KM-HN-1. The appropriate recombinants were then digested with *Pme*1 and cotransformed into BJ5183 *E.coli* strain with the pAdEasy adenoviral backbone vector. The obtained recombinant adenoviral plasmids were transfected to 293 cells to generate KM-HN-1 expressing adenovirus. The expression of recombinant GFP fused KM-HN-1 was confirmed by immunoblotting using anti-GFP monoclonal antibody (JL-8, Clonetech).

Antibodies for indirect immunofluorescence staining

For indirect immunofluorescence staining the following proteins were visualized with the indicated antibodies and dilutions: KM-HN-1, 1:200 diluted affinity-purified anti KM-HN-1 antibody; γ-tubulin, 1:1,000 diluted GTU-88 mouse monoclonal antibody (Sigma). Secondary antibodies for immunofluorescence staining were AlexFluoro 488-conjugated goat anti rabbit IgG (Molecular Probes), Texas red conjugated goat anti-mouse IgG (Jackson Immunological Laboratories).

IF staining and confocal microscopy

U-2 OS cells were grown on Poly D-lysine (Sigma P0899) coated glass coverslips. After washing with PBS, the cells were fixed with PBS containing 3.7% formaldehyde solution, then the cells were permeabilized with PBS containing 0.5% Triton X-100. After blocking with PBS containing 5% BSA solution, the coverslips were incubated with KM-HN-1 antibody, anti γ-tubulin antibody for 2 h. After washing of coverslips, fluorescence-conjugated secondary antibodies were treated for 2 h. For nuclear visualization Heoschst 33452 (Sigma) dye was used after seconary antibody incubation. The

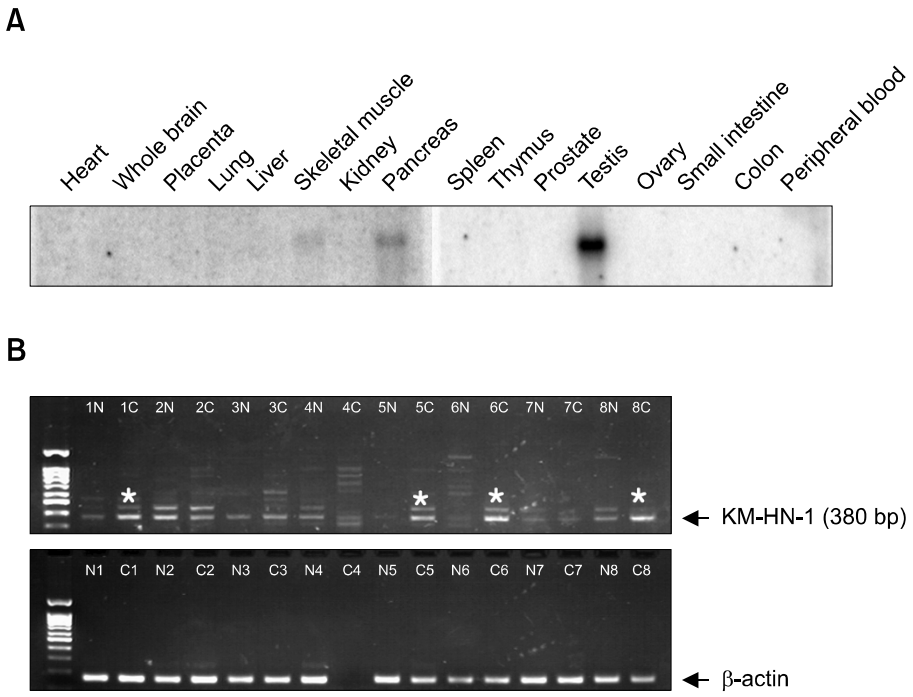

Figure 2. Northern blot and RT-PCR analysis of KM-HN-1. (A) Two kinds of humun MTN blots covering 16 different human tissues were used. The KM-HN-1 cDNA was labeled with P^{32} and probed. After hybridization, the membranes were washed and autoradiography was carried out. (B) RNAs of 8 samples of colon cancer tissues and surrounding normal tissues were used for RT-PCR analysis. The asterisk (*) represents KM-HN-1 messenger RNA increased lane.

stained coverslips were mounted on glass slides with semi-solidifying mounting solution (Polyscience). Confocal fluorescence images were collected by Carl Zeiss LSM 510 Meta confocal microscope.

