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RESEARCH ARTICLE

HAI-1 Over-Expression Promotes Autophagy of Prostate Cancer PC-3 Cells

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Abstract

This study examined the potential effect of HAI-1 over-expression on autophagy of prostate cancer PC-3 cells and its possible mechanism. PC-3 cells were transfected with plasmids to generate HAI-1 stably over-expressing PC-3/HAI-1 and control cell lines. The relative levels of HAI-1 mRNA transcripts in control PC-3 and PC-3/HAI-1 cells were determined by RT_PCR. The levels of Beclin1 expression in both cell lines were determined by Western blot. The levels of autophagic vesicles and autophagosomes in these cell lines were characterized by Cyto-ID® Autophagy Detection Dye Staining and transmission electronic microscopy. In comparison with the control PC-3 cells, HAI-1 mRNA transcripts and Beclin1 expression significantly increased in PC_3/HAI1 cells. There were many autophagic vesicles and autophagosomes detected in PC-3/HAI-1 cells. In conclusion, HAI-1 over-expression may promote autophagy of prostate cancer PC-3 cells in vitro.

Keywords: HAI-1 over-expression; Autophagy; Prostate cancer

Introduction

Prostate cancer is a common malignancy with a high mortality. Its incidence is increasing in the world (1). Previous studies have shown that the imbalance of cell proliferation, cell cycle, apoptosis, autophagy and senescence is crucial for the development of prostate cancer (2). However, the pathogenesis of prostate cancer has not been fully understood. Further understanding its pathogenesis may be valuable for the management of patients with prostate cancer.

Hepatocyte growth factor activator inhibitor type 1 (HAI-1) is a membrane-associated serine protease inhibitor expressed in the placenta and epithelial tissues. Our previous study has revealed that HAI-1 expression is down-regulated in prostate cancer tissues and associated inversely with pathogenic degrees and survival of patients with prostate cancer (3). Furthermore, inhibition of HAI-1 activity enhances proliferation and invasion of prostate cancer cells (Sanders AJ, et al. Int. J Mol. Med, 2007). However, the precise role of HAI-1 in the pathogenesis of prostate cancer remains an open question.

Autophagy is a natural process to destroy unnecessary or dysfunctional components in cells. During the process autophagy, signals from amino acid sensing, oxidative stress and growth factors can activate the AMPK and mTOR, which induce Unc-51-like kinases ULK1 phosphorylation at different sites to activate or inhibit ULK1. The activated ULK1 further phosphorylates Beclin1 to initiate the autophagosome formation and eventual fusion with lysosome to form autolysosomes. Functionally, autophagy can respond to cellular stress, such as nutrition defect and oxidative stress, to promote the survival and apoptosis of cells (4,5). However, there is no information about whether HAI-1 regulates autophagy of prostate cancer cells.

In this study, we induced HAI-1 over-expression in prostate cancer PC-3 cells and determined the effect of HAI-1 over-expression on autophagy of PC-3 cells in vitro. Our findings indicated that HAI-1 over-expression enhanced autophagy of PC-3 cells, which may provide insights into pathogenesis of prostate cancer.

Materials and Methods

Cell culture

Human prostate cancer PC-3 cells were purchased from Shanghai Institutes of Biological Science (Shanghai, China), and cultured in RPMI-1640 supplemented with 10% fetal bovine serum (FBS), 100 Units/ml of penicillin, 100 µg/ml of streptomycin and gentamycin at 37˚C in a humidified incubator of 5% CO_2. All the procedures were performed in accordance with the Ethical Committee of the Gongli Hospital.

Construction of HAI-1 expression vector and stable transfection

Human HAI-1-expression vector was constructed. Briefly, human HAI-1 cDNA was amplified by PCR suing a set of primers carrying restriction sites (EcoRI/SalI). The sequences

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of the primers were 5'-TGAGGAAGAGCAGCAGTGCC-3' (forward) and 5'-GGCTACCACCACCACAATGC-3' (reverse). The PCR product was digested with the restriction enzymes and sub-cloned into the EcoRI/SalI site of an expression vector pCI-neo (Promega, Madison, WI) to generate pCI-HAI-1, followed by sequencing. The plasmid pCI-HAI-1 or control pCI-neo was linearized by digestion with XmnI and transfected into PC-3 cells using Lipofectamine 2000 reagent (Gibco BRL, Gaithersburg, MD), according to the manufacture's instruction. Subsequently, the transfected cells were treated with 1 mg/ml of G418 antibiotic (Sigma, St. Louis, MO). The G418-resistant cell clones were selected and screened for the expression of HAI-1 by PCR. The pCI-neo-transfected clones served as controls.

