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Oncosuppressor protein p53 and cyclin-dependent kinase inhibitor p21 regulate interstitial cystitis associated gene expression

Susan Keay[a], Shreeram C. Nallar[b,d], Padmaja Gade[b], Chen-Ou Zhang[c], Dhan V. Kalvakolanu[b,c,*]

[a] Department of Medicine, University of Maryland School of Medicine, Baltimore, MD 21201, United States
[b] Department of Microbiology & Immunology, University of Maryland School of Medicine, Baltimore, MD 21201, United States
[c] Department of Pathology, University of Maryland School of Medicine, Baltimore, MD 21201, United States
[d] Greenebaum NCI-Comprehensive Cancer Center, University of Maryland School of Medicine, Baltimore, MD 21201, United States

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ABSTRACT

Interstitial cystitis (IC) is a chronic syndrome that affects the urinary bladder. The etiology of this disease is unclear, and no effective therapies are available at this time. Although inflammation is suspected, no clear evidence for a role of conventional mediators of inflammation, such as cytokines and their downstream molecules, has been obtained to date. Our previous studies indicated that primary cell cultures derived from IC urothelium abnormally express molecules associated with cell adhesion. Here we describe a mechanism by which transcriptional changes in tight junction and adhesion molecules are mediated. Oncosuppressor proteins p53 and cyclin-dependent protein kinase inhibitor p21 directly associate with regulatory sites on the ZO-1 and E-cadherin genes, identifying important roles for p53 and p21 in driving non-oncogenic pathologies. These data also suggest that interference with these factors offers a potential therapeutic opportunity.

1. Introduction

Interstitial cystitis/bladder pain syndrome (IC/BPS) is a chronic syndrome characterized by pain with increased urinary frequency and urgency [1] that affects several million people in the USA [2–4]. Since the etiology for IC/BPS is unknown, and moderately effective treatment for patients with IC/BPS has not been found, greater understanding of the pathogenesis of this debilitating syndrome may be necessary for developing effective therapies. Our lab is interested in understanding the etiology and mechanisms of bladder epithelial thinning and/or denudation in IC/BPS, which are cardinal histopathologic findings in biopsies from IC/BPS patients [5–7]. Using immunohistochemical and gene-expression microarray analyses, we and others have found [8–11] that epithelial gene expression was consistently abnormal in primary cells derived from epithelial biopsies of IC/BPS patients suggesting that an abnormal program of epithelial gene expression and/or differentiation may occur. These cells grown from biopsies of patients with IC/BPS who fulfilled modified National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) criteria (without measurement of bladder capacity) [12] display stable and heritable abnormalities similar to those found in patient biopsies *in vivo*, including profoundly reduced rates of cell proliferation (which could result in thinning or ulceration) [13], specifically altered cell differentiation markers including higher E-cadherin and lower ZO-1, occludin, and vimentin [14–16], and increased paracellular permeability [16], as compared to cells grown from biopsies of age- and gender-matched controls (*i.e.*, normal bladder or NB cells). These explanted IC/BPS cells also uniquely produce a frizzled 8 protein-related antiproliferative factor (APF) that is not made by cells from age- and gender-matched asymptomatic controls [17,18]. Antiproliferative activity is found in urine samples from ≥90% of patients with IC/BPS including patients with Hunner's lesions [19–22]. Indeed, primary NB epithelial cells treated with HPLC-purified and/or a synthetic APF derivative in our laboratory displayed an IC/BPS cell phenotype in every manner examined including decreased proliferation [13,18], increased E-cadherin production [14,16], decreased ZO-1, occludin, and vimentin production [14,16], and increased paracellular permeability [16]. Since the regulation of these genes during IC is poorly understood, we have investigated this aspect in the current report. Here we show that tumor suppressor proteins p53 and p21, which were both previously shown to be increased in IC/BPS cells as compared to matched normal control cells [27], participate in regulating the expression of ZO-1 and E-cadherin genes. These data present an unconventional role for onco-suppressor proteins in non-tumor chronic pathologies.

* Corresponding author at: Greenebaum Cancer Center, University of Maryland School of Medicine, 660 W. Redwood St, Baltimore, MD 21201, United States.
E-mail address: dkalvako@umaryland.edu (D.V. Kalvakolanu).

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Table 1
Primers used fro PCR in this study.

Primer	Sequence (5′→3′)	purpose
ZO-1 Fwd:	cggtcctctgagcctgtaag	mRNA
ZO-1 Rev:	ggatctacatgcgacgacaa	quantification
β-Actin Fwd:	acattaaggagaagctgtgc	
β-Actin Rev:	ttctgcatcctgtcggcaat	
E-Cad Fwd:	cgggaatgcagttgaggatc	
E-cad Rev:	aggatggtgtaagcgatggc	
p21-Fwd:	gagcgatggaacttcgactt	
p21-Rev:	ggtacaaga cagtgacaggt	
p53 Fwd:	agtctagagccaccgtcca	
p53 Rev:	aggtctgaaaatgtttcctgactca	
ZO-1 P8F:	gcccagtgaaggaaacaact	Quantitative
ZO-1 P8R:	agccgggtaacccaagtaac	ChIP assays
E-cad P3F:	agtcccacaacagcataggg	
E-cad P3R:	ttctgaactcaaggcgatcct	

2. Materials and methods

2.1. Cell culture

NB and IC/BPS cells were grown in DMEM-F12 containing 10%FBS. Cells were serum starved for 2 days prior to ChIP assays, RNA, and/or Protein extraction. ChIP grade antibodies specific for p53 (Mouse mAb #48818), p21 (Rabbit mAb #2947) and H3-K9-Ac (Rabbit mAb #9649) were obtained from Cell Signaling Technologies, Danvers, MA).