Results

Expression of KM-HN-1 mRNA in normal tissues and colon cancers

To detect the tissue-specific distribution pattern of KM-HN-1 in human tissue, human multiple tissue northern blots (hMTN) were used for northern hybridization analysis. This analysis detected a single band of approximately 4 kB in the testis, pancreas and skeletal muscle. However, we failed to detect KM-HN-1 transcripts in the whole brain, heart, placenta, thymus, and ovary tissues. The expression of KM-HN-1 was exceptionally high in the testis (Figure 2A). The high expression levels of KM-HN-1 in the testis were consistent with previous Northern blot data from initial cloning report of KM-HN-1 (Monji *et al.,* 2004). Due to the high proliferation activity in the testis, the high level of KM-HN-1 in testis suggests that it may play a crucial role in cell proliferation. We also analyzed the KM-HN-1 expression in several colon cancers. 4 out of 8 samples of colon cancer evidenced increased KM-HN-1 expression (Figure 2B).

Production of antisera and immunoprecipitation of KM-HN-1

In order to generate antisera against KM-HN-1, either the N-terminal (1-417) or C-terminal domain (418-833) of KM-HN-1 ORF was cloned into pGEX-4T and the resultant fusion proteins were purified. Each GST-N-terminal KM-HN-1 and GST-C-terminal KM-HN-1 was used for production of antisera. The purified antisera detected the overexpressed KM-HN-1 and endogenous KM-HN-1 (Figure 3A). When competing with immunogen (GST-KM-HN-1 or C-terminal peptide) the binding of antisera to KM-HN-1 was effectively diminished (Data not shown). As is shown in Figure 3A, the antisera against the N-terminal half of KM-HN-1 is capable of recognizing endogenous KM-HN-1 as a 105 kDa protein. We also verified the existence of KM-HN-1 via immunoprecipitation with the C-terminal antibody followed by the immunoblotting of immnuoprecipitates using the N-terminal antisera (Figure 3B).

Colocalization of KM-HN-1 and hCdc14A

hCdc14A is known to localize to the centrosome and to perform crucial functions in centrosome duplication and mitosis (Kaiser *et al.,* 2002; Mailand *et al.,* 2002; Trautmann and McCollum, 2002). As we obtained the KM-HN-1 gene via yeast two-hybrid screening using hCdc14A as bait, we tried to see the colocalization of KM-HN-1 with

