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13. ABSTRACT (Maximum 200 Words)

Precise biochemical pathways have evolved in eukaryotic cells to coordinate the multiple events needed to ensure genomic stability. Fundamental to these biochemical pathways are checkpoints which serve to monitor the integrity of chromosomes and cell cycle progression. Defects in cell cycle checkpoints can result in gene mutations, chromosome damage and aneuploidy, all of which can contribute to tumorigenensis. The mitotic spindle checkpoint monitors spindle microtubule structure, chromosome alignment on the spindle, and chromosome attachment to kinetochores during mitosis. The spindle checkpoint delays the onset of chromosome segregation during anaphase until any defects in the mitotic spindle are corrected.

Human and mouse Mps1 homologs (hMps1/PYT/TTK and mMps1/Esk) were identified and recently reported to function in spindle checkpoint and centrosome duplication. Our results show that overexpression of hMps1 activates the spindle checkpoint. hMps1 kinase can also autophosphorylate. We hypothesize that the autophosphorylation of hMps1 is linked to its function in spindle checkpoint by activation of apoptosis that follows mitotic arrest.

We found that the overexpression of hMps1 also results in mitotic arrest. Based on the results that Mps1 works upstream of the mitotic spindle signaling pathway in yeast and that induction of mitotic arrest results in apoptosis in mammalian system. Thus, hMps1 might act as a upstream signaling of mitotic checkpoint, initiating cascades of events leading to apoptosis upon induction of mitotic checkpoint in mammalian system.

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FINAL REPORT FOR AWARD #DAMD17-03-1-0683

Title: Genetic instability of breast cancer cells induced by aberrant expression of hMpS1

PI: Shalom Avraham, M.D., Ph.D.

Introduction: The mitotic spindle is essential for the maintenance of genetic stability. In budding yeast, the assembly and function of the mitotic spindle is dependent on the MpS1 protein kinase. The components of mitotic spindle checkpoints were first identified in yeast and their homologs of higher organisms were then identified and characterized. These include Bub1, Bub2, Bub3, Mad1, Mad2, Mad3 and MpS1. MpS1 is a dual specificity kinase. Human and mouse MpS1 homologs (also termed hMpS1, PYT, TTK or Esk) were identified and were reported to function in spindle checkpoint and centrosome duplication (1-6). Although several recent reports demonstrated that vertebrate MpS1 proteins regulate the spindle checkpoint, there were conflicting reports regarding their role in centrosome duplication.

Thus, we hypothesized that there is increased expression of hMpS1 which may lead to increased centrosome numbers in breast cancer cells, leading to aneuploidy and to transformation. Therefore, our goals were to:

1) Examine the expression of hMpS1 in breast cancer cells
2) Examine the interaction of hMpS1 with the centrosome component, centrosomin (CNN).
3) Evaluate the oncogenic activity of CNN in normal breast epithelial cells and breast cancer cells.

While our studies were in progress, several reports have been published (7, 10-12) that showed hMpS1 is required for centrosome duplication and normal mitotic progression (7). In addition, MpS1 was shown to interact with microtubules and that microtubule binding could contribute to the regulation of kinase activity of hMpS1 (10). Human MpS1 is also required for the spindle assembly checkpoint but not for centrosome duplication (11). Lastly, hMpS1 kinase was reported to be required for mitotic arrest induced by the loss of kinesis-like protein, CENP-E for kinetochores (12).

BODY

Key Research Accomplishments

A. Generation of specific polyclonal antibodies for hMpS1 and plasmid constructs

Since specific antibodies for hMpS1 were not available, we generated fusion proteins of GST-hMpS1 that contain the N-terminus. We purified this fusion protein and used the protein to immunize rabbits. We purified IgG fraction from the rabbit serum using protein-A beads (Fig. 1). Western blot analysis revealed that these antibodies were specific to hMpS1 kinase (Fig. 2).

B. Expression and localization of hMpS1

We have generated wild type hMpS1 N-terminally-flag tagged as well as tagging GFP at the N-terminus of hMpS1 (Fig 2B-2C). We also generated kinase-dead hMpS1 by mutating codon 664 to Ala (Fig. 2C).

