VHZ is a novel centrosomal phosphatase associated with cell growth and human primary cancers

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Abstract

Background: VHZ is a VH1-like (member Z) dual specific protein phosphatase encoded by DUSP23 gene. Some of the dual specific protein phosphatases (DSPs) play an important role in cell cycle control and have shown to be associated with carcigenesis. Here, the expression of VHZ associated with cell growth and human cancers was investigated.

Results: We generated a mouse monoclonal antibody (mAb clone#209) and rabbit polyclonal antibodies (rAb) against VHZ. We performed cell proliferation assay to learn how VHZ is associated with cell cycle by retroviral transduction to express VHZ, VHZ(C95S), and control vector in MCF-7 cells. Overexpression of VHZ but not VHZ(C95S) in MCF-7 cells promoted cell proliferation compared to control cells. shRNA-mediated knockdown of VHZ in MCF-7 cells showed that reduction of VHZ resulted in increased G1 but decreased S phase cell populations. Using indirect immunofluorescence, we showed that both exogenous and endogenous VHZ protein was localized at the centrosome in addition to its cytoplasmic distribution. Furthermore, using immunohistochemistry, we revealed that VHZ protein was overexpressed either in enlarged centrosomes (VHZ-centrosomal-stain) of some invasive ductal carcinomas (IDC) Stage I (8/65 cases) or in entire cytoplasm (VHZ-cytosol-stain) of invasive epithelia of some IDC Stage II/III (11/47 cases) of breast cancers examined. More importantly, upregulation of VHZ protein is also associated with numerous types of human cancer, in particular breast cancer. VHZ mAb may be useful as a reagent in clinical diagnosis for assessing VHZ positive tumors.

Conclusions: We generated a VHZ-specific mAb to reveal that VHZ has a novel subcellular localization, namely the centrosome. VHZ is able to facilitate G1/S cell cycle transition in a PTP activity-dependent manner. The upregulation of its protein levels in primary human cancers supports the clinical relevance of the protein in cancers.

Introduction

Aberrant protein tyrosine phosphorylation can result from the dysregulated expression of protein tyrosine kinases (PTKs) or protein tyrosine phosphatases (PTPs) [1]. Protein phosphorylation and dephosphorylation are major regulatory events in many cellular and pathogenic processes [2]. Considerable attention has centered on protein kinases in cancer development, however the role of protein phosphatases in cancer is still an under-explored area.

In recent years, emerging evidence indicates that members of the phosphatase of regenerating liver (PRL) subgroup of PTPs are linked to multiple human cancers [3]. The PRL-PTP family comprises three members: PRL-1, PRL-2, and PRL-3. Compelling evidence suggests that each PRL-PTP member might individually participate in the process of cancer development and metastases [1]. In particular, PRL-3 is the most thoroughly investigated member of this subgroup; and was first shown to be a metastasis-associated phosphatase that is consistently overexpressed in metastatic colorectal cancer (CRC) [4]. In an attempt to identify more PRL-PTP-related phosphatases, we used the human PRL-3 amino acid sequence to perform BLAST database searches and found VHZ that shares about 28% amino acid sequence identity with...
human PRL-PTPs. Indeed, VHZ was cloned and reported by three independent groups in 2004 [5-7].

There are 107 known human protein tyrosine phosphatases (PTPs) [8]. Among these PTPs, there is a class called dual-specific or VH1-like PTPs (http://www.ptphome.net/resource/resource.html[8]). The VH1-like family consists of 63 members, which comprises 19 Atypical Dual-Specific Phosphatases (DUSP), 16 Myotubulins, 11 Map Kinase Phosphatases (MKPs), 5 PTEN members, 4 CDC14 members, 3 PRLs (PRL-1, PRL-2, and PRL-3), and a few others. VHZ belongs to the atypical DUSP and has also been referred to as DUSP23, DUSP25, FLJ20442, LMW-DSP3. The HUGO accepted nomenclature for this gene is DUSP23, we have referred to DUSP23-encoded protein as VHZ since it is a member of the VH1-like phosphatase family. VHZ is expressed in many tissues and is located in both the cytosol and in nucleoli [5]. It is the smallest (calculated 16-kDa) of the catalytically active protein-tyrosine phosphatases (PTP) [5]. The crystal structure of VHZ has recently been determined at 1.93 Å resolution [9]. Despite VHZ’s remarkably high degree of conservation throughout evolution with orthologues in frogs, fish, fly, and the Archaea, the physiological role of VHZ is still largely unknown since it was first cloned and identified six years ago [5-7].