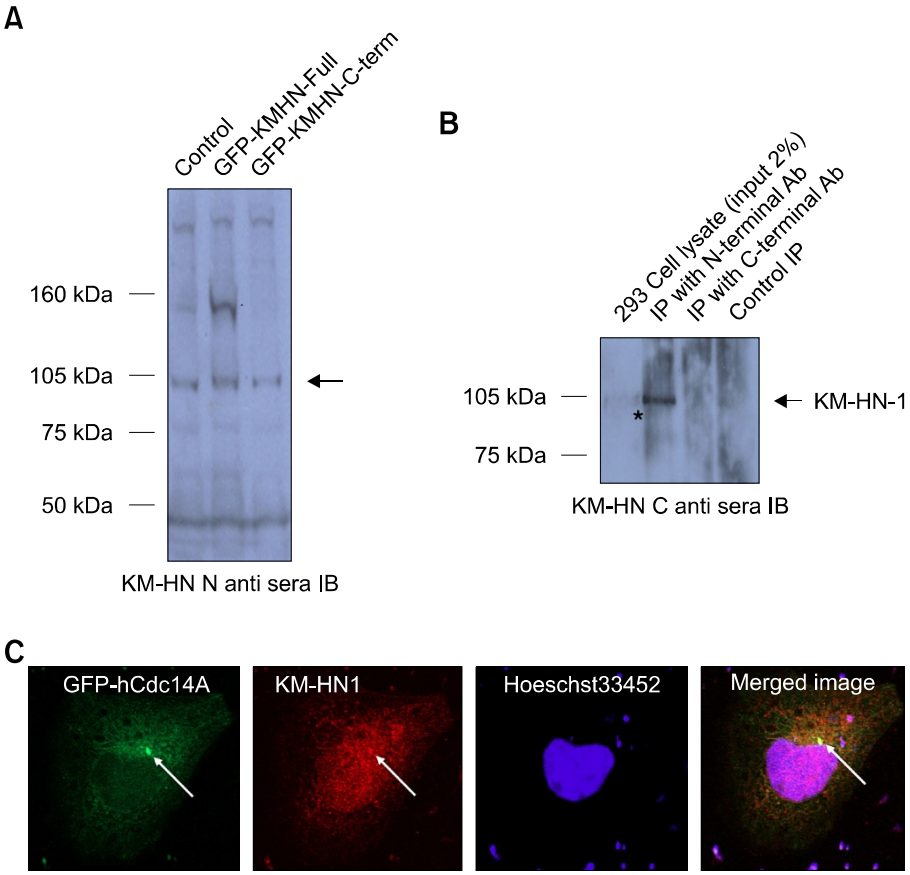

Figure 3. Production of KM-HN-1 antisera and identification of KM-HN-1. Two kinds of antisera were raised-one is against the N-terminal half of KM-HN-1 (anti-KM-HN-N antisera), and the other is against the C-terminal half of KM-HN-1 (anti-KM-HN-C antisera). (A) Immunoblotting using anti-KM-HN-N antisera, 293 cells were transfected with pShuttleCMV-GFP-KM-HN-full and pShuttleCMV-GFP-KM-HN-C. After 36 h of transfection, the cell lysates were electrophoresed and transferred to NC membranes, after which immunoblotting was carried out. Control means vector transfected cell. The arrowhead marks the GFP tagged full length of KM-HN-1, the arrow marks endogenous KM-HN-1. (B) Immunoblotting of immunoprecipitated KM-HN-1 protein. For immunoprecipitation 293 human embryonic kidney cells were lysed and was pulled down with indicated antisera. The immunoprecipitated samples were electrophoresed and Western blot analysis was carried out using anti-KM-HN-C antisera. The anti-KM-HN-C antisera cannot pull down endogenous KM-HN-1. The asterisk (*) represents two bands of endogenous KM-HN-1 (full length and splicing variant). (C) Colocalization of EGFP-hCdc14A with KM-HN-1. For colocalization analysis of KM-HN-1 and hCdc14A pEGFPC1-hCdc14A was transfected to U-2 OS cells. After 16 h of transfection, the cells were fixed in 3.7% formaldehyde then permeabilized with 0.5% Triton X-100 for 5 min. For the staining of KM-HN-1 the rabbit polyclonal anti-KM-HN-1 antisera was used. Green, EGFP-tagged full length of hCdc14A; red, KM-HN-1; Blue, Hoeschst 33452 dye for nuclear staining. Arrows mark the colocalization signal of KM-HN-1 and hCdc14A

hCdc14A. As is shown in Figure 3C, the EGFP-Tagged hCdc14A showed colocalization with KM-HN-1 in the centrosome area. However, we failed to observe apparent binding of KM-HN-1 with hCdc14A.

Generations of GFP tagged KM-HN-1 deletion mutants

The C-terminal region of KM-HN-1 harbored a coiled-coil region, which contains the SMC motif and shared a conserved region of homology with yeast spindle pole proteins Spc110p and Rad51p. In addition to the SMC motif, KM-HN-1 harbors a bipartite NLS motif and a leucine zipper motif in its internal 1/3 fragment (Figure 1A and B). The KM-HN-1 protein was shown to contain more than five leucine zipper repeats (Figure 1B). This region appeared to be important to proper cellular localization and function. To determine the function of each region in KM-HN-1, we expressed various forms of KM-HN-1 in U-2OS cells using adenoviral system (Figure 4A). These recombinant proteins were N-terminally fused with GFP to facilitate

