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A novel heme oxygenase-1 splice variant, 14kDa HO-1, promotes cell proliferation and increases relative telomere length

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ABSTRACT

Alternative splicing is a routine phenomenon which greatly increases the diversity of proteins in eukaryotic cells. In humans, most multi-exonic genes are alternatively spliced and their splice variants confer distinct functions. Heme oxygenase-1 (HO-1, 32 kDa) is an inducible stress responsive protein, which possesses multiple functions in many cellular processes. In the current study, we identified a novel alternative splice isoform of 14 kDa HO-1 generated through exclusion of exon 3, and it is highly expressed in immortalized cells. In contrast to nuclear accumulation of the full-length 32 kDa HO-1, the novel 14 kDa HO-1 isoform is retained in the cytoplasm under ultraviolet (UV) irradiation. Interestingly, the 14 kDa HO-1 is shown to promote cell proliferation and an increase in relative telomere lengths *in vivo* and *in vitro*. Thus, we are pioneer to report and confirm the presence of a novel splice form of HO-1 and its distinct role in modulating telomere length and tumor growth.

1. Introduction

Heme oxygenase-1 (HO-1) derived from the full-length mRNA transcript of *HMOX1* gene is a 32 kDa protein [1]. The transcription is induced by exposure to various agents that promote oxidative stress including UVs, H_2O_2, heat and hemin [2–4]. HO-1 is an essential enzyme and catalyzes the conversion of heme into biliverdin, carbon monoxide, and ferrous iron [5].

The expression levels of HO-1 in many human cancers, particularly breast [6], thyroid [7] and oral cancers [8], are usually correlated with disease stage and poor prognosis in patients. HO-1 promotes cancer cell proliferation and prevents apoptosis triggered by chemotoxic agents or UV irradiation [9–11]. However, the controversial roles of HO-1 including involvement in tumorigenesis had been explained [12]. Moreover, HO-1 also regulates many other biological responses including autophagy, apoptosis, intracellular signal transduction, cell proliferation, and angiogenesis [13–16]. Recently, the subcellular localization of HO-1 has been investigated, since HO-1 can localize to the smooth endoplasmic reticulum [17,18], the nucleus [19], mitochondria [20], and caveolae [21]. In response to cellular stressors, HO-1 has been reported to translocate to the nucleus where it can have a plethora of important roles particularly transcriptional regulation independent of its enzymatic activity [19,22]. Although different cellular localizations could be important for the multiple functions of HO-1, additional modifications of HO-1 may also be of importance, including regulation of splice isoforms with distinct functions. About 95% of human multi-exonic genes are alternatively spliced [23], and the role of alternative splicing in a given gene appears to be quite independent. Dozens of differentially spliced variants of human Telomerase Reverse Transcriptase have been reported, and some lacking reverse transcriptase activity have different functions independent of the enzyme activity [24,25]. However, no HO-1 alternative splice isoforms have been analyzed up to now.

In this study, an alternative splice form of the *HMOX-1* gene was identified generated by exon 3 skipping, resulting in expression of a 14-kDa HO-1 protein. We provide experimental evidence for 14 kDa HO-1 expression pattern along with its role in cell proliferation and

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telomere length, which are significantly different to the well-known functions of full-length 32 kDa HO-1.

2. Materials and methods

2.1. Cell culture and treatment

All cells used in the study were cultured in Dulbecco's modified Eagle's medium (DMEM) (HyClone) containing 10% fetal bovine serum and maintained at 37 °C in a humidified 5% CO_2 incubator. The medium was exchanged every 2 days with fresh medium to maintain cell activity. For treatment, cells were seeded at 5×10^5/6-cm dish. After overnight culture, cells were exposed to UVR (0 kJ/m^2, 30 kJ/m^2, 60 kJ/m^2 and 90 kJ/m^2) or to H_2O_2 (0 μM, 50 μM and 100 μM), and after 4 h RNA was extracted.

2.2. Reverse transcription and PCR

Total RNA was extracted from cell lines using TRIzol reagent (TaKaRa) and were reverse-transcribed using GoScript™ Reverse Transcription System (Promega). PCR was performed using Ex Taq polymerase (Takara). The PCR primers are as follows: #1 5′-GAGGAACTTTCAGAAGGGCCAG-3' (forward), #2 5′-GGTCATCCCCTACACACCAGCCA-3' (forward), #3 5′-GACGGCTTCAAGCTCTTTGAGGA-3' (forward), and shared 5′-ATAAAGCCCTACAGCAACTGTCGCC-3' (reverse). Primers #4 are 5′-GGTCATCCCCTACACACCAGCCA-3' (forward) and 5′-AGGGCTTTCTGGGCAATCTTTTTGA-3' (reverse). qRT-PCR was conducted as previously described [26].

