Title

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Authors

Choi, In-Seon
Yu, Kevin
Kim, Jayoung
et al.

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Alterations in the DNA Methylation Patterns of *Calca, Timp3, Mmp2, and Igf2r* are Associated with Chronic Cystitis in a Cyclophosphamide-induced Mouse Model

In-Seon Choi[1], **Kevin Yu**[1], **Jayoung Kim**[2], **Erika De Guzman**[1], **Daniel J. Weisenberger**[3], **Shirley Oghamian**[4], **Hee Ju Kim**[4], **Kyung Hwa Lee**[1], **Cindy Carroll**[4], **Binh N. Trinh**[4], **Myungjin Kim**[4], **Sahar Houshdaran**[4], **Peter W. Laird**[3,4], **Peter A. Jones**[5], **David Warburton**[1,5], and **Chester J. Koh**[1,5]

[1]Developmental Biology, Regenerative Medicine and Surgery Program and the Division of Pediatric Urology, Saban Research Institute, Children's Hospital Los Angeles

[2]Division of Cancer Biology and Therapeutics, Samuel Oschin Comprehensive Cancer Institute, Cedars-Sinai Medical Center

[3]USC Epigenome Center, USC/Norris Comprehensive Cancer Center, Keck School of Medicine of USC

[4]Department of Surgery, Keck School of Medicine of USC

[5]USC Institute of Urology and USC/Norris Comprehensive Cancer Center, Keck School of Medicine of USC, Los Angeles, California

Abstract

Objectives—To determine whether epigenetic changes occurred during cyclophosphamide (CYP)-induced chronic bladder inflammation in mice. Epigenetics plays a role in the regulation of inflammatory genes in non-cancer diseases such as asthma and COPD. However, epigenetic (DNA methylation) changes during chronic bladder inflammation have not been described previously.

Methods—Chronic cystitis was induced in three groups of adult CD-1 male mice using multiple weight-based intraperitoneal cyclophosphamide (CYP) injections over a period of three months. Histopathologic and MethyLight assays were performed on chronic bladder inflammation specimens at multiple time points to monitor cystitis progression and DNA methylation changes in comparison to control specimens, respectively.

Results—Histopathological analysis showed the most extensive edema and urothelial sloughing at the 1-month time point. MethyLight analyses revealed statistically significant changes in DNA methylation associated with the *Calca, Timp3, Mmp2, and Igf2r* genes in the chronic bladder injury model. The changes in DNA methylation associated with chronic cystitis were noted to be

Corresponding Author: Chester J. Koh, MD, FACS, FAAP, Developmental Biology, Regenerative Medicine, and Surgery Program, Saban Research Institute, CHLA; Division of Pediatric Urology, CHLA; USC Institute of Urology, Keck School of Medicine, USC, 4650 Sunset Boulevard, Mailstop 114, Los Angeles, CA 90027, Phone: 323-361-2247, Fax: 323-361-8034, ckoh@chla.usc.edu.

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DNA hypomethylation of the *Calca* gene in the control tissue, and DNA hypermethylation for the *Calca, Timp3, Mmp2*, and *Igf2r* genes, in comparison to control tissue.

Conclusions—DNA methylation changes were noted in *Calca, Timp3, Mmp2, and Igf2r* genes during chronic cystitis in a murine model. Epigenetics appears to play a role in the regulation of inflammatory bladder genes during chronic cystitis; however, further studies are needed to elucidate the pathways associated with these genes.