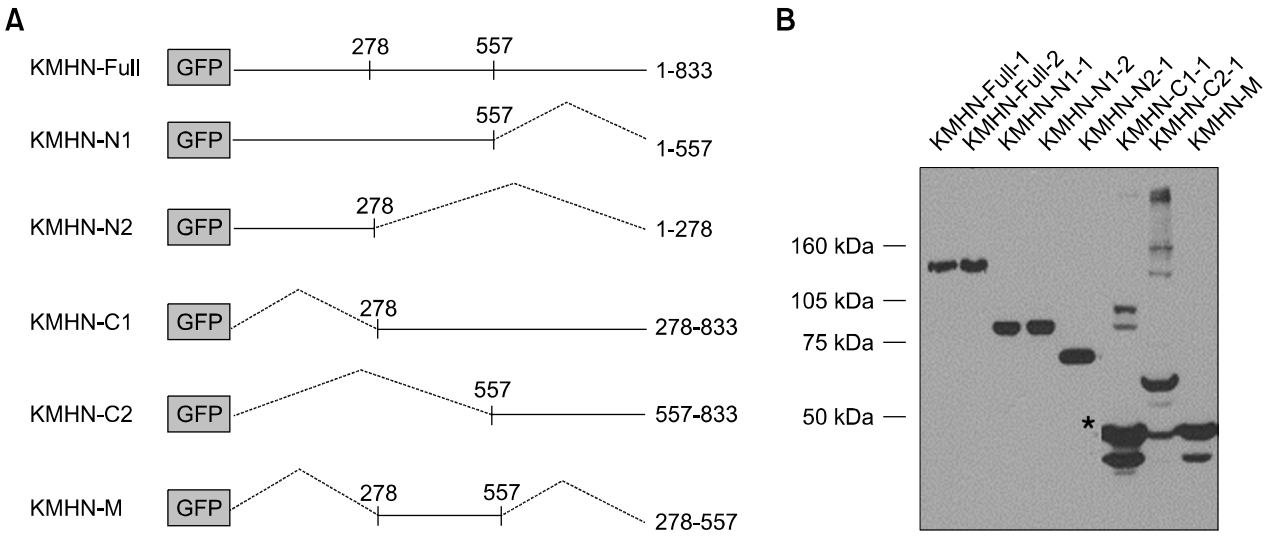

Figure 4. Generation of KM-HN-1 deletion mutant. (A) For the expression of five kinds of deletion mutants (N1, N2, C1, C2 and M) and full length of KM-HN-1 were fused with GFP and expressed via an adenoviral system. (B) Immunoblotting analysis of each virus infected cell. The recombinant adenovirus was infected into U-2 OS cell. The cell lysates were analyzed with Western blot analysis using GFP antibody (JL-8, Clonetech). In KM-HN-C1 immunoblotting the band marked with * is proteolytic cleavage fragment.

protein detection and subcellular localization. After 24 h of virus infection, the total cellular proteins were prepared for immunoblotting with anti-GFP antibody. As shown in Figure 4B, each GFP-fused proteins were expressed according to their assuming molecular weights. However in GFP-KM-HN-C1 infected cell lysates the proteolytic cleavage products were dominant (marked * in Figure 4B) than the uncleaved product.

Intracellular localization of endogenous KM-HN-1 and various deletion mutants

To determine the intracellular localization of KM-HN-1 we carried out immunofluorescence staining using affinity-purified KM-HN-1 antibody. To validate the antibody specificity we also carried out immunofluorescence staining in the presence of competing immunogen (GST-KM-HN-1). As is shown in Figure 5, the endogenous KM-HN-1 (endo-KMHN in Figure 5) showed nuclear localization and colocalization with γ-tubulin, which make γ-TuRC and localize to the centrosomes. The competition of GST-KM-HN-1 with the endogenous KM-HN-1 effectively blocked the centrosomal signal of KM-HN-1 antibody (Competition). We also used C-terminal peptide (800-815) antibody for immunofluorescence staining. The employing of C-terminal peptide competition during immunofluorescence staining using C-terminal peptide antibody also blocked the centrosomal localization of KM-HN-1 (data not shown). The GFP-tagged full length of KM-HN-1 also showed similar localization with endogenous KM-HN-1 protein (KMHN-full in Figure 5). The centrosomal localization signal of GFP-KM-HN-full was more prominent than that of the endogenous protein. The C-terminal 1/3 (KM-HN-C2) of KM-HN-1 also evidenced centrosomal localization, but the nuclear localization intensity was diminished (KMHN-C2 in Figure 5). Due to the abundance of the cleavage product, the GFP signal in GFP-KM-HN-C1 expressed cells was greatly superimposed with the cleavage product. As the anti-GFP antibody detects the cleavage product of GFP-KM-HN-C1 (Figure 4B) and the N-terminal Ser65-Tyr66-Gly67 of GFP is essential for proper fluorescence emission (Heim *et al.,* 1994), the major cleavage product of GFP-KM-HN-C1 seems to be C-terminally cleaved and similar with GFP-KM-HN-M. Without the contaminating cleavage product of GFP-KM-HN-C1, the centrosomal localization signal of GFP-KM-HN-C1 might have been enhanced more. The N-terminal 2/3 of KM-HN-1 (KMHN-N1 in Figure 5) showed similar level of nuclear and cytoplasmic signal, the centrosomal signal of this protein was no less than that of KM-HN-C2. The N-terminal 1/3 of KM-HN-1 (KMHN-N2 in Figure 5) exhibited weakened centrosomal localization, but interestingly it showed strong nuclear localization. The internal 1/3 fragment (KM-HN-M), which harbors putative NLS and leucine zipper motif, showed weakened centrosomal and nuclear localization (KMHN-M in Figure 5). The KM-HN-M over-

