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POH1 Knockdown Induces Cancer Cell Apoptosis via p53 and Bim[1,2,3,4,5]

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Abstract

The ubiquitin-proteasome system is implicated in cell apoptosis that is frequently dysregulated in human cancers. POH1/rpn11/PSMD14, as a part of the 19S proteasomal subunit, contributes to the progression of malignancy, but its role in apoptosis remains unclear. Here, we showed that POH1 expression was increased and associated with poor outcomes in three independent cohorts of patients with hepatocellular carcinoma (HCC), esophageal cancer (EC), and colorectal cancer (CRC). The knockdown of POH1 significantly inhibited tumor cell proliferation and induced apoptosis mediated by the mitochondrial pathway *in vitro*. Intratumoral injection of POH1 small interfering RNA (siRNA) significantly reduced the progression of tumor growth and induced apoptosis *in vivo*. Furthermore, p53 or Bim siRNA markedly attenuated the apoptosis induced by POH1 depletion. POH1 depletion resulted in cell apoptosis by increasing the stability of p53 and Bim and inhibiting their ubiquitination. Overall, POH1 knockdown induced cell apoptosis through increased expression of p53 and Bim via enhanced protein stability and attenuated degradation. Thus, POH1 may serve as a potential prognostic marker and therapeutic target in human cancers.

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Introduction

Apoptosis is a programmed cell death, which is a highly regulated and controlled process essential for organisms to remove the damaged, dysfunctional, or excess cells [1]. Dysregulation in apoptosis may lead to a variety of diseases such as cancer. In general, there are two apoptotic pathways: intrinsic and extrinsic pathway. The intrinsic pathway of apoptosis is controlled by the B-cell lymphoma-2 (Bcl-2) family of anti-apoptotic (such as Bcl-2 and Bcl-xL) and pro-apoptotic

Abbreviations: Bcl-2, B-cell lymphoma-2; HCC, hepatocellular carcinoma; EC, esophageal cancer; CRC, colorectal cancer; POH1, Pad1 homologue, proteasome 26S subunit, non-ATPase 14; IHC, Immunohistochemistry; qRT-PCR, quantitative reverse transcription polymerase chain reaction; TMA, tissue microarray; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; PARP1, poly (ADP-ribose) polymerase 1; TUNEL, terminal deoxynucleotidyl transferase (TdT) dUTP nick-end labeling; siRNA, small interfering RNA.

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[2] Consent for publication: Not applicable.

[3] Ethics approval and consent to participate: This study has been approved by the Institutional Review Board and Human Ethics Committee of SYSUCC. Written consent for using the samples for research purposes was obtained from all patients prior to surgery. The project has been examined by Animal Ethical and Welfare Committee of SYSU and is in compliance with animal protection, animal welfare, and ethical principles and the relevant provisions of the National Laboratory Animal Welfare Ethics.

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proteins (such as Bad, Bid, Bax and Bim) [2]. When the cells are suffering severe cellular damage or stress, the Bcl-2 family of proteins regulates apoptosis by controlling mitochondrial permeability and the release of cytochrome *c*. Released cytochrome *c* binds Apaf1 and forms an activation complex with caspase 9, which gives rise to the formation of apoptosome, which is responsible for induction of mitochondrial apoptosis [3–5]. Bim is a member of Bcl-2 family proapoptotic proteins, which translocates to mitochondria in response to death stimuli, including survival factor withdrawal [6,7]. In addition, p53 is involved in the regulation of mitochondrial apoptotic pathway, mainly through the transcriptional regulation of the mitochondrial membrane Bcl-2 family proteins [8,9]. Upon exposure of cells to certain stimuli (DNA damage, oxidative stress, etc.), the mitochondrial membrane permeability increases, resulting in the release of cytochrome *c* and the subsequent induction of mitochondrial apoptosis [10–12]. Cell apoptosis is frequently dysregulated in human cancers, and emerging evidence indicates that cancer cells adopt various strategies to override apoptosis [13,14].

The proteasome is an abundant multienzyme complex that provides the main pathway for the degradation of intracellular proteins in eukaryotic cells. The 26S proteasome consists of one 20S core complex for proteolysis and two 19S regulatory complexes for protein degradation [15–17]. Accumulating evidence indicates that the loss of control over the ubiquitin proteasome system may induce cell apoptosis [18,19]. POH1, a deubiquitinating enzyme within the 19S proteasomal subunit, is responsible for substrate deubiquitination during proteasomal degradation [20,21]. POH1 functions in various biological processes, including protein stability [22,23], aggresome clearance and disassembly [24], cellular proliferation [25] double-strand DNA break responses [26], and embryonic stem cell differentiation [27]. In normal cells, POH1 small interfering RNA (siRNA) may induce reduction in cell proliferation [28]. POH1 is also known to play an important role in the progression of tumors. For instance, siRNA-mediated knockdown of POH1 had a considerable impact on cell viability and induced cell arrest in the G0-G1 phase, ultimately leading to senescence [28]. Wang et al. [29] proposed that the aberrant upregulation of nuclear POH1-mediated E2F1 stabilization promotes tumor formation in hepatocellular carcinoma (HCC). It is suggested that targeting POH1 may overcome proteasome inhibitor (such as bortezomib) resistance in multiple myeloma by inducing cell apoptosis [30]. Whether POH1 deregulation contributes to the intrinsic pathway of apoptosis in cancer is questionable.

