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Peroxiredoxin 1 is involved in disassembly of flagella and cilia

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ABSTRACT

Cilia/flagella are evolutionarily conserved cellular organelles. In this study, we demonstrated that *Dunaliella salina* Peroxiredoxin 1 (DsPrdx1) localized to the flagella and basal bodies, and was involved in flagellar disassembly. The link between DsPrdx1 and flagella of *Dunaliella salina* (*D. salina*) encouraged us to explore the function of its human homologue, *Homo sapiens* Peroxiredoxin 1 (HsPrdx1) in development and physiology. Our results showed that HsPrdx1 was overexpressed, and cilia were lost in esophageal squamous cell carcinoma (ESCC) cells compared with the non-cancerous esophageal epithelial cells Het-1A. Furthermore, when HsPrdx1 was knocked down by short hairpin RNA (shRNA) lentivirus in ESCC cells, the phenotype of cilia lost can be reversed, and the expression levels of tumor suppressor genes LKB1 and p-AMPK were increased, and the activity of the oncogene Aurora A was inhibited compared with those in cells transfected with scrambe-shRNA lentivirus. These findings firstly showed that Prdx1 is involved in disassembly of flagella and cilia, and suggested that the abnormal expression of the cilia-related gene including Prdx1 may affect both ciliogenesis and cancernogenesis.

1. Introduction

Cilia (also known as flagella) are microtubule-based signaling platforms that protrude as filiform organelles from the surface of cells, and are highly conserved in organisms ranging from *Chlamydomonas reinhardtii* (*C. reinhardtii*), *Drosophila melanogaster*, *Caenorhabditis elegans* and to all vertebrates so far examined [1]. Formerly, the cilia have been viewed as an evolutionary vestige in vertebrates. But, recent studies revealed that the cilia are sensory organelles, which act as machinery and chemical receptors [2–3]. Additional evidences showed that the cilia play important roles in several crucial signaling pathways of cancernogenesis, tissue development and cell homeostasis, such as, PDGFαα, Hedgehog, Wnt and mTOR [4–7]. Furthermore, it has been indicated that cilia are predominantly lost or disassembly in cancers including renal cell, breast, glioblastoma cells and pancreatic cancers in comparison with their normal cellular counterparts [8–11]. Until now, evidence has not been reported on whether the cilia are lost in esophageal squamous cell carcinoma (ESCC) cells.

Previous studies on *Chlamydomonas* and *Dunaliella* have begun to dissect the process of flagellar disassembly or assembly [12–15], i.e., identified that *C. reinhardtii* Aurora-like kinase (CALK) was an essential factor for flagellar disassembly [16], End-binding protein 1 (EB1) localized to flagellar tip and basal bodies of *C. reinhardtii* [17] and was required for cilia assembly in fibroblasts [18], and S-adenosylhomocysteine hydrolase (SAHH) accelerated flagellar regeneration of *D. salina* [19]. Additional investigations also showed that Liver kinase B 1 (LKB1) and its target AMP-activated protein kinase (AMPK) localize to the cilium or basal body of epithelial cells [20], and it negatively regulates HEF1-Aurora A-HDAC6 signaling pathway [21,22], which contributes to ciliary disassembly in hTERT-RPE1 cells [23].

Peroxiredoxin 1 (Prdx1) is an ubiquitously expressed protein for scavenging reactive oxygen species (ROS) in cells, and overexpressed in many cancers [24–28], but its biology functions in cancers remain enigmatic. In this study, we found that *D. salina* Peroxiredoxin 1 (DsPrdx1) localized to the flagella and basal bodies, and was involved in flagellar disassembly in *D. salina*. The link between DsPrdx1 and flagella of *D. salina* encouraged us to explore the function of *Homo sapiens* Peroxiredoxin 1 (HsPrdx1) in development and physiology. The results showed that HsPrdx1 was overexpressed and the cilia were lost in ESCC cells compared with the non-cancerous esophageal epithelial cells Het-1A cells. The ciliogenesis and the expression levels of LKB1 and p-AMPK were increased, while the activity of Aurora A was inhibited in HsPrdx1-knockdown ESCC cells. These findings suggest that Prdx1 may play important roles in disassembly of cilia, and this study establishes a link between ciliary disassembly mediated by cilia-related genes

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and cancernogenesis, which is likely to provide not only new sights into the molecular mechanism of cancernogenesis but also potential therapeutic or diagnostic targets for cancer patients.

2. Materials and methods

2.1. Cell line and cell cultures

ESCC cell lines Eca109 and EC9706 were purchased from the State Key Laboratory of Molecular Oncology, Chinese Academy of Medical Sciences. The control cell was non-cancerous esophageal epithelial cell Het-1A, which immortalized by transfection with plasmid pRSV-T containing the RSV-LTR promotor and SV40 T-antigen [29] was kindly provided by Professor Zhenyu Ji (Henan Academy of Medical and Pharmaceutical Sciences, Zhengzhou University, Henan, PR China). Each of the cell lines was cultured in RPMI 1640 medium (Gibco-BR2, USA) supplemented with 10% fetal bovine serum (HyClone Laboratories, USA), at 37 °C in the presence of 5% CO_2.