Keywords

Bladder Inflammation; Cystitis; Cyclophosphamide; Epigenetics; DNA; Methylation

Introduction

Bladder inflammation is a physiological process that characterizes many bladder diseases such as urinary tract infections and hemorrhagic cystitis. Cyclophosphamide (CYP) is a chemotherapeutic agent with well-described urological sequelea, including hemorrhagic cystitis, hematuria, and resulting urothelial damage, edema, and necrosis.[1] While CYP administration is a well-studied acute bladder injury model to investigate bladder inflammation and wound healing,[2] repeated exposure to CYP has also served as an effective animal model for chronic cystitis.[3]

Epigenetics involves the investigation of the meiotically and mitotically heritable changes in gene activity that occur independently of the changes in an organism's primary DNA sequence.[4] One main epigenetic modification identified in humans is the methylation of cytosine residues that are located within the CpG DNA sequence context. Methylation of the cytosine residues within CpG islands can be associated with loss of gene expression via transcriptional repression.[4]

While there is increasing recognition in recent literature that epigenetics may play an important role in the regulation of inflammatory genes in several organ systems, the relationship between DNA methylation and chronic inflammation of the bladder has not been described yet. Our study investigated whether epigenetic / DNA methylation changes occur as a result of CYP-induced chronic cystitis in murine urothelium in cancer-related genes, with the intention of identifying possible prognostic markers for bladder cystitis.

Materials and Methods

Animal Use and Cyclophosphamide Injections, and Histological Examination

All animal experimentation described was performed in accordance to protocols approved by the Institutional Animal Care and Use Committee. Adult (two month old) male mice (CD-1) with a body weight of 25–35 g were used in the experiments. Of note, only male mice were used to avoid the possible effects of hormonal variations that occur during the menstrual cycle in females. CYP (150 mg/kg) was injected intraperitoneally for the three groups of experimental animals under 2% isoflurane anesthesia. Experimental animals received the CYP injections every four days for 1, 2, or 3 months. The control animals were injected intraperitoneally with saline (0.1 ml/100 g). A total of 36 mice were sacrificed after CYP injection, including three mice from each of the three groups. The bladder tissues were then retrieved after sacrifice and fixed in 10% formalin for further histological analysis via H&E staining.

Proliferating Cell Nuclear Antigen (Pcna) Expression Detection in Chronic Cystitis Bladders

Detection of Pcna expression was performed on bladder tissue fixed in 10% formalin, then paraffin-embedded and sectioned to produce 5-μm-thick samples. Pcna expression was detected using a primary monoclonal antibody from Santa Cruz Biotechnologies (Santa Cruz, CA) at a dilution of 1:200 and a Cy-5-labelled anti-mouse secondary antibody from Jackson ImmunoResearch (West Grove, PA). Sections were incubated overnight with mouse monoclonal anti-Pcna (1:200, SantaCruz, CA) antibodies. An Alexa Fluor 647-labelled secondary antibody was used to detect the signal.

DNA Extraction

DNA from the urothelium in microdissected specimens using a laser capture microscope was isolated using a commercial kit (QIAamp® DNA Micro Kit, Qiagen Inc., Valencia, CA, USA) and stored at −80°. A 2-μg quantity of DNA from each sample was then treated with bisulfite using the Zymo EZ DNA Methylation kit (Zymo Research, Irvine, CA) according to manufacturer instructions. Bisulfite-treated DNA was stored at −30°.

Quantitative Methylation-Sensitive Real-Time Polymerase Chain Reaction (MethyLight Assay)

DNA methylation analysis on the bisulfite-treated DNA was performed by MethyLight assay[5] at the USC Epigenome Center using specifically designed MethyLight primer and probe sequences for 49 mouse gene regions (Supplemental Table 1). The MethyLight primer and probes sequences for the *Calca, Timp3, Mmp2, and Igf2r* genes are listed in Table 1. All MethyLight reactions used probes with a 5'-FAM fluorophore and either a 3'-BHQ-1 or a 3'-MGB (minor groove binder) quencher. We used the MGuca2a- C1B as a control reaction. The gene regions were selected for their known DNA methylation changes in human cancers. We *in vitro* methylated mouse tail DNA with the M.*Sss*I methylase (New England Biolabs (Ipswich, MA) according to the manufacturer's specifications. We tabulated the mean M.*Sss*I-DNA cycle threshold (C(t)) values for the 49 genes evaluated. The Percent of Methylated Reference (PMR) values were calculated as previously described.[6] The *Guca2a* control reaction primers are the same as previously reported,[7] except that, in our study, the probe contained a 5'-FAM fluorophore and a 3'-BHQ-1 quencher.