Both wild type and mutant GFP-hMps1 were expressed in 293 cells and the expression was determined by Western blotting and by fluorescence microscopy. Two days after the transient tranfection, cells transfected with wild type hMps1 showed higher expression than mutant hMps1 (Fig 3). Also cells transfected with wild type hMps1 were arrested at mitosis revealed by the localization of hMps1 at the condensed chromosomes in many cells. Furthermore Western blotting supported the result of immunofluorescence of higher expression of wild type hMps1 (Fig 4A). Higher expression of wild type hMps1 can be explained in two ways. It was reported that expression of hMps1 is regulated during cell cycle and the level increases as cells approach mitosis. Indeed, one explanation is overexpression of wild type hMps1 arrests cells at mitosis, thus hMps1 was stabilized at mitosis. This is also supported by Fig. 3 which shows that mitotic cells which have condensed chromosomes that express wild type

hMps1showed higher GFP signal than cells in interphase. Another explanation is that wild type hMps1 can be stabilized by some mechanism including autophosphorylation. Overexpressed wild type hMps1 showed not only higher protein levels but also slower mobility than mutant hMpS1 in SDS-polyacrylamide gel. The mobility change was revealed as phosphorylation (Fig 4B). The band of low mobility disappeared by the treatment of lambda phosphatase and the disappearance was blocked by the treatment of phosphatase inhibitors. Based on the results by other groups that Mps1 can phosphorylate itself in vitro and our result that mutant form was not phosphorylated, we suggest that the phosphorylation of wild type hMpS1 might be due to autophosphorylation of hMpS1.

Previous studies have shown that mutations of genes in spindle checkpoint results in the abrogation of spindle checkpoint. We analyzed the sequences of hMpS1 isolated from several breast cancer cell lines (MCF-7 and T47D cells) but found no mutations in hMpS1. Next, we analyzed the expression of hMpS1 in normal breast epithelial cells (MCF-10A), breast cancer cell lines, and breast cancer tumor specimens. All samples expressed hMpS1 by Western blotting (Fig. 5).

Using these antibodies, we then attempted to find colocalization and/or association of endogenous CNN (8-9) with endogenous hMpS1. Although we observed an association of CNN when we overexpressed hMpS1 in 293T cells, we could not confirm this association in the endogenous levels of both hMpS1 and CNN in breast cancer cells (MCF-7 and T47D cells). However, interestingly, we observed an interaction of hMpS1 with BRCA1 (Fig. 6). This interaction may regulate genomic stability.

Based on these results, we hypothesized that the autophsophorylation of hMpS1 in linked to its function in spindle checkpoint and the downstream target of hMpS1 kinase might be involved in the activation of apoptosis that follows mitotic arrest in the presence of microtubules disrupting drugs. Furthermore, the interaction of hMpS1 with BRCA1 may contribute to genomic stability.

Reportable Outcomes

Currently, we are continuing this project and we aim to define the residues which are autophsophorylated by hMpS1. We plan to study the function of autophsophorylation by analyzing the localization and interaction of hMpS1 with other proteins involved in the spindle checkpoint using autophosphorylation mutant and kinase-dead mutant of hMpS1. We will test whether hMpS1 can phosphorylate proteins of the apoptotic pathway such as Bcl2, which are known to be phosphorylated after mitotic arrest. Understanding the role of hMpS1 in mitotic checkpoint in-vivo will greatly contribute to the application of microtubule disrupting drugs to breast cancer patients.

Conclusions:

1. hMpS1 function at mitotic checkpoints
2. hMpS1 autophosphorylation may be linked to its function in spindle checkpoint
3. hMpS1 expression levels were not altered in breast cancer cells.
4. No endogenous interaction was observed between hMpS1 and CNN, while endogenous interaction of hMpS1 was observed with BRCA1 in breast cancer cells.

Further studies: We will define the role of autophosphorylated hMpS1 in spindle checkpoints.

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Fig1. Purification of GST-hMpS1-N-terminus

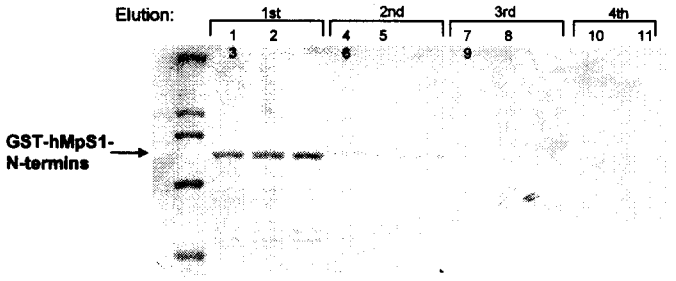

Fig 1: GST-fusion protein of hMpS1 that contain the N-terminus was generated and purified according to company protocol (Pharmacia).