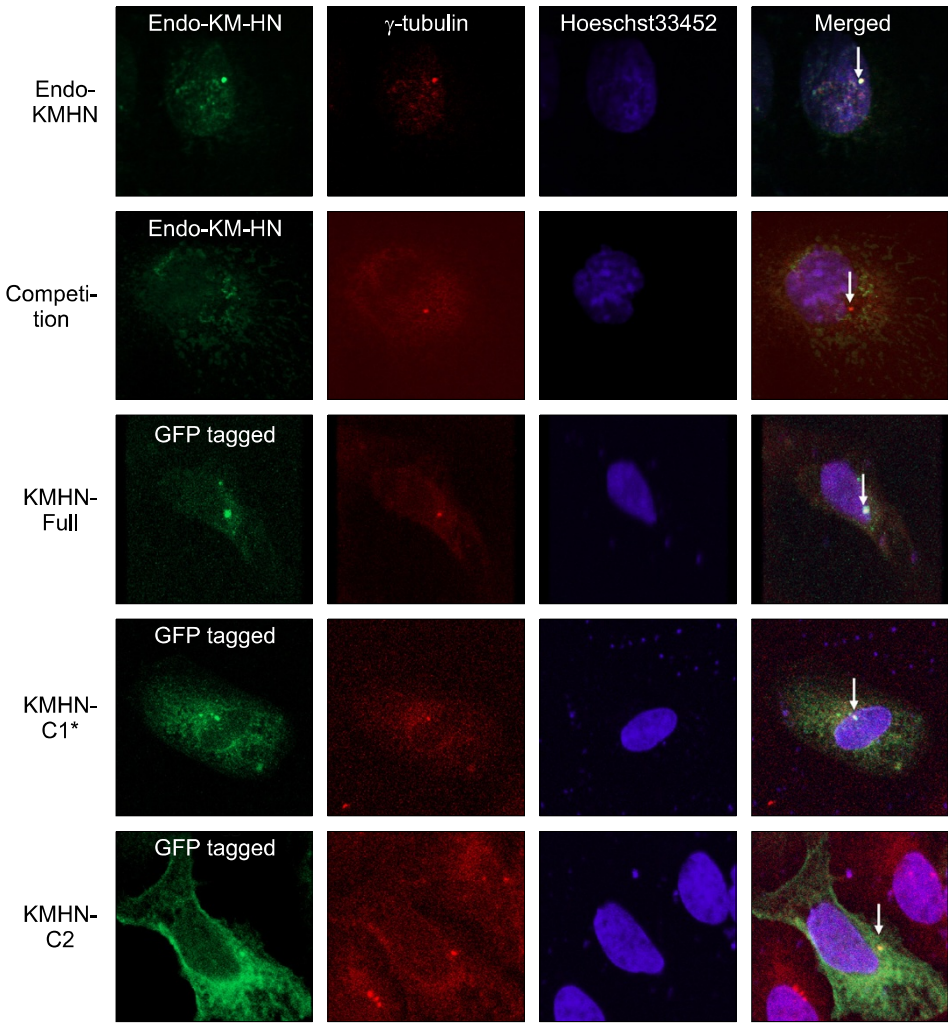

Figure 5. Intracellular localization of various kinds of KM-HN-1 deletion mutants and endogenous KM-HN-1. For expression of GFP-KM-HN-1 adenovirus was transduced into U-2 OS cells. After 16 h of infection cells were fixed in 3.7% formaldehyde then permeabilized with 0.5% Triton X-100 for 5 min prior to the incubation of γ-tubulin antibodies. Endo-KMHN, endogenous KM-HN-1 protein probed with N-terminal affinity purified antisera; Competition, To see effective blocking of KM-HN-1 antibody purified GST-KM-HN-N was preincubated with KM-HN-1 antisera; Arrow marks the colocalization signal of KM-HN-1 and γ-tubulin. *As KM-HN-C1 contained cleaved product as majority, the localization profile cannot directly represent the localization of C-terminal 2/3 of KM-HN-1.

expressed cell evidenced a decreased γ-tubulin signal. The localization profiles of various KM-HN-1 constructs are summarized in Table 1. These results indicate that the cytoplasmic and centrosomal localization-determining region resides in the C-terminal region, and the nuclear localization-determining region is in the N-terminal region.

Forced overexpression of KM-HN-1 can generate aberrant γ-TuRC-like foci

We attempted to determine whether the centrosomal localization of KM-HN-1 requires intact microtubule polymers. The nocodazole-treated cells also showed distinct localization to the centrosome (Figure 6), much like the untreated cells (endo-KMHN in Figure 5). This result indicates that the centrosome localization is not dependent on microtubule integrity and dynamics. We also investigated whether overexpressed KM-HN-1 foci can recruit γ-TuRC. For prolonged overexpression we incubated for 36 h after infection of adv-GFP-KM-HN-full, then fixed and immunofluorescence staining was carried out. As is shown in Figure 7A and B, the ectopically overexpressed KM-HN-1 foci could induce γ-TuRC-like foci. Unlike endogenous KM-HN-1, superfluously overexpressed GFP tagged KM-HN-1 showed punctuate pattern (Figure