Statistical Analysis

Statistical analysis was performed using one-way ANOVA test with SPSS 19.0 statistical software (SPSS Inc., Chicago, USA). A level of $P<0.05$ was considered statistically significant. All data was expressed a mean ± standard deviation (S.D.), with percentages when appropriate.

Results

Histopathological Validation of the CYP-mediated Chronic Cystitis Model

Histological examination of the retrieved murine bladders revealed an intact urothelial layer with normal caliber blood vessels and a lack of edema or infiltrate in the control bladders at the 1-month, 2-month, and 3-month time periods (Figures 1A – C). However, extensive urothelial inflammation was noted in the bladders of the CYP-injected mice with extensive edema in the lamina propria, bladder mucosa and submucosal layers, inflammatory cell accumulation, and urothelial sloughing at the 1-month mark (Figure 1D). Similar histologic changes were seen at the 2-month and 3-month time periods in the bladders of the CYP mice, but of decreasing severity where the histologic characteristics of the control and

experimental murine bladders at the 3-month time period appeared to be similar (Figures 1E and 1F).

Increased urothelial cell proliferation and regeneration was seen by Pcna nuclei staining patterns in the bladder samples of the CYP-injected mice at the 1-month, 2-month, and 3-month time periods (control - Figure 2, panel A1 – A3; experimental - Figure 2, panel A4 – A6). Pcna labeling index analysis (the percentage of positivestained cells) in each group revealed a near-doubling of Pcna nuclei staining at the 1- month mark ($P<0.001$), with decreasing severity at the later time points (Figure 2B).

Epigenetic (DNA Methylation) Changes Occur during Chronic Bladder Inflammation

MethyLight assay (quantitative methylation-sensitive real-time PCR)[5] was used to obtain quantitative estimates for DNA methylation levels of a standard panel of 49 cancer-related genes and repetitive elements (Supplemental Table 1) in the murine bladder samples in order to assess for changes associated with the 1-month, 2-month, or 3-month time periods of CYP-induced chronic cystitis. Of these cancer-related targets, the *Calca, Timp3, Mmp2, and Igf2r* genes showed significant changes in DNA methylation levels compared to controls at the different time points (Figure 3).

For the *Calca* gene, lower DNA methylation levels by MethyLight assay associated with CYP-induced cystitis as compared to controls occurred at the 1-month time point (control: 82 ± 12, cystitis: 19.5 ± 11.5). A trend toward decreased DNA methylation levels was seen throughout the time course for the control samples. However, *Calca* exhibited increased DNA methylation levels at the 3-month time point in cystitis samples compared to controls.

The *Timp3* gene showed significant increases in DNA methylation associated with CYP-induced cystitis at the 1-month time point as compared to controls. In addition, the DNA methylation levels were significantly higher than at the 2-month ($P<0.05$) and 3-month time points ($P<0.05$). Interestingly, no differences in the DNA methylation levels were noted between the cystitis samples and the controls at the 2-month and 3- month time points.

For *Mmp2*, DNA methylation levels associated with cystitis exhibited a consistent increase at the three time points when compared to the controls. At the 1-month time point, no significant differences between the DNA methylation levels were seen between the control samples and the samples with induced cystitis. However, there were significant increases in DNA methylation levels at the 2-month and 3-month time points as well as a significant increase at the 3-month time point as compared to the controls at the 3-month time point ($P<0.05$). Moreover, the control samples did not show any appreciable changes in DNA methylation throughout the three-month time course.

For *Igf2r*, DNA methylation levels associated with cystitis were unchanged at the 1-month time point, but increased at the 2-month time point ($P<0.001$). However, the DNA methylation levels of *Igf2r* at the 3-month time point were lower when compared to controls.