Reverse transcription–polymerase chain reaction (RT-PCR) analysis

Total RNA was extracted from individual groups of cells using TRIzolTM (Invitrogen, Carlsbad, CA, USA), according to the manufacturers' instruction. After being quantified by measuring the absorbance at 260 nm, 2μg total RNA of each sample were reversely transcribed into cDNA using the reaction system of 5×RT reaction buffer plus 0.005 M DTT, 1 mM of each dNTP, 20 U RNase inhibitor, 50 μM oligo (dT) primer, and 200 U of DiaStar™ RTase (SolGent, Daejeon, South Korea) at 50˚C for 50 min and then at 70˚C for 10 min. The relative levels of HAI-1 mRNA transcripts to the control β-actin were determined by PCR using PCR Master mixture, specific primers, and 1 μl cDNA (sample) or serially diluted standard cDNA. The sequences of primers were forward, 5'-TGAGGAAGAGCAGCAGTGCC-3' and reverse, 5'-GGCTACCACCACCACAATGC-3' for HAI-1; forward, 5'-GGGGAGCCAAAAGGGTCATCATCT-3' and reverse, 5'-GAGGGGCCATCCACAGTCTTCT-3' for β-actin. The PCR amplifications were performed in triplicate for 32 cycles of 10 sec at 95˚C; 5 sec at 57˚C and 15 sec at 72˚C. The PCR products were analyzed by electrophoresis on 1.2% agarose gels. The relative levels of HAI-1 to the control β-actin expression were analyzed by ImageJ software.

Western blot analysis

The different groups of PC-3 cells were washed twice with phosphate-buffered saline (PBS), and were lysed in an ice-cold RIPA lysis buffer (Beyotime Institute of Biotechnology, Haimen, Jiangsu, China). After being centrifuged, the protein concentrations were determined using a bicinchoninic acid kit (Pierce Biotechnology, Inc., Rockford, IL, USA). The cells lysate samples (30 μg/lane) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 12% gels, and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, US). The membranes were blocked with 5% fat-free dry milk in TBST and incubated at 4˚C overnight with anti-β-actin and anti-Beclin-1 (company name, city, country). After being washed, the bound antibodies were detected with secondary horseradish peroxidase-conjugated goat anti-rabbit antibodies (1:5,000, Beyotime Institute of Biotechnology) and visualized using the enhanced chemiluminescent reagents. The relative levels of target protein to the control β-actin were determined by Bio-Rad Quantity One software (Bio-Rad Laboratories, Hercules, CA, USA).

Cyto-ID® Autophagy Detection Dye Staining

The presence of punctate vesicles in different groups of cells was examined using Cytp-ID autophagy detection kit, according to the manufacturers' instruction (ENZO). Briefly, PC-3 cells (5×104 cells/well) were cultured overnight in 24-well plates and treated with chlorophyll-f for one hour. The cells were stained with Cyto-ID® Autophagy Detection dye (ENZO) and Hoechst 33342 nuclear dye (ENZO) at 37°C for 30 min in the dark. The stained cells were photographed under a confocal laser scanning microscope (CLSM, TCS-SP8, Leica).

Transmission electron microscopy

The different groups of PC-3 cells were harvested and fixed in 2.5% glutaraldehyde for 2 h, washed three times with 0.1 M PBS, and further fixed with 1% O_sO_4 buffer. Subsequently, the samples were dehydrated and then immersed in pure acetone with embedding liquid (2:1) overnight, followed by pure embedding liquid for 3 h. The samples were solidified and cut into ultrathin sections in an ultra-microtome (KB-I). The ultrathin sections were stained with 3% lead citrate plus uranyl-acetate and observed by an electron microscope (Philips CM20).

Statistical analysis

Data are expressed as the mean ± SEM. The difference between groups was statistically analyzed by the Student-Newman-Keuls test using the SPSS 17.0. A P-value of less than 0.05 was considered statistically significant.