VHZ and VHR belong to the same subgroup of VH1-like PTPs [8]. Since VHR has been reported to have a function in regulating cell cycle progression [10], we attempt to investigate if VHZ also plays a role in regulating cell cycle. We revealed that VHZ has an additional novel centrosomal localization and show that VHZ has a capacity to enhance G1/S phase transition. Furthermore, many dual specific protein phosphatases (DSPs), such as MKPs [11], are associated with carcinogenesis. We also investigated the expression levels of VHZ in multiple human primary cancers and showed that VHZ is overexpressed in human cancers especially in breast cancers.

Materials and methods

Generation of VHZ-EGFP, VHZ(C95S)-EGFP, GST-VHZ expression constructs

The human Universal Quick-clone II cDNA library (BD, Cat#637260) was used as template in the generation of VHZ cDNA. Forward primer A; 5’gcgaattcaccatgggcgagtgcaccccccaacttctcc3’ and reverse primer B; 5’gtggatc-ccgtttcgttcgctggtag 3’ were used to perform PCR. The VHZ PCR fragment was then inserted into the EcoR1 and BamH1 sites of the pEGFP-N1 vector, resulting in VHZ-EGFP. The VHZ PCR product was also inserted into pGEX-KG to form GST-VHZ. The authenticity of all the clones was confirmed by DNA sequencing of the coding region.

Generation of mouse monoclonal VHZ antibody (clone #209)

We used the ClonaCell™-HY Hybridoma Cloning Kit (Stemcell Technologies Inc.) to generate VHZ hybridomas [3]. The procedures were followed according to the manufacturer’s directions. Briefly: 1. Immunization of BALB/c mice with GST-VHZ fusion protein. 2. Growth of BALB/c parental myeloma cells SP2/0. 3. Preparation of BALB/c mice for spleenocytes from immunized mice 4. Fusion of spleenocytes with SP2/0 cells: 5. Selection and characterization of the hybridoma clones. Over 500 surviving hybridoma clones were isolated and grown. By ELISA, 75 clones showed good reactions with VHZ. We searched for the best hybridoma clone that could be used for multiple applications such as ELISA, WB, IF, IHC. Finally, VHZ clone (#209) with the strongest reactivity was selected for further studies. Rabbit polyclonal anti-VHZ serum was generated (Genemed Synthesis, Inc.) by immunizing rabbits with a synthetic peptide C-RRLRPG-SIETYEQEK corresponding to amino acid residues 126-140 of human VHZ. Antibodies were purified using Protein A followed by peptide affinity chromatography.

Generation of ascetic fluids

Hybridoma cells (5 × 10^6) were suspended in 200 μl of serum-free DMEM medium and injected with a 26-gauge needle into the peritoneal cavity. After 10 days, the mouse developed a large quantity of ascetific fluid, and the abdomen was greatly distended. The mouse was sacrificed and a small shallow was cut to open the abdominal cavity. The ascetic fluid was drawn with 10 ml syringe fitted with an 18-gauge needle. The fluid was centrifuged at 200 g for 10 min at 4°C. The supernatant fluid was collected and frozen at -70°C till further use.

Elisa assay

GST-VHZ or pure GST antigen stocks were made in carbonated buffer (pH 9.6). Solution (100 μl) containing an indicated amount of antigen was added to each well and incubated at 4°C for overnight to coat the antigen onto the plate. The plate was then blocked with 3% bovine serum albumin in PBS containing 0.05% Tween 20 for 30 min at 37°C. The plate was then washed thrice with PBS. Culture medium or monoclonal antibodies were added to each well and incubated for 40 min at 37°C. The secondary antibody, anti-mouse IgG conjugated with horseradish peroxidase (Pierce Cat. No 31430) diluted at 1: 3000 in
PBS was added to each well and incubated at 37°C for 40 min. The plate was rinsed with PBS containing 0.05% Tween-20 thrice followed by 3 washes with sterile water. The substrate, 100 μl of Turbo-TM B® (Pierce product number 34022), was added to each well and incubated for 10 min at room temperature. The reaction was stopped by adding 100 μl of concentrated H₂SO₄. Absorbance was measured at 450 nm (Elisa Reader DYNATECH MR7000).