Discussion

In this present study, we sought to use the CYP-induced chronic cystitis model in mice over a period of 1 month, 2 months, or 3 months to investigate, for the first time, possible alterations in the DNA methylation status of 49 cancer-related genes in urothelial cells as a result of benign chronic bladder disease. Using the MethyLight assay, we have identified four genes, *Calca, Timp3, Mmp2,* and *Igf2r*, where significant changes were identified in the DNA methylation status of their respective CpG islands in association with chronic cystitis.

Although the recent epigenetics literature has focused mainly on cellular development and differentiation, as well as cancer,[8] there is increasing recognition that epigenetics may also play an important role in the regulation of inflammatory genes in diseases such as asthma and COPD.[9] For the bladder, although DNA methylation has been shown to play an important role during bladder tumorigenesis and has been considered as tumor markers for diagnosis,[10] DNA methylation associated with bladder inflammation and its potential as a diagnostic / response marker has not been well-described as of yet.

Calca DNA Methylation Status in Relation to Chronic Inflammation

The results from the MethyLight assay performed in this study showed the CpG island of the *Calca* gene to be decreased in cystitis samples compared to control samples at the 1-month and 2-month time periods. However, *Calca* DNA methylation levels appear to steadily decline over the time course in the control samples, while substantial increases in *Calca* DNA methylation were evident in the 3-month time point in the cystitis samples. *Calca* is a gene that codes for three related, but different, polypeptides, calcitonin, katacalcin and α-calcitonin gene-related peptide (α-CGRP).[11,] One alternatively-spliced version of the *Calca* gene, α-CGRP, has been shown to have a potent vasodilatory effect on vascular tone, with increasing cell migration, adhesion, growth, and differentiation, which are important characteristics of inflammation.[12] In our experiments, the Pcna results at the 1-month and 2-month time periods revealed increased cell proliferation associated with the induced cystitis.

Moreover, α-CGRP has been shown to enhance angiogenesis through its interactions with Calca receptor components on vascular endothelial cells that stimulate endothelial cell growth and migration as well as promote capillary-like tube formation *in vitro*.[13] While the process of angiogenesis can be beneficial in wound healing, it can also occur pathologically as a result of chronic inflammation. Since α-CGRP plays a role in inflammation, hypomethylation of the CpG island in the promoter area of the *Calca* gene as a result of chronic inflammation may be a potential future biomarker for the diagnosis or prognosis of benign bladder diseases such as chronic cystitis.

Timp3 DNA Methylation in Relation to Chronic inflammation

MethyLight analyses revealed that *Timp3* underwent significant changes in DNA methylation status at the 1-month mark. *Timp3* was hypermethylated at one month compared to the controls and the 2- and 3-month time periods. *Timp3* is a member of a family of proteins called the tissue inhibitors of metalloproteinases, which are physiological inhibitors of matrix metalloproteinases (MMPs) and A disintegrin and metalloproteinases (ADAMs), and especially of a particular member of the ADAM family, ADAM17, which is also known as TNF-á converting enzyme (TACE).[14] TACE is a protease that generates soluble TNF-á from the cell surface form of the cytokine which in turn plays a central role in the regulation of inflammatory responses. Of note, *Timp3*$^{-/-}$ mice displayed a marked increase in the expression of TNF-á which led to inappropriate induction of inflammation and TNF-mediated cell death in the liver.

The DNA hypermethylation at the 1-month time period of CYP-induced cystitis may reflect a reduced expression of the *Timp3* gene, which may subsequently result in an increase in TNF-á production, and may help explain the severe inflammation seen on histopathological analysis while less severe inflammation was seen at the 2- and 3-month time points. This is the first time that the promoter DNA methylation status of *Timp3* has been shown to change as the result of CYP-induced chronic cystitis with severe urothelial inflammation. Further studies are necessary to determine the precise role that *Timp3* DNA hypermethylation plays in chronic cystitis and the possibility of whether the methylation status of *Timp3* can serve as a prognostic or diagnostic marker of benign bladder diseases.