Results

To the potential role of HAI-1 in regulating autophagy of prostate cancer cells, PC-3 cells were transfected with pCI-HAI-1 or control pCI-neo, respectively and treated with G418 for 3 weeks to establish HAI-1 stable expressing PC-3/HAI-1 and control PC-3 cells. The relative levels of HAI-1 mRNA transcripts were determined by semi-quantitative RT-PCR (Figure 1). While there was no detectable HAI-1 mRNA transcript in the control PC-3 cells a strong HAI-1 DNA band with expected size was detected in PC-3/HAI-1 cells, demonstrating high levels of HAI-1 expression in PC-3 cells.

Beclin1, a Bcl-2 homology3 (BH3) domain-only protein, is a key regulator of autophagosome formation and autolysosome fusion (6). To determine the effect of HAI-1 on autophagy of prostate cancer cells, we examined the relative levels of Beclin1 expression in control PC-3 and PC-3/HAI-1 cells by Western blot. While there was a little Beclin1 expression in PC-3 cells the relative levels of Beclin1 significantly increased in PC-3/Hai-1 cells (Figure 2).

To further examine the effect of HAI-1 over-expression on autophagy of PC-3 cells, we characterized the vesicle

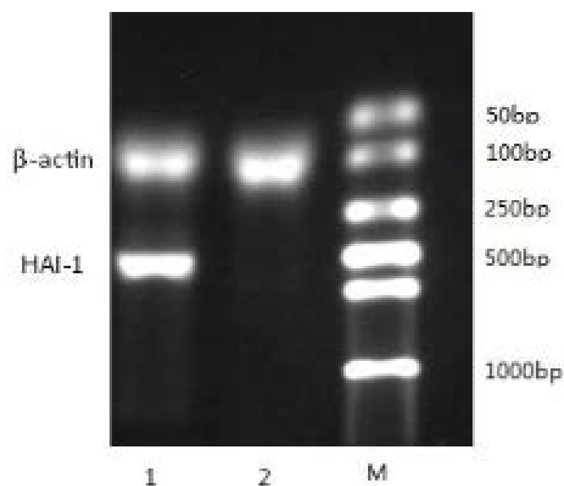

Figure 1: High levels of HAI-1 mRNA transcripts were detected in PC-3/HAI-1 cells.

Total RNA was extracted from control PC-3 and PC-3/HAI-1 cells and the relative levels of HAI-1 mRNA transcripts to control β-actin were determined by semi-quantitative RT-PCR. Data are representative image of three separate experiments. M: DNA markers.

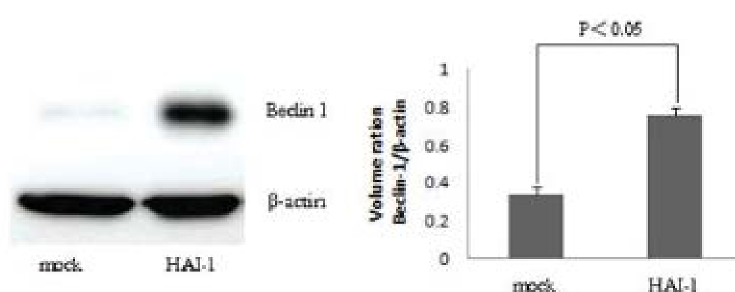

Figure 2: HAI-1 enhances Beclin1 expression in PC-3 cells.

The relative levels of Beclin1 expression win control PC3 and PC3-HAI-1 cells were determined by Western blot. Data are representative images or expressed as the mean ± SD of each group of cells from three separate experiments.

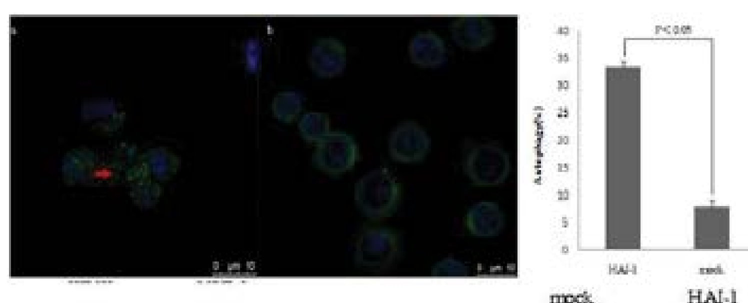

Figure 3: HAI-1 enhances the formation of autophagic vesicles in PC-3 cells.