2.2. Transcript quantification

Real-time PCR analyses were performed with specific primers using the JumpStart SYBR Green Master Mix (Sigma-Aldrich) in a Stratagene Mx3005P real time PCR machine. Expression differences of specific transcripts were quantified using the ACTB as an internal control by the ΔΔCT method. Each PCR reaction was had 5 replicates and each experiment was repeated with atleast 2 independent batches of RNA preparations. Primer sequences for real-time PCR are described in Table 1.

2.3. Chromatin Immunoprecipitation (ChIP) assays

Standard ChIP assays were performed using a commercially available kit from the Upstate Biotech, Inc as recommended by the manufacturer and the ChIPed DNA was used for normal or real time PCR as described in our previous studies [23,24]. After cross linking the chromatin with 1% formaldehyde for 5 min, cells were collected. The cells were sonicated for 15 s for 7 times with 30 s interval under ice with Bronson sonicator. The average fragment size was 500 bp under these conditions. An equal quantity of chromatin from each sample was incubated with at least 5 µg of either protein-specific IgG or non-specific IgG (Cell Signaling Technologies) at 4 °C overnight. IP products were collected after incubating with magnetic beads coated with protein G (Active motif, Inc). The beads were washed; protein-DNA cross-links were reversed; and DNA was purified after phenol-chloroform extraction and ethanol precipitation. Purified DNA from the input and IP samples were subjected to qPCR with specific primers (Table 1).

2.4. RNAi-mediated knockdown

To deplete the indicated transcripts, commercially available (Open Biosystems, Huntsville, AL) lentiviral vectors expressing target-specific short hairpin RNAs (shRNAs) under the control of human U6 promoter were employed. These vectors also carried a puromycin resistance gene, which allows the selection of stably-transfected cells. To deplete p21 protein, lentiviral vector (pLKO1) encoding a sequence (TRCN0000040123) specific to human p21 mRNA was used. p53 was knocked down using a previously published [25] shRNA (pLKO-p53-shRNA-427) obtained from Addgene, Cambridge, MA.

Lentiviral particles were prepared and used as described in our earlier studies [23]. Briefly, each shRNA expression plasmid (3 µg) was mixed with pCMV-dR8.2dvpr (2.7 µg) and pCMV-VSVg (0.3 µg) vectors and transfected into HEK-293 T cells using the Fugene 6 reagent (Roche) as described earlier (49). Medium from these cultures were collected daily for 5 days, pooled and passed through a 0.45 µm filter and used as source for lentiviral shRNAs. Knockdown of the target gene expression was assessed by performing Western blot analyses or qPCR. The empty vector and scrambled shRNA as controls to ascertain the specificity of targeting in all experiments. No significant differences in the transcript levels were observed with these two controls. Hence only one of these controls presented.

3. Results

3.1. Promoter analyses of the ZO-1 and E-cadherin genes

To identify potential transcriptional regulators for ZO-1 and E-cadherin, we selected from +1 to −1000 bp of the human ZO-1 and E-cadherin genes from the NCBI genome database and analyzed this region for the binding of potential transcription factors (TF) using publicly available search tools (http://molbiol-tools.ca/Transcriptional_factors.htm ; http://gene-regulation.com/pub/databases.html http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3/). Data from these searches were then used to generate potential binding site maps for both genes (Fig. 1). Approximate coordinates of the TFs with binding at multiple sites were notated. We noticed that both genes had high preponderance of TP53 (p53) binding sites. Sp1 and Jun B and Jun D binding sites were also found in these response elements.

3.2. Increased binding of p53 and p21 to response elements for the ZO-1 gene in IC cells

Since ZO-1 and E-cadherin are regulated in a diametrically opposing manner during IC/BPS, and a high number of p53 binding sites were found for both genes, we next examined if p53 directly bound to these enhancers using quantitative Chromatin Immunoprecipitation (ChIP) assays. Multiple primer sets (n = 8) spanning the length (between −1000 to +1 bp) of ZO-1 and E-cadherin genes were synthesized and PCR was performed to determine a specific amplification of the target sites. In the initial ChIP assays p53-specific antibodies were tested for specificity against two control ChIP reactions with: (1) no antibody; and (2) a non-specific IgG control antibody. Parallel control reactions, where total DNA from soluble chromatin was used as a template for

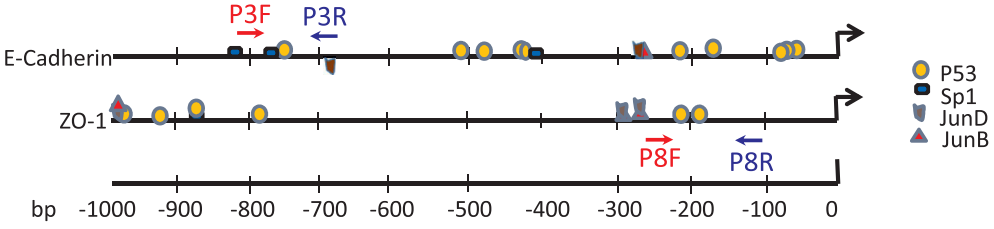

Fig. 1. Line diagrams showing the organizations of human ZO-1 and E-cadherin genes. Approximate distance of DNA-binding sites of various transcription factors upstream of the transcriptional start sites (arrow at the 3′ end) are indicated. Locations of specific primers used for the ChIP assays are indicated.