2.3. Vector construction and lentivirus transfection

The full-length coding regions of 32kDa/14 kDa HO-1 were amplified by PCR and cloned into vector pLJM1 (Addgene #19319) for expression. The specific primers were as follows: 5′-ATACCGGTCACGAACGAGCCCAGCACC-3′ (forward) and 5′-GCATGCCTGAATTCACATGGCATAAAGCC-3′ (reverse). The plasmid was co-transfected with psPAX2 envelope and pCMV-VSV-G packaging plasmids into actively growing HEK-293T cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Virus-containing supernatants were collected 48 and 72 h after transfection, respectively, and filtered to eliminate cells. Target cells were infected in the presence of 8 μg/ml polybrene and 24 h later, cells were selected with 1 μg/ml puromycin and analyzed 10 days after infection. To knock down endogenous HO-1 expression, the plasmids were established with pLKO.1 (Addgene #8453) according to the manufacturer's protocol. The target sequences were as follows: 5′-AACTCCCTGGAGATGACTCCC-3′ for 32 kDa HO1 and 5′-GACGGCTTCAAGCTCTTTGAG-3′ for 14 kDa HO1. Packaging of these knockdown lentiviruses is the same as with that of expression lentiviruses.

2.4. DNA extraction and telomere length assay

Extraction of genomic DNA of cells and tumors was performed as with the Gentra Puregene Cell Kit (Qiagen), according to the manufacturer's protocol. Telomere lengths were determined by qPCR as previously described [26,27]. The telomere lengths of cells were measured after 25 days of cultivation.

2.5. Flow cytometry

Treated cells were collected and then fixed in precooled 70% ethanol at 4 °C overnight. The cell cycle analysis was performed on a flow cytometer (Influx, BD).

2.6. Cell proliferation assays

Cell proliferation assays were performed with the CCK-8 Kit and Crystal Violet Staining Kit (Beyotime) according to the manufacturer's protocol.

2.7. Western blot and immunofluorescence

Western blot and immunofluorescence analyses were performed as previously described [26]. The antibodies used in western blot were anti-β-Actin (Santa, sc-130065) and anti-HO-1 (Abcam, ab13248). The antibodies used in immunofluorescence were anti-HA (Abcam, ab137838) and Cy3-labeled Goat Anti-Rabbit IgG (Beyotime).

2.8. Xenografted tumor model

Male BALB/c nude mice (5–6 weeks of age, 17–20 g body weight) were purchased from the Laboratory Animal Center of Chongqing Medical University. The study protocol was approved by the local Ethics Committee of the Third Military Medical University (Chongqing, China). A375-sh-14 kDa HO-1, A375-14 kDa HO-1 or control cells (2×10^6) were subcutaneously injected into the flanks of each individual mouse. Formation of tumors was examined every 5 days, length and width were measured with calipers, and the tumor volumes were calculated. On day 30, the mice were euthanized and the resulting tumors were excised, photographed and analyzed by routine histology.

2.9. Bioinformatics analysis

The protein structures were visualized and modified with DeepView and pyMOLTM software. Branch point sequence analysis was predicted with the online software Human Splicing Finder (http://www.umd.be/HSF3/HSF.shtml).

2.10. Statistical analysis

All values were expressed as mean ± S.D. Statistical analyses of the data were performed by two-tailed Student's *t*-test (*, $p < 0.05$; **, $p < 0.01$).

3. Results

3.1. Identification of a novel HO-1 human splice variant

In order to clone the human *HO-1* gene, we amplified the HO-1 coding sequence (CDS) from several cell lines. Unexpectedly, smaller bands around 400bp in length were amplified from A375, OSC-19 and SCC-15 cDNA but not from FEK4 cDNA (Fig. 1A). When sequenced, we found that the longer band with 867bp in length represented the full-length human *HO-1* gene (GenBank: BT019785), but in addition, shorter PCR fragment of 375bp in length revealed a 492-bp deletion (from 145 to 636 bp) caused by omission of exon 3 in the coding sequence when compared to the full-length *HO-1* gene (Fig. 1A and B). The corresponding coding protein sequence can be fully matched to the full-length HO-1 protein except for a 164-aa deletion (from amino acid positions 49 to 212). To distinguish both forms of HO-1, we named the full-length HO-1 protein 32 kDa HO-1 and the new alternative splice product 14 kDa HO-1 according to their molecular mass (Fig. 1B). Based on the human 32 kDa HO-1 structure (PDB ID: 4WD4) [28], the structure of 14 kDa HO-1 was predicted by a model in which the 14 kDa HO-1 is missing the heme pocket region that binds heme (Fig. 1B). As a result, we postulate that the 14 kDa HO-1 protein can perform