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As a novel p53 direct target, bidirectional gene HspB2/αB-crystallin regulates the ROS level and Warburg effect

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ABSTRACT

Many mammalian genes are composed of bidirectional gene pairs with the two genes separated by less than 1.0 kb. The transcriptional regulation and function of these bidirectional genes remain largely unclear. Here, we report that bidirectional gene pair HspB2/αB-crystallin, both of which are members of the small heat shock protein gene family, is a novel direct target gene of p53. Two potential binding sites of p53 are present in the intergenic region of HspB2/αB-crystallin. p53 up-regulated the bidirectional promoter activities of HspB2/αB-crystallin. Actinomycin D (ActD), an activator of p53, induces the promoter and protein activities of HspB2/αB-crystallin. p53 binds to two p53 binding sites in the intergenic region of HspB2/αB-crystallin *in vitro* and *in vivo*. Moreover, the products of bidirectional gene pair HspB2/αB-crystallin regulate glucose metabolism, intracellular reactive oxygen species (ROS) level and the Warburg effect by affecting metabolic genes, including the synthesis of cytochrome c oxidase 2 (SCO2), hexokinase II (HK2), and TP53-induced glycolysis and apoptosis regulator (TIGAR). The ROS level and the Warburg effect are affected after the depletion of p53, HspB2 and αB-crystallin respectively. Finally, we show that both HspB2 and αB-crystallin are linked with human renal carcinogenesis. These findings provide novel insights into the role of p53 as a regulator of bidirectional gene pair HspB2/αB-crystallin-mediated ROS and the Warburg effect.

1. Introduction

Activated oncogenes and loss of tumor suppressors change metabolism and induce the Warburg effect [1–3]. In most cancers, the tumor suppressor p53 is modified through mutations or changes in its expression, leading to the altered regulation of hundreds of genes that are directly influenced by this sequence-specific transcription factor [4,5]. In turn, p53 regulates key processes, including DNA repair, cell-cycle arrest, senescence, apoptosis and metabolism [6–8]. Non-mutant p53 promotes oxidative phosphorylation and inhibits the Warburg effect by affecting metabolism-related genes, thereby influencing the synthesis of cytochrome c oxidase 2 (SCO2), hexokinase II (HK2), and TP53-induced glycolysis and apoptosis regulator (TIGAR). These proteins regulate metabolic pathways that control mitochondrial respiration and glycolysis. In contrast, the loss and/or mutation of p53 results in an increase in glycolysis and intracellular reactive oxygen species (ROS) levels [1,7,9–11].

As members of the small heat shock protein gene family, αB-crystallin (αBC) and heat shock protein B2 (HspB2) share an approximate 0.9 kb intergenic promoter region in the human, mouse and rat genomes [12,13], indicating that αB-crystallin and HspB2 make up a bidirectional gene pair. The bidirectional sequences of the αB-crystallin and HspB2 genes contain an orientation-dependent enhancer that differentially directs expression of the αB-crystallin gene in different tissues, including the eye lens, heart and skeletal muscle [12,14]. Furthermore, the core

Abbreviations: αBC, αB-crystallin; ActD, Actinomycin D; ChIP, chromatin immunoprecipitation; EMSA, Electrophoretic Mobility Shift; HK2, hexokinase II; HspB2, heat shock protein B2; RCC, renal cell carcinoma; ROS, reactive oxygen species; SCO2, cytochrome c oxidase 2; sHSPs, small heat shock proteins; TIGAR, TP53-induced glycolysis and apoptosis regulator.

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promoter and enhancer recognition complexes, along with their cofactors, may have a critical regulatory function in driving cell-specific transcription [15,16]. Several transcription factors have been identified in the proximal promoter and enhancer cis-elements that regulate αB-crystallin gene expression, while little is known about the regulation of HspB2 gene expression [17–24].

Bidirectional genes are defined as two genes arranged head to head on opposite strands with less than 1000 base pairs between their transcription start sites (TSSs) [25–27]. Generally, approximately 10% of the protein-coding genes in the human genome are organized in this manner [25,26]. Bidirectional promoters, a critical driver for the specific transcript, are often coupled with protein-coding genes that are involved in the same biological processes, thereby enabling the coordination of temporal environmental responses, and cancer [25,26,28–31]. Motifs that correspond to the binding sites of transcriptional factors in bidirectional gene pairs are over-represented [28,32,33] and the combinational binding of transcription factors in diverse cell types is associated with gene expression and cell-specific biology [34]. The motifs of several transcription factors have been identified in bidirectional promoter regions [28,33,34], however, whether transcription factors, such as p53, control bidirectional genes like αB-crystallin and HspB2 remains unclear.

In the present study we show that the shared intergenic regulatory region of the bidirectional gene HspB2/αBC contains two potential p53 binding sites. We demonstrate that HspB2/αB-crystallin is a direct target of the transcription factor p53 and the products involved in metabolism by the regulation of p53.