2.2. Antibody preparation and Western blotting of D. salina

The DsPrdx1 cDNA fragment (GenBank accession number KC999111) was subcloned into the prokaryotic protein expression vector pET28a (+), and expressed in competent cells of *Escherichia coli* (*E. coli*) BL21 to produce fusion DsPrdx1 proteins. Transformants were cultured in Luria–Bertani medium for 3 h, and then isopropyl β-d-1-thiogalactopyranoside (IPTG) at a final concentration of 1 mM was added to induce the expression of the fusion proteins. The inclusion bodies were purified using Ni-IDA Sefinose Kit (BBI, Canada), and the anti-DsPrdx1 polyclonal antibodies were prepared in rabbit using purified recombinant fusion proteins. In Western blotting, protein samples were subjected to 12% SDS–PAGE gels and transferred to PVDF membranes (Millipore, USA), which were blocked in 5% skimmed milk in TBST buffer, and then incubated with the anti-DsPrdx1 polyclonal antibody (1:2000), and the horseradish-peroxidase labeled secondary antibodies (1:3000, Sangon Biotech, China). Finally, the bands of specific proteins on the membranes were detected by Enhanced Chemiluminescence Kit (Santa Cruz, USA).

2.3. Protein extraction and treatment of D. salina cells

The *D. salina* cells were treated with 3-isobutyl-1-methylxanthine (IBMX, Sigma Aldrich, USA) at a final concentration of 1 mM to induce flagellar disassembly [30,31]. Flagella were detached from *D. salina* cell bodies by pH shock method, and purified by sucrose density gradient centrifugation as described previously [13]. Total proteins were extracted from cells of *D. salina* at particular times during IBMX treated, and the DsPrdx1 protein in cell bodies (CP) and flagella (FL) was examined by Western blotting.

2.4. Examination of DsPrdx1 localization

The *D. salina* cells were collected and fixed by 2% glutaraldehyde in the centrifuge tube, and then they were washed with PBS at room temperature (RT). The cells were smeared on the slides and air-dried, and the slides were treated with 0.5% Nonidet P 40 (NP-40) for 5 min and washed by water and ice-Acetone three times with every time for 5 min. Subsequently, the slides were incubated with anti-DsPrdx1 antibody of 1:200 diluted with antibody diluent at 37 °C for 2 h. The slides were rinsed with PBS and incubated with FITC-conjugated goat anti-rabbit IgG (1:100, Sangon Biotech, China) at 37 °C for 1 h. Stained cells were viewed under confocal laser scanning biological microscope (LEICA, TCS-SP2).

2.5. Lentiviral production and transduction

Small interfering RNA (siRNA) targeting HsPrdx1 (GenBank accession number: NM_181696) sequence (TGTCTGACTACAAAGGAAA) and a scrambe sequence (TTCTCCGAACGTGTCACGT) were transformed into short hairpin RNA (shRNA) (stem-loop-stem structure) and were cloned into GV112-Lentivirus vectors with *Age* I/*EcoR* I sites, respectively. All the recombinant lentiviral particle and transduction were prepared as described previously [32]. A final concentration of 5 μg/mL Polybrene and 10 μL of concentrated packaged lentiviral particle were added to the cells, allowed to incubate for 12 h, and then the medium was changed for fresh growth medium and the cultures were collected for experiments at 72 h after transfection.

2.6. Detection of cilia by immunofluorescence in ESCC cells

To induce visible cilia, the cells were grown to 80% confluence in 24-well plate, and then replaced for fresh medium without serum for 24 h. The cells were fixed with 4% paraformaldehyde for 30 min, and incubated with 200 μL glycine (2 mg/mL) for 5 min. The cells were permeabilized with 0.5% Triton-X 100 in PBS, and then blocked in blocking solution (5% goat serum and 0.5% Triton-X 100 in PBS) for 1 h. The cells were incubated with the monoclonal antibody Acetyl-α-Tubulin (1:500, Cell Signaling Technology) for 2 h. After cells were washed with PBS three times, followed incubated with secondary antibody Goat Anti-Rabbit IgG/FITC (1:500, Sangon Biotech, China). Nuclei were stained by Hoechst 33342 at RT for 30 min in the dark. Stained cells were viewed under inverted immunofluorescence microscope (OLYMPUS I, X71). Quantitation was performed on at least three independent experiments, counting about 100–200 cells per sample each time.

2.7. Western blotting of ESCC cells

Protein concentrations were determined using Bradford protein assay kit (Sangon Biotech, China) as a standard. Typically, 10–15 μg of proteins were subjected to each lane. Each of the antibodies sera against HsPrdx1 (Cell Signaling Technology, USA), LKB1 (Cell Signaling Technology, USA), phospho-AMPKα^{Thr172} (Cell Signaling Technology, USA), phospho-Aurora A^{Thr288} (Cell Signaling Technology, USA) and GAPDH (Cell Signaling Technology, USA) were used at 1:1000, and the horseradish-peroxidase labeled secondary antibodies (Sangon Biotech, China) at 1:3000. Finally, the bands of specific proteins on the PVDF membranes were detected by Enhanced Chemiluminescence Kit (Santa Cruz, USA).

2.8. Statistical analysis

All experiments were repeated at least three separate experiments, and the data were performed using SPSS version 17.0 (SPSS, Chicago, USA). Results were expressed as means ± standard deviations. In all statistical analyses, a P value <0.05 was considered statistically significant and was two-sided.

3. Results

3.1. Function and localization of DsPrdx1

Polyclonal antibodies of DsPrdx1 were prepared against the purified prokaryotic expressed protein and used in Western blotting and immunolocalization experiments. As shown in Fig. 1B, DsPrdx1 antibodies specifically recognized a single band of the predicted molecular weight of DsPrdx1 at ~22 kDa, and the Western blotting result also showed that DsPrdx1 existed in the