Overexpression or shRNA-mediated knockdown of VHZ in MCF7 cells by retroviral transduction

The amphotropic Phoenix packaging cells (kindly provided by Nolan Lab at Stanford University) were transduced with the retroviral vector overexpressing VHZ, VHZ-mutant (pBABEpuro) or shRNAs in pSUPER.retro.puro vector (OligoEngine) using Effectene according to manufacturer’s instruction (Roche). The short hairpin RNA sequences (shRNAs) to suppress VHZ expression were purchased from Origene Technologies (Rockville, MD). The sequences for VHZ-KD-214, VHZ-KD-215, and VHZ-KD-216 are 5'CCATGCTGGCCTGCTGCTG, 5'GGCTGGCCCATCGAGAGGAG, and 5'AAGCAGTCTTCCAGTTTACACCAGCGAAGG3' respectively. After 48 h, the retroviral supernatants were collected, filtered (0.45 μm; Millipore) and then added onto the target MCF7 cells (HTB-22), which is a human breast cancer cell line purchased from American Type Culture Collection (ATCC; Manassas, VA), in the presence of 5 μg/ml of polybrene (Sigma-Aldrich) for 6-8 h. Infection was done twice. The cells were then selected with puromycin (1 μg/ml) followed by western blot to show overexpression of VHZ or VHZ-mut in MCF7 cells. These cell clones were seeded out in 6-well plates and the numbers of cell were counted from second day to fifth day. The VHZ knockdown MCF7 cells were analyzed using bromodeoxyuridine (BrdU) incorporation assay to assess the population of cells in the S phase. Briefly, the cells were incubated with BrdU for 15 mins and the incorporated BrdU was directly visualized under fluorescence microscopy. Confocal imaging was performed (Zeiss LSM 510 image Browser).

Analysis of exogenous VHZ-EGFP subcellular localization in NRK cells

NRK cells (ATCC CRL-6509) transfected with VHZ-EGFP expressing constructs were grown on coverslips and washed once with PBS (PBS containing 1 mM MgCl₂ and 1 mM CaCl₂). Cells were then fixed with 100% methanol for 15 mins at -20°C. The methanol fixation can reduce the cytosomal VHZ to clearly reveal the centrosomal VHZ signals. After two washes with PBS, the cells were permeabilized for 15 mins with 0.12% Saponin in PBS and incubated with rabbit anti-pericentrin antibody from Covance’ Inc (Princeton, NJ) for 1 hour at room temperature (RT), and then overnight at 4°C. The cells were gently washed three times with PBS and incubated with anti-mouse IgG conjugated with Texas Red (Sigma) for 4 hrs at RT. The VHZ-EGFP was directly visualized under fluorescence microscopy. Histopathologic Analyses with Immunohistochemistry (IHC)

The immunohistochemistry was performed as previously described [3]. Positive signals were detected by staining with 3,3'-diaminobenzidine chromogen (Dako, Carpinteria, CA). 80 formalin-fixed and paraffin-embedded surgical specimens of primary human breast cancer samples were collected from the archives of the Pathology Department of the Henan Medical Hospital. In addition, 32 human breast carcinoma tissues (CC08-02) were purchased from Cybrdi (Frederick, MD). Majority of the tissues were purchased from Cybrdi, Inc. (Rockville, Maryland 20850 USA: http://cybrdi.com/index.php) and include a major solid tumors tissue array (CC00-01-006); squamous cell carcinoma (CC00-01-009); lung carcinoma (CC04-01-006); colon adenocarcinoma (Grade I–III) with normal tissue controls (CC05-01-001); pancreatic
carcinoma (Duct adenocarcinoma/islet cell carcinoma/mucinous carcinoma) with normal controls (CC14-01-001).