2. Materials and methods

2.1. Reagents, antibodies and cell cultures

Human breast adenocarcinoma cancer cells MCF7 (HTB-22), human cervix adenocarcinoma cancer cells HeLa (CCL-2), mouse muscle myoblast cells C_2C_{12} (CRL-1772), and monkey kidney fibroblast-like cells COS7 (CRL-1651) were purchased from ATCC and maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum. Mouse αTN4 lens epithelial cells were maintained as previously described [21]. Actinomycin D (ActD) was from Sigma-Aldrich (St. Louis, MO). Anti-p53 antibody (DO-1 and Ab-1) was from Santa Cruz Biotechnology Inc. (Santa Cruz, CA) and Calbiochem (Gibbstown, NJ). Anti-αB-crystallin antibody was from Stressgen (Michigan, USA). Anti-β-actin antibody was from Sigma (St. Louis, MO). Anti-HspB2 (ab91258) was purchased from Abcam (Cambridge, MA). Human recombinant p53 was from Active Motif (Carlsbad, CA).

2.2. Plasmids and site-directed mutagenesis

The p53 expression plasmid (pCMV-Sport6-p53) was from Open Biosystems (Huntsville, AL). The dual-directional reporter plasmid pFL-HspB2αB-RL (RL is Renilla luciferase and FL is firefly luciferase) is described elsewhere [14]. Site-directed mutagenesis was performed with the QuikChange Site-Directed Mutagenesis kit (Stratagene; La Jolla, CA). The primers containing mutated sequences were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA) as follows: HSPB2-mu: 5′-CCTGCTCTGCAGGAGGGAAGGAGAGGACTAGTTGGGCC and 5′-GGCCCAACTAGTCCTCTCCTTCCCTCCTGCAGAGCAGG; αB-mu: 5′-GCTA GTGAAACAAGACCATGAAAGTCACCGGTCAGCTCAG and 5′-CTGAGCTG ACCGGTGACTTTCATGGTCTTGTTTCACTAGC.

2.3. Transfection and reporter gene/luciferase assay

Cells were grown in 24-well plates to 40–50% confluent and co-transfected with pCMV-Sport6-p53 and $pHSPB_2FL\alpha BRL$ reporter plasmids using Fugene6 (Roche Applied Science, Indianapolis, IN). pSV40-β-gal (Clontech, Mountain View, CA) was co-transfected to normalize transfection efficiency. ActD or DMSO (0.01%) was added into the culture medium 24 h after transfection and the cells were incubated for another 24 h. Luciferase activities were determined in 20 μl of cell lysate by a Dual Luciferase Assay kit (Promega Corporation, Madison, WI) on a GloMax™ 96 microplate luminometer (Promega Corporation, Madison, WI). Relative luciferase activities were expressed as ratios of the activities generated by the reporter plasmid to that of the control plasmid. Fold change was recorded as the ratio of relative luciferase activity from experimental group *versus* control group.

2.4. In vitro transcription and translation, Electrophoretic Mobility Shift (EMSA) and chromatin immunoprecipitation (ChIP) assays

One microgram of pCMV-Sport6-p53 was and translated *in vitro* using a rabbit reticulocyte lysate expression kit (Promega) following the manufacturer's instruction. The reaction mixtures were combined and incubated with probes as indicated. 5 μl of each lysate was used for gel shift experiments. The assays of EMSA and ChIP were followed as previously described [22].

2.5. Western immunoblotting

Protein lysates were quantified using a BCA Protein Assay kit (Pierce). Equal amounts of protein were subjected to 4–12% Bis-Tris SDS-PAGE (Invitrogen) and transferred electrophoretically onto a nitrocellulose membrane. The membrane was blocked with 5% fat-free milk/PBST, sequentially incubated with primary antibody overnight at 4 °C and secondary antibodies for 1 h at room temperature, and autoradiographed by the ECL detection kit (Pierce).

2.6. RNA interference

For knockdown of p53, the same pmol of shRNA control (pSUPER) and shRNA p53 (sip53) [10] was transfected into the cells respectively by DharmaFECT transfection reagents (Dharmacon). For knockdown of αB-crystallin and HspB2, shRNA αB-crystallin (siαBcrystallin, Cat# PIEE102001218), shRNA HspB2 (siHspB2, Cat#PIEE248019991) and related control shRNAs were purchased from GeneChem. The same pmol of shRNA control and target shRNA that was targeted to three different sequences was transfected into the cells as indicated with DharmaFECT transfection reagents (Dharmacon).

2.7. Reverse transcription and real-time PCR

Cells were transfected with the specified siRNAs for 48 h and harvested with Trizol (Invitrogen). cDNAs were synthesized with SuperScript II (Invitrogen) according to the manufacturer's protocol. Real-time PCR analysis was performed using the Applied Biosystems 7500 Real-Time PCR System, according to the manufacturer's instructions. The reactions were performed in duplicate for three to four independent experiments; the results were normalized to GAPDH. The primer sequences used are listed in Supplement table 1. The mean ± SD of three to four independent experiments is shown.

2.8. Immunohistochemical analysis

Human clear cell renal cell carcinoma (RCC) tissues from routine diagnostic biopsy specimens were obtained from the Department of Pathology, Xiangya Hospital, Changsha, China for immunohistochemical analysis. We obtained permission from the patients to use the related tissues for our current research. The presence of antigen was detected with the primary antibody (anti-p53 antibody, used at 1:500, Santa Cruz; anti-αBC, 1:500, Stressgen; anti-HspB2, 1:500, Abcam) and then incubated with the secondary antibody, which was horseradish peroxidase conjugated with anti-rabbit or mouse IgG. Two pathologists quantified the images of sections respectively.