Mmp2 DNA Methylation in Relation to Chronic Inflammation

MethyLight analysis also revealed significant changes in DNA methylation levels of *Mmp2*, where an increase in DNA methylation levels was noted from the 1-month time period to the 3-month time period. The DNA methylation levels of *Mmp2* in cystitis samples were higher than controls at the 2-month time point and were significantly higher at the 3-month time point, which may correlate with increased regulation of *Mmp2* expression at the transcriptional level at these time points.

Mmp2 is a member of a family of proteases, MMPs, which functions primarily in the remodeling of the extracellular matrix (ECM) through the degradation of various extracellular components.[15] In particular, the *Mmp2* gene codes the metalloprotease, Gelatinase-A, which degrades collagen IV, a critical remodeling factor in inflammatory responses and angiogenesis.[16] Previous studies have suggested that Gelatinase-A acts upon other substrates that are directly related to the inflammatory response.[1617] The relative amounts of intact and cleaved CCL7 present after pathophysiological cleavage regulate chemotaxis and the extent of inflammation.

Furthermore, *Mmp2* appears to have a protective effect that aids in wound healing in other disease profiles. *Mmp2* promotes eosinophil egression into the alveolar lumen during the inflammatory allergic responses of the lungs, and therefore prevents prolonged and heightened inflammatory responses that could lead to tissue damage.[18] In addition, *Mmp2* expression has also been shown to play a role in other inflammatory diseases such as emphysema,[9] cardiovascular inflammatory diseases,[19] and inflammatory bowel disease.[20]

Changes to Igf2r DNA Methylation with Chronic Inflammation

Although *Calca, Timp3*, and *Mmp2* DNA methylation changes have previously been described in various inflammatory responses of non-urologic organ systems, our study presents, for the first time, alterations in the DNA methylation status as a result of chronic bladder inflammation. In addition to these three genes, we also identified DNA methylation changes for the *Igf2r* gene, which has not been previously described in benign inflammatory diseases. Further studies are necessary to better delineate the significance on the changes in DNA methylation levels of this gene to ascertain the significance of the observed changes.

Chronic Inflammation As An Early Risk Factor for Bladder Cancer

Although this study is aimed at finding possible changes in DNA methylation in benign bladder diseases, accumulated epidemiologic studies are beginning to support that chronic inflammatory diseases are frequently associated with increased risk of cancers in other organ systems such as the liver and the intestines.[21–24] Chronic inflammation has also been linked to various steps that are involved in tumorigenesis, which include cellular transformation, promotion, survival, proliferation invasion, angiogenesis, and metastasis.[21]

MethyLight assay showed marked DNA hypermethylation of the CpG islands of the promoter region of the *Calca* gene in the cystitis sample at the 3-month time period compared to the other time periods and the control sample at 3 months. It has been shown previously that *CALCA* was hypermethylated in the promoter regions in a variety of human cancers that includes breast cancer, non-small cell lung cancer, leukemia, colon cancer, cervical cancer, and bladder cancer.[25–30] Also, angiogenesis is an important event that is integral to tumorigenesis and it has been shown previously that the *Calca* gene facilitates angiogenesis.[13] In the light of these previous findings, the DNA hypermethylation of the *Calca* gene found in the present study could provide a possible link between chronic bladder inflammation and the development of bladder cancer, but further studies are necessary to further investigate this.

Conclusions

Epigenetic (DNA methylation) changes were noted in four cancer-related genes, *Calca, Timp3, Mmp2, and Igf2r,* in this non-cancer chronic bladder inflammation animal model. Similar to other organ systems, epigenetics most likely plays a role in the regulation of inflammatory bladder genes. However, further pre-clinical and clinical studies are necessary to elucidate the pathways associated with these genes during chronic bladder inflammation, which may lead to future novel diagnostic and therapeutic advances.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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