Findings and Discussion
Generations of VHZ mAb (clone #209)
There is a high degree of homology within the DUSP protein family, it is therefore difficult to generate specific antibodies that do not cross-react with other DUSP proteins. ClonaCell™-HY Hybridoma Cloning Kit http://www.biowish.com/system/uploadtechN/DC22L44J9P.pdf was used to generate the VHZ hybridoma. After fusing spleenocytes derived from mouse immunized with GST-VHZ protein with SP2/0 myeloma cells, 502 surviving hybridoma clones were isolated and grown in culture. By ELISA, 75 clones showed good reactions with VHZ were subjected to evaluation by several critical tests. Only one clone (#209) was finally selected as the best since it can be used for multiple applications (ELISA, WB, IF, and IHC) (Figure 1A). The mAb secreted by clone #209 hybridoma was extensively characterized. The property and its uses (folds of dilution of ascitic fluid) in various applications were summarized. The multiple applications of the VHZ mAb were shown throughout this study (Elisa data in Additional file 1, Additional file 2). The VHZ specific antibody will be beneficial to the community of the researchers in this field.

Overexpression of VHZ enhances cell proliferation in MCF-7 cells
To test if VHZ plays a role in cell cycle regulation, we generated three MCF-7 stable pools that express control vector, VHZ, and VHZ(C95S) respectively by retroviral transduction. Lysates of puromycin-selected cells derived from the cells were analyzed by western blot using VHZ (#clone 209) mAb (Figure 1B) to show the levels of VHZ and VHZ-mut were comparable (Figure 1B, lane 2-3) and the sizes of exogenous VHZ and VHZ-mut protein (lane 2-3) were identical to the size of endogenous VHZ (lane 1). The three stable pools were then subjected to assess for their cell proliferation (Figure 1C), the number of cells (×1000) were plotted over the time of culture. VHZ-expressing cells displayed enhanced growth as compared to control and VHZ-mut cells. D. Colony forming assay. 10,000 cells as indicated were plated onto 6-well plates and grown for 2 weeks. The cells were then stained and images were taken. Cells expressing VHZ displayed enhanced ability of colony formation as compared to control and VHZ-mut expressing cells.

Knockdown of VHZ retards G1/S transition in MCF-7
We then carried out knockdown of VHZ in MCF-7 cells which were transduced with the control (Figure 2A, lane 1) and various shRNAs (lanes 2-4) targeting different sites of VHZ mRNA. Cell lysate was assessed by western blot to evaluate the efficiency of knockdown. The western blot (with VHZ mAb clone #209) results demonstrated that the levels of VHZ protein in KD-215 and KD-216 cells were much reduced compared to KD-214 cells (Figure 2A, lane 2). Interestingly, cells with efficient VHZ knockdown (KD-215 and KD-216 Figure 2A, lane 3-4) exhibited decrease in DNA replication as assessed by BrdU incorporation (Figure 2B), which was accompanied with increased population of cells in the G1 phase and decreased population of cells in the S phase (Figure 2C). These results indicate that knockdown of VHZ retarded cell cycle progression from G1 to S phase and are consistent with the observation that VHZ overexpression enhanced cell proliferation (Figure 1). Overall, the data support that VHZ may facilitate G1-S transition during cell cycle, and have the ability to promote cell proliferation.
VHZ is localized at the centrosome and the cytoplasm