Fig2. Analysis of hMpS1 rabbit antibodies by Western Blotting

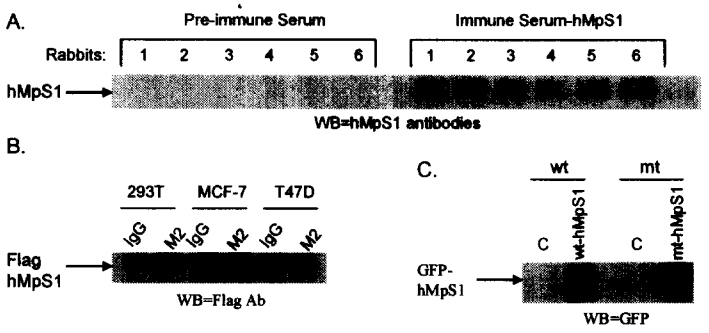

Fig 2A: Six rabbits were immunized with GST-hMpS1. IgG fractions were purifed from each rabbit antiserum using protein-A beads, and analyzed by.Western Blotting.

Fig 2B+2C: Generation and Expression of wild type hMpS1 tagged with Flag (Panel B) and with GFP (Panel C). Mutated hMpS1 was also generated and tagged to GFP. These constructs were transfected into 293T cells, MCF-7 and T47D cells as indicated. Total cell lysates were prepared and analyzed by Western blotting as indicated. Ab=antibody; wt=wild type; mt=mutated.

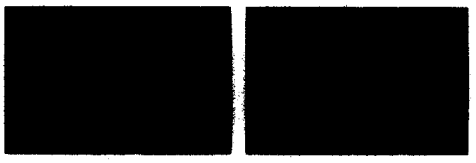

Fig 3: GFP-hMps1 expression and localization in 293T cells.
(A) Wild type GFP-hMps1 expression. (B) Kinase dead GFP-hMps1(D 644 to A) expression.

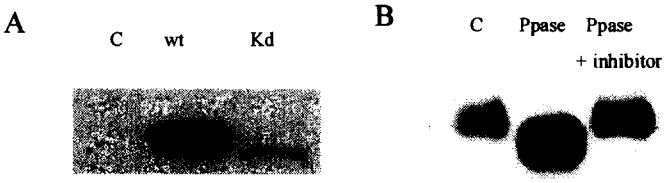

Fig 4: Overexpression of GFP-hMps1 in 293 cells and phosphorylation of hMps1. (A) Western blotting of GFP-hMps1 after transient transfection. C: control vector, wt: wild type GFP-hMps1, kd: kinase dead GFP-hMps1. (B) wild type GFP-hMps1 was immunoprecipitated and then treated with lambda phosphatase or with lambda phosphatase and phosphatase inhibitor.

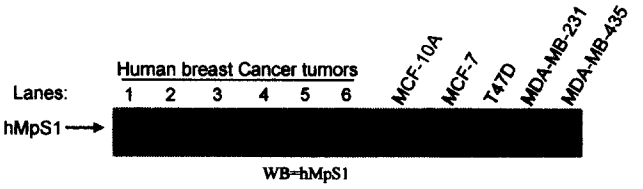

Fig 5: Total cell lysates were immunoprecipitated hMpS1 antibodies. The samples were then analyzed by SDS-PAGE and immunoblotted with hMpS1.

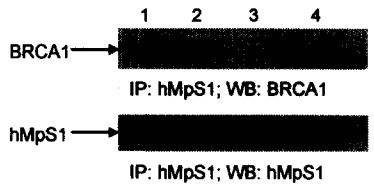

Fig 6: Total cell lysates from MCF-7 cells were immunoprecipated with control antibodies (lanes 3 and 4) or with hMpS1 antibodies (lanes 1 and 2). The samples were analyzed by SDS-PAGE and immunoblotted with hMpS1 or BRCA1 antibodies as indicated.