The distribution of autophagic vesicles in control PC-3 and PC-3/HAI-1 cells were characterized by Cyto-ID fluorescence staining and imaged under a fluorescent microscope. Data are representative images or expressed as the mean ± SD of each group of cells from three separate experiments. Scale bar: 10 μm.

distribution in both control PC3 and PC-3/HAI-1 cells by staining with Cyto-ID® Green autophagy dye. As shown in Figure 3, there were many green punctate vesicles in the perinuclear regions and focally throughout the cytoplasm of PC-3/HAI-1 cells (Figure 3a), but very few in the control cells (Figure 3b). These further support that induction of HAI-1 over-expression enhances autophagy of PC-3 cells.

Finally, we examined the distribution of autophagosomes in the control PC-3 and PC-3/HAI-1 cells by TEM (7,8). As shown in Figure 4, there were double membrane-bound vacuoles, marked by the arrowheads, in the PC-3/HAI-1 cells (Figure 4a), but not in the control PC-3 cells (Figure 4 b). The vacuoles displayed the engulfed bulk cytoplasm and cytoplasmic organelles, a hallmark of autophagosomes. Collectively, the

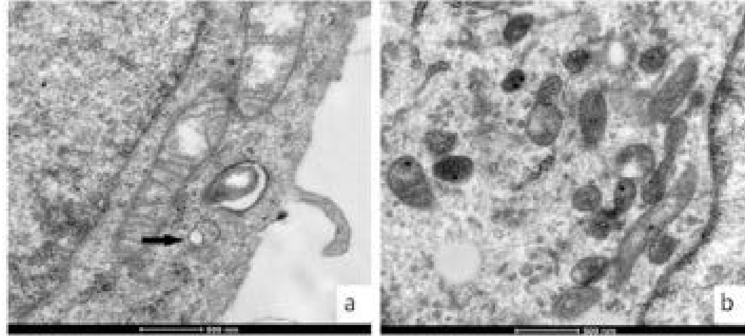

Figure 4: TEM analysis of autophagosomes in PC-3 cells.

The control CP-3 and experimental PC-3/HAI-1 cells were subjected to TEM analysis. Data are representative images of each group of cells from three separate experiments. The arrowhead indicates the typical appearance of double membrane-bound autophagosomes with engulfed bulk cytoplasm and cytoplasmic organelles in PC-3/HAI-1 cells. Scale bar: 500 nm.

increased levels of Beclin1 expression, autophagic vesicles and autophagosomes in PC-3/HAI-1 cells indicated that HAI-1 over-expression enhanced autophagy in prostate cancer cells.

Discussion

Recently, the levels of serine proteinase inhibitors are associated with cancer progression and poorer prognosis in cancer patients (9,10). In this study, we generated HAI-1 over-expressing PC-3 cells and found that induction of HAI-1 over-expression enhanced Beclin1 expression and increased the numbers of autophagic vesicles and autophagosomes in PC-3 cells. These novel data indicated that HAI-1 over-expression promoted autophagy of prostate cancer PC-3 cells. Given that autophagy is associated with survival of cancer cells our findings extended previous observation that high levels of HAI-1 expression are associated with poor prognosis of prostate cancer patients (xxx, xxx).

Beclin-1 is an essential element in the autophagic process and an important signal for its initiation (11). In this study we found that HAI-1 over-expression increased levels of Beclin1 expression in PC-3 cells. This suggests that HAI-1 through an unknown pathway, together with active ULK1, to initiate the autophagic process, leading to formation of autophagosomes and autolysosomes. Indeed, we found that HAI-1 over-expression increased the number of vesicles and autopahgosomes in PC-3 cells. It is notable that HAI-1 over-expression can inhibit matriptase-induced PI3K/AKT activation and malignancy in mice (Karin List et al. Genes & develop. 2005). Accordingly, it is possible that HAI-1 over-expression may inhibit the PI3K/AKT and mTOR signaling, which promotes ULK1 and Beclin1 activation as well as autophagy formation in PC-3 cells. We are interested further investigating the molecular mechanisms underlying the regulatory role of HAI-1 in the autophagic process in prostate cancer. Autophagy plays an important role in a wide variety of physiological and pathological processes, such as adaption of starvation (12), embryonic development (13), cell development (14) and tumor suppression (15). In contrast, excessive autophagy can promote apoptosis (16). It is important to further determine the action of HAI-1 in regulating the proliferation, invasion and drug resistance of prostate cancer cells in the future studies.