In this study, we detected the expression of POH1 at both mRNA and protein levels in HCC, esophageal carcinoma (EC), and colorectal cancer (CRC) tissues and determined the relationship between POH1 and clinicopathological features of patients with these cancers. Furthermore, we observed that POH1 silencing induced cell apoptosis through an increase in the expression of p53 and Bim mediated by enhanced protein stability. Our study, therefore, describes a previously unknown mechanism that p53 and Bim expression is regulated by POH1 and its implication in apoptosis.

Materials and Methods

Patients, Tissue Specimens, and Follow-Up

A total of 461 paraffin-embedded HCC specimens, 216 paraffin-embedded EC specimens and 314 paraffin-embedded CRC specimens were obtained from the archives of the Department of Pathology of the Sun Yat-sen University Cancer Center (SYSUCC) between January 2000 and December 2015. Fifty-nine cases of paired fresh HCC and adjacent nontumorous liver tissues, 12 cases of paired fresh EC and adjacent nontumorous esophageal tissues, and 20 cases of paired fresh CRC tissues and adjacent nontumorous colon tissues were collected from patients at the time of surgical resection for the determination of POH1 mRNA and protein expression. None of the patients received any chemotherapy or radiotherapy before the surgery. The follow-up period was defined as the interval from the date of surgery to the date of death or the last follow-up. This study was approved by the Institutional Review Board and Human Ethics Committee of SYSUCC.

Tissue Microarray (TMA) Construction and Immunohistochemistry (IHC)

Using a tissue array instrument (Minicore Excilone, Minicore, UK), a tissue core (0.6 mm in diameter) was punched from the marked areas and re-embedded. All specimens were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer for 24 hours and embedded in paraffin wax. The paraffin-embedded tissues sections were sliced into 4-μm sections and mounted onto glass slides. After dewaxing, the slides were treated with 3% hydrogen peroxide in methanol and blocked with a biotin-blocking kit (DAKO, Germany). After blocking, the slides were overnight incubated with POH1 monoclonal antibody (1:50, Abcam, US), p53 monoclonal antibody (1:50, Santa Cruz, US), and Bim monoclonal antibody (1:50, CST, US) in a moist chamber at 4°C. After washing thrice in phosphate-buffered saline (PBS), the slides were incubated with biotinylated goat anti-rabbit antibodies for 1 hour. The slides were stained with DAKO liquid 3,3′-diaminobenzidine tetrahydrochloride (DAB), followed by their counterstaining with Mayer's hematoxylin and observation under a microscope.

The protein level of POH1 was determined by semiquantitative IHC detection. The positively stained samples were scored as follows: 1, ≤25% of positively stained cells; 2, >25%-≤50% of positively stained cells; 3, >50%-≤75% of positively stained cells; 4, >75% of positively stained cells. The intensity of staining was scored according to the following standard: 0, negative staining; 1, weak staining; 2, moderate staining; and 3, strong staining. The final score was calculated by multiplying the percentage score by the staining intensity score. The scores were independently determined by two pathologists (Dr. Jing-Ping Yun and Dr. Yong Li). The median IHC score was chosen as the cutoff value for defining high and low expression.

Chemicals and Reagents

Dulbecco's modified Eagle's medium (DMEM) was obtained from Gibco (Gibco, Gaithersburg, MD). The radioimmunoprecipitation assay (RIPA) buffer supplemented with a protease inhibitor cocktail (P8340) and cycloheximide (CHX) was purchased from Sigma-Aldrich (MA, USA), and MG132 was from Millipore. Phosphatase inhibitors were supplied by Roche Diagnostics (Shanghai, China), and monoclonal antibodies against POH1 were purchased from Abcam (USA). Monoclonal antibodies against Bim, Bad, Bid, Bak, Bax, puma, Noxa, p21, Bcl-xl, caspase-3, and caspase-9 were purchased from Cell Signaling Technology (USA). Monoclonal antibodies against β-actin, p53, Mcl-1, hemagglutinin (HA), PARP1, ubiquitin (Ub), a nd horseradish peroxidase (HRP)–conjugated anti-rabbit and anti-mouse antibodies were obtained from Santa Cruz Biotechnology Inc (CA, USA). Cytochrome *c* was purchased from Affinity Biosciences (USA), and Annexin V-FLOUS