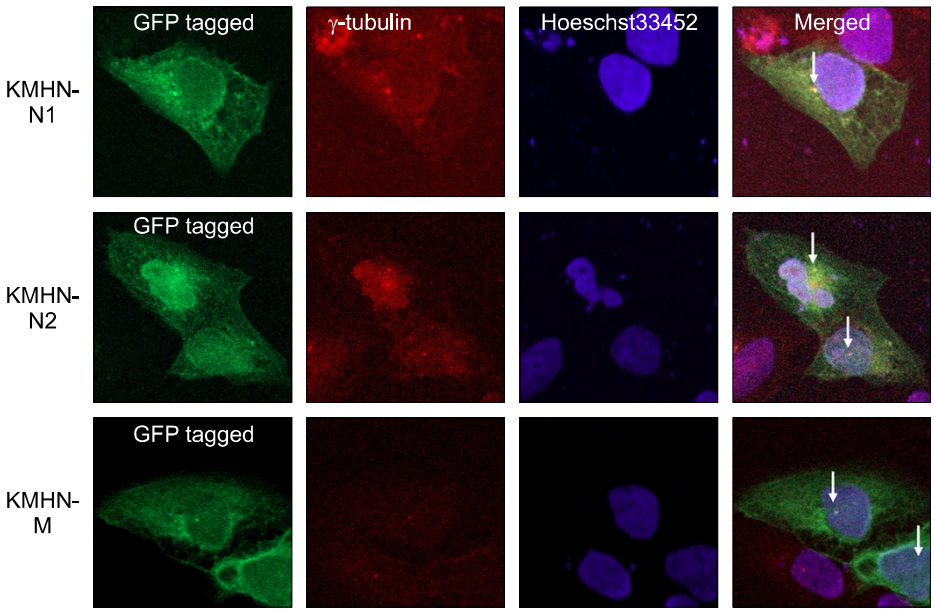

Figure 5. Continued.

Table 1. Summary of various GFP-KM-HN-1 protein localizations.

	Centrosome	Cytoplasm	Nucleus
KM-HN1 Full	++++	+	+
KM-HN1 N1	++	++	+
KM-HN1 N2	+/–	+	++
KM-HN1 C1*	+++	++	–
KM-HN1 C2	++	++	–
KM-HN1 M	+	++	+/–

The relative intensity of centrosomal and nuclear localization of GFP-tagged KM-HN-1 mutants was measured and graded. ++++, most strong; +/–, same as average of surrounding intracellular structures; –, not detectable. More than 15 cells were counted for each sample. *As KM-HN-C1 contained cleaved product as majority, the localization profile cannot wholly reflect the localization of C-terminal 2/3 of KM-HN-1.

7A). This is due to the self-aggregation characteristics of overexpressed KM-HN-1 and does not represent the characteristics of intracellular localization pattern of endogenous KM-HN-1.

Discussion

Although KM-HN-1 has previously been identified as a cancer/testis antigen, the study of KM-HN-1 has mainly been limited to mRNA expression level analyses using several cancer tissues and cell lines (Monji *et al.,* 2004; Condomines *et al.,* 2007). KM-HN-1 has been shown to localize to the nucleus in interphase cells and chromosomes in mitotic cells, but the characterization of this protein at the protein level has not been addressed yet.

We isolated KM-HN-1 as an hCdc14A interacting protein from a yeast two-hybrid screening. We identified this protein as a 105 kDa protein via immunoblotting and immunoprecipitation. The immunofluorescence analyses using GFP-KM-HN-1 and N-terminal antisera revealed that KM-HN-1 mainly localize to centrosomes as well as nucleus. No chromosomal localization during mitosis was observed. Immunofluorescence staining using the C-terminal peptide antibody against the C-terminal 800-815 peptide residues of KM-HN-1 also showed similar centrosomal and nuclear localization (data not shown). Based on these results we concluded that the cancer/testis antigen KM-HN-1 is another member of centrosomal localizing proteins.

The centrosome is the principal microtubule-organizing center of the cell and is surrounded by pericentriolar material, which tethers a variety of proteins, including γ-tubulin and hundreds of other proteins (Doxsey *et al.,* 2005). The centrosome is crucial with regard to microtubule nucleation and anchorage, cell cycle regulation, cytokinesis, protein phosphorylation and signal transduction (Rieder *et al.,* 2001; Doxsey *et al.,* 2005). The hCdc14A localizes to the centrosomes and plays important role in centrosome cycle, chromosome segregation and anaphase progression (Bembenek and Yu, 2001; Kaiser *et al.,* 2002; Mailand *et al.,* 2002).