Conclusion

In conclusion, our data indicated that HAI-1 over-expression promoted autophagy of prostate cancer PC-3 cells. Our findings may provide new insights into pathogenesis of prostate cancer.

Acknowledgments

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Interest conflict

The authors declare no conflicts of interest.

Author's contribution

CYH, JC, JCZ and JZ designed the study. CYH, JC, JCZ, JZ, GZW and NJ collected and analyzed the data. CYH and JC advised on histological staining and analysis. JCZ and JZ contributed samples collection and intellectual input. CYH and JC drafted and wrote the manuscript. JCZ, JZ, GZW and NJ revised the manuscript critically for intellectual content. All authors gave intellectual input to the study and approved the final version of the manuscript.

References

1. Siegel R, Ma J, Zou Z, Jemal A (2014) Cancer statistics. *CA Cancer J Clin* 64:9-29. [View Article]
2. Hanahan D, Weinberg RA (2011) Hallmarks of cancer: the next generation. *Cell* 144: 646-74. [View Article]
3. Hu C, Jiang N, Wang G, Zheng J, Yang W, et al. (2014) Expression of hepatocyte growth factor activator inhibitor-1 (HAI-1) gene in prostate cancer: clinical and biological significance. *J BUON* 19: 215-20. [View Article]
4. Ouyang L, Shi Z, Zhao S, Wang FT, Zhou TT, et al. (2012) Programmed cell death pathways in cancer: a review of apoptosis,

autophagy and programmed necrosis. *Cell Prolif* 45: 487-98. [View Article]

5. Eisenberg-Lerner A, Bialik S, Simon HU, Kimchi A (2009) Life and death partners: apoptosis, autophagy and the cross-talk between them. *Cell Death Differ* 16: 966-75. [View Article]
6. Maejima Y, Kyoi S, Zhai P, Liu T, Li H, et al. (2013) Mst1 inhibits autophagy by promoting the interaction between Beclin1 and Bcl-2. *Nat Med* 19:1478-1488. [View Article]
7. Eskelinen EL, Reggiori F, Baba M, Kovacs AL, Seglen PO, et al. (2011) Seeing is believing: the impact of electron microscopy on autophagy research. *Autophagy* 7: 935-956. [View Article]
8. Eskelinen EL, Kovacs AL (2011) Double membranes vs. lipid bilayers, and their significance for correct identification of macroautophagic structures. *Autophagy* 7:931-932. [View Article]
9. Kataoka H, Itoh H, Koono M. (2002) Emerging multifunctional aspects of cellular serine proteinase inhibitors in tumor progression and tissue regeneration. *Pathol Int* 52: 89-102. [View Article]
10. Devoogdt N, Hassanzadeh Ghassabeh G, Zhang J, Brys L, De Baetselier P, et al. (2003) Secretory leukocyte protease inhibitor promotes the tumorigenic and metastatic potential of cancer cells. *Proc Natl Acad Sci U S A* 100: 5778-82. [View Article]
11. Cao Y, Klionsky DJ (2007) Physiological functions of Atg6/Beclin 1: a unique autophagy-related protein. *Cell Res* 17: 839-849. [View Article]
12. Ryter SW, Cloonan SM, Choi AM (2013) Autophagy: a critical regulator of cellular metabolism and homeostasis. *Mol Cells* 36: 7-16. [View Article]
13. Qu X, Zou Z, Sun Q, Luby-Phelps K, Cheng P, et al. (2007) Autophagy gene-dependent clearance of apoptotic cells during embryonic development. *Cell* 128: 931-946. [View Article]
14. Takagi H, Matsui Y, Sadoshima J (2007) The role of autophagy in mediating cell survival and death during ischemia and reperfusion in the heart. *Antioxid Redox Signal* 9: 1373-1381. [View Article]
15. Gozuacik D, Kimchi A (2004) Autophagy as a cell death and tumor suppressor mechanism. *Oncogene* 23: 2891-2906. [View Article]
16. Qian W, Liu J, Jin J, Ni W, Xu W, et al. (2007) Arsenic trioxide induces not only apoptosis but also autophagic cell death in leukemia cell lines via up-regulation of Beclin-1. *Leuk Res* 31:329-339. [View Article]

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