To understand the role of VHZ in cell cycle regulation, we assessed the subcellular localization of VHZ by generating NRK cells that stably overexpress VHZ-EGFP. It is worthy to point out that truncated EGFP protein is often artificially located to the nuclei. Therefore, we observed the VHZ-EGFP localized to the nuclei of interphase, prophase, and telophase cells and appeared to redistribute to the cytoplasm of cells undergoing metaphase (Figure 3A). Importantly, the VHZ-EGFP was enriched in the centrosomes of cells at all stages of the cell cycle, as established by its co-localization with the pericentrin (a centrosomal marker) (Figure 3A). The localization of endogenous VHZ was determined by double immunofluorescence labeling with affinity-purified rabbit polyclonal anti-VHZ antibody in conjunction with mouse monoclo-
VHZ is overexpressed in some human breast cancers. Many human tumors show centrosomal aberrations. The centrosome is an organelle that plays a key role in cell-cycle progression and cell division. It organizes microtubule arrays throughout the cell cycle and plays a pivotal role in regulating cell division in meiotic and mitotic cells. Deregulation of the centrosome organelle is linked to human genetic diseases and cancer. Indeed, many human tumors show centrosome aberrations [12]. We hypothesized that like MKPs or PRLs; VHZ phosphatase might have similar functions in modulating the progression of some cancers. We investigated the expression levels of VHZ in 112 human breast cancer specimens and found that 8 out of 65 breast cancer samples (IDC/ILC stage I) expressed high levels of VHZ protein in the enlarged centrosome (VHZ-centrosomal-stain), as shown by double immunofluorescence with rabbit anti-VHZ and mouse anti-γ-tubulin on the same section of the cancer sample (Figure 4A) and by single staining with either rabbit anti-VHZ antibody or mouse anti-γ-tubulin antibody on two adjacent sections of the same sample (Figure 4B, a-b). In addition to VHZ-centrosomal-staining, we also observed a VHZ-cytoplasmic-staining pattern in a different subset of breast cancer samples that were diagnosed as IDC Stage II/III. 11 out of 47 of this type of breast cancer samples showed high levels of VHZ protein distributed throughout the cytoplasm of epithelial tumor cells that displayed a fibroblast-like morphology (Figure 4C a-b). In the latter, it is possible that a fair amount of VHZ might still be centrosomal but the staining could be masked by the increased cytoplasmic staining in these tumor cells. Tumor cells infiltrate the surrounding tissue matrices in diverse patterns including both individual- and collective-cell-migration strategies [13,14]. In our study, we showed that there were both individual-VHZ- (Figure 4C, a) and collective-VHZ-cytosol-positive cells (Figure 4C, b). Mouse VHZ mAb was used to reconfirm some of the 112 breast cancer specimens. Both rabbit and mouse VHZ antibodies show similar results. These phenomena might represent a relatively early onset of local invasion within microenvironments in vivo. However, by IHC, 24 normal breast tissues used as control in human breast carcinoma tissues (CC08-02) purchased from Cybrdi (Frederick, MD) were shown to be all negative for VHZ.

VHZ is a phosphatase that is involved in cell signaling pathways. Its expression and activity have been linked to various cellular processes, including cell division and migration. The study of VHZ expression in breast cancer tissues can provide insights into the mechanisms of tumor progression and metastasis.

Figure 3. Exogenous and endogenous VHZ are localized in the centrosome and the cytoplasm.

A. VHZ-EGFP was transfected into NRK cells and visualized in cells at various cell cycle stages: Interphase (a), Prophase (b), Metaphase (c), and Telophase (d). Pericentrin is labeled in red and nuclei are labeled with To-pro-3 iodide in blue (a-d). The images were merged as shown (a’-d’). Bar, 10 μm. B. Endogenous VHZ was detected in NRK (a-c, bar, 10 μm) cells by double staining with affinity-purified rabbit anti-VHZ (rAb) and mouse anti-γ-tubulin antibodies followed by anti-rabbit IgG conjugated with anti-rabbit-FITC (green) and anti-mouse IgG conjugated with anti-mouse-Texas Red. Endogenous VHZ was also detected in MCF10A and MCF-7 cells (d-f, bar, 20 μm and g-i, bar, 10 μm) by double staining with mouse anti-VHZ (mAb) and rabbit anti-pericentrin antibodies followed by anti-mouse IgG conjugated with anti-mouse-FITC (green) and anti-rabbit IgG conjugated with anti-rabbit-Texas Red.

Figure 4. Tumor cells infiltrate the surrounding tissue matrices in diverse patterns. A subset of breast cancer samples showed high levels of VHZ protein distributed throughout the cytoplasm of epithelial tumor cells that displayed a fibroblast-like morphology. The localization of VHZ to the centrosomal regions, which is of particular interest, gives some insight to the potential role of this phosphatase.
the conventional wound-healing or Transwell chamber assays, we used an alternative 'Inverted Coverslip' assay previously described [15]. As shown (S. Figure 2B, a' white arrows), MCF7-VHZ cells migrated out from the coverslip, while MCF-VHZ(C95S) cells remain within the coverslip (S. Figure 2B, b' white arrows). The results suggest that VHZ is able to promote cell motility. Most importantly, we obtained 4 pairs of human samples (breast cancers matched with their respective normal breast tissues) from 4 individuals to compare the expression of VHZ expression levels, all 4 breast tumors showed robust VHZ upregulation while 3 normal breast tissues showed no expression with 1 showed low expression (Figure 4D), indicating that VHZ is specifically overexpressed in these breast cancers examined. This result also further confirmed the specificity of VHZ mAb in recognizing endogenous protein. The data suggest that VHZ may potentially be a useful therapeutic target for breast cancer.