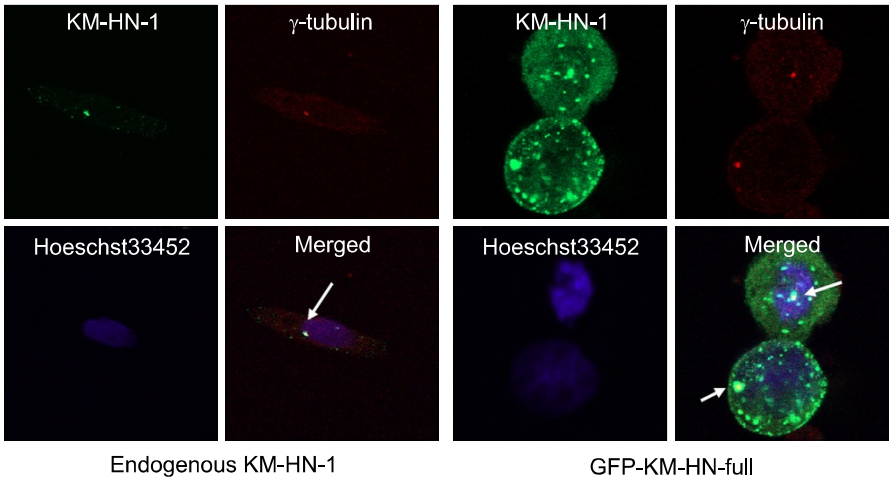

Figure 6. Microtubule depolymerization by nocodazole treatment does not alter centrosomal localization of KM-HN-1. For microtubule depolymerization, nocodazole U-2OS cells were treated at 200 nM for 16 h. For visualization of GFP-KM-HN-full under the microtubule depolymerized status the KM-HN-1 adenovirus and nocodazole was treated simultaneously to U-2OS cells. After 16 h of treatment the cells were fixed and permeabilized and immunofluorescence staining was performed. The arrows mark the centrosomal colocalization of KM-HN-1.

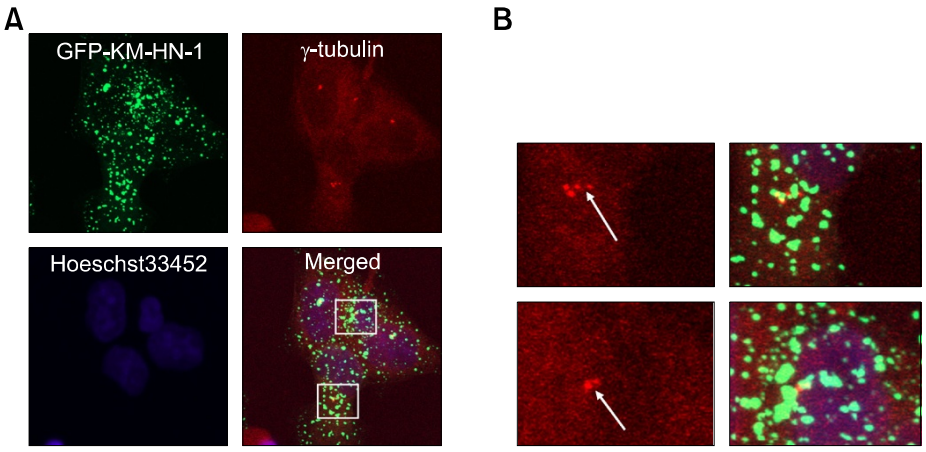

Figure 7. Forced overexpression of GFP-KM-HN-1 can induce aberrant γ-TuRC foci formation. (A) After 36 h of advGFP-KM-HN-1 infection, the cells were fixed and stained for γ-tubulin antibody. Green, overexpressed GFP tagged KM-HN-1; red, γ-tubulin; blue, Hoeschst 33452 dye for DNA staining. (B) The rectangular area of Figure 7 A was magnified for the visualization of γ-TuRC foci, and KM-HN-1. The arrow represents aberrant γ-TuRC foci.

The endogenous KM-HN-1 showed colocalization with GFP tagged hCdc14A (Figure 3C).

KM-HN-1 has three coiled-coil regions in the C-terminal half of the protein. The coiled-coil region is comprised of more than two or three α-helices wrapped around each other and forms helical structures (Lupas, 1996; Burkhard *et al.,* 2001). The coiled-coil regions function as protein-protein interaction domains for further assembly of macromolecular structures. These coiled-coil region containing proteins play diverse biological functions as components of cytoskeleton, nuclear matrix, centrosomes, spindle pole bodies, centromeres, and Golgi apparatus (Rose and Meier, 2004). A lot of centrosome-localizing proteins have been shown to harbor coiled-coil motifs, and the integrity of these motifs appears to be important for centrosomal localization (Andersen *et al.*, 2003; Rose and Meier, 2004). In addition to the coiled-coil motif in C-terminal half region, the KM-HN-1 harbors leucine zipper and NLS motif in internal 1/3 of this protein. To determine which region in KM-HN-1 is

responsible for centrosomal and nuclear localizations, we generated various GFP-fused KM-HN-1 deletion mutants.

We observed that the GFP-KM-HN-1 C-terminal fragment which harbors coiled-coil region efficiently localized to the centrosomes, while the mutants lacking the coiled-coil region localized to centrosomes marginally. These results suggest that the centrosomal localization-determining region resides within the C-terminal coiled-coil region. In addition to centrosomal localization, the GFP-KM-HN-C2 existed in cytoplasm. The cytoplasmic localization of this protein was due to the putative NES (Nuclear Export Signal) between amino acid residues 611-616. This region showed homology with conserved motif of NES (L-X-X-L-X-L, where L can be either L, I, V, F or M.) (la Cour *et al.,* 2004). As well as centrosomes the endogenous KM-HN-1 also localized to the nucleus. The nuclear distribution of endogenous KM-HN-1T is concord with previous KM-HN-1 report (Monji *et al.,* 2004). Among the various kinds of KM-HN-1 constructs, the N-terminal 1/3 of it (KM-HN-N2) exhibited the most prominent nuclear localizations. However, the N-terminal 1/3 region of this protein harbors no distinct nuclear localization signal, suggesting that this region contains a yet unidentified NLS motif. Protein target site prediction using Internet WoLF PSORT (http://wolfpsort.org/) program (Horton *et al.,* 2007) showed that the N-terminal 1/3 shared similar nuclear localization value with internal 1/3 of KM-HN-1 even in the absence of putative NLS.

The KM-HN-1 harbors a leucine zipper motif in the internal 1/3 region followed by coiled-coil region. For the dimerization of the leucine zipper-containing protein, the minimal number of leucine zipper repeats is four (Landschulz *et al.,* 1988). In the case of KM-HN-1, the number of leucine zipper repeats is six. The leucine zipper motif has been known as protein dimerization region of various kinds of DNA binding proteins. Thus far, speriolin (Goto and Eddy, 2004), ninein (Bouckson-Castaing *et al.,* 1996), Nek2 kinase (Fry *et al.,* 1999) are known as leucine zipper-containing centrosomal proteins. The centrosomal localizing Nek2 kinase dimerizes via the leucine zipper region (Fry *et al.,* 1999). The N-terminal leucine zipper of speriolin is required for the targeting of this protein to centrosomes (Goto and Eddy, 2004). In ninein, one of the essential components of pericentriolar centrosomal material, the coiled-coil domain harboring three leucine zippers is essential for centrosomal localization (Stillwell *et al.,* 2004). The leucine zipper containing KM-HN-N1 showed centrosomal localization despite the impaired C-terminal coiled-coil region. This may be due to the fact that the leucine zipper region can act as a dimerization domain with other leucine zipper containing centrosomal localizing proteins or KM-HN-1 itself. From these results, we could conclude that the leucine zipper motif also contributes the centrosomal localization of KM-HN-1. Although the leucine zipper fragment of KM-HN-1 with impaired coiled-coil region can lead this protein to centrosomes, the coiled-coil region seems to have more responsibility for centrosomal localization. The centrosomal signal of KM-HN-M, in which leucine zipper harbors but coiled-coil is disrupted was not so strong as C-terminal fragment of KM-HN-1 or full length of it. The coiled-coil fragment of KM-HN-1 lacking leucine zipper or leucine zipper motif without coiled-coil region can act as dominant negative or inhibitor of organization of centrosomes through binding and sequestration of other leucine zipper or coiled-coil motif containing centrosomal localization proteins.

The centrosomal localization of KM-HN-1 was not related with microtubule integrity or microtubule dynamics. Microtubule depolymerization by nocodazole treatment could not alter the centrosomal localization of KM-HN-1. The overexpression of ninein, a centrosome localizing leucine zipper and coiled-coil region containing protein, exhibited a mislocalization of γ-TuRC (Stillwell *et al.,* 2004). The ectopic overexpression of GFP-KM-HN-full induced abnormal γ-TuRC foci. Forced overexpression of KM-HN-1 also resulted in multiply dotted signals among which the γ-TuRC signal appeared. The multiple dot signals are likely due to the inter-molecular interactions between KM-HN-1 proteins or other coiled-coil domain containing proteins.

In conclusion, the cancer/testis antigen KM-HN-1 is a new member of centrosome localizing proteins and the C-terminal coiled-coil region and the leucine zipper motif are important for proper centrosomal localization. Further studies on the protein interaction and the function of KM-HN-1 at the centrosomes may provide new insights into the organizations and functions of centrosomes.

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