**VHZ is elevated in a significant fraction of several types of human cancers**

In addition to 112 breast cancers examined with rabbit VHZ antibodies, we also reconfirmed the data with VHZ mAb (clone #209) and obtained similar results; we further used the specific VHZ mAb (clone #209) to examine 448 multiple human cancers (Figure 5) to determine the expression of VHZ in other types of human cancers by immunohistochemistry (IHC) [3]. VHZ level was elevated in 17.5% (25 out of 143 cases) of colon cancers, 24.0% (20 out of 82 cases) of lung cancers, 35.0% (22 out of 63 cases) of squamous carcinoma (such as the lip, pharynx, vulva, cervix, and penis etc), 33.3% (17 out of 51 cases) of pancreatic cancers, 17.5% (7 out of 40 cases) of breast cancers, 35.3% (4 out of 12 cases) of esophageal cancers, 35.7% (5 out of 14 cases) of stomach cancers, 2 out of 6 cases of bladder cancers, 2 out of 11 cases of kidney cancers, 2 out of 6 cases of skin cancers, 2 out of 6 cases of esophageal cancers, 3 out of 8 cases of prostate cancers, and 2 out of 6 cases of testis cancers. These observations imply that VHZ overexpression may be associated with the development or progression of various human cancers, although the functional and clinical implications need to be addressed by future experiments. Our VHZ mAb will be useful as a reagent in clinical diagnosis for VHZ expression and for research community in the field of DUSP protein family.

Our findings reveal new insight into VHZ centrosomal localization and elucidate its role in promoting cell proliferation by participating in G1-S transition. The observed overexpression of VHZ in human cancers especially in breast cancer indicates that VHZ may have a proliferative role in human cancer. Further investigation of this phosphatase will help to increase understanding of the molec-
ular mechanism responsible for its pro-proliferative role. The mAb developed and characterized here will be an important reagent to the scientific community for further studies on VHZ phosphatase.

Additional material

Additional file 1 VHZ (clone #209) mAb and Rabbit polyclonal antibodies are specific against VHZ antigen by Elisa. To assess the specificity of VHZ antibodies, 96-well plate was coated with indicated GST or GST-VHZ antigen (10 ng). Both antibodies showed 9-11 times more reactivity to 10 ng of GST-VHZ antigen than to pure GST antigens.

Additional file 2 A. VHZ is not expressed in normal human breast tissue by IHC. B. Overexpression of VHZ in MCF-7 cells enhances cell migration. Cell motility was assessed by growing MCF-7 cells overexpressing VHZ-EGFP or VHZ (C95S)-EGFP to form a confluent monolayer on a coverslip. The cell-coated coverslip was then inverted with the cell side down onto a fresh culture dish. Images were taken at 0 and 48 h of the MCF-7-VHZ-EGFP cells (a, a') and the MCF-7-VHZ (C95S)-EGFP (b, b'). Panel a shows MCF-7-VHZ-EGFP cells moving out (indicated by the arrows) from underneath the coverslip. Immunofluorescent images (a, b). Phase-contrast images (a, b'). Magnification x200.

Abbreviations
VHZ: VHI-like member Z; PRL: phosphatase of regenerating liver; PT: protein tyrosine phosphatase; WB: western blot; IF: immunofluorescence; IHC: immunohistochemistry; FACS: fluorescence-activated cell sorter; EGFP: enhanced green fluorescent protein; MCF10A: human breast epithelia cell line; MCF-7: human breast cancer cell line; NRK: normal rat kidney cell line; IDC: invasive ductal carcinoma.

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
QZ designed and drafted the manuscript. JPT did molecular cloning, transfection, IF, CPT, MMS, and HZY carried out PCR and western blot, LJ generated mAb (#209) against VHZ and performed IHC, JEP and SWC carried out the experiments for cell proliferation, knockdown, and FACS. GJ, LWC, and CJ analyzed data from IHC on human cancer samples. WNT helped in discussion. All authors approved the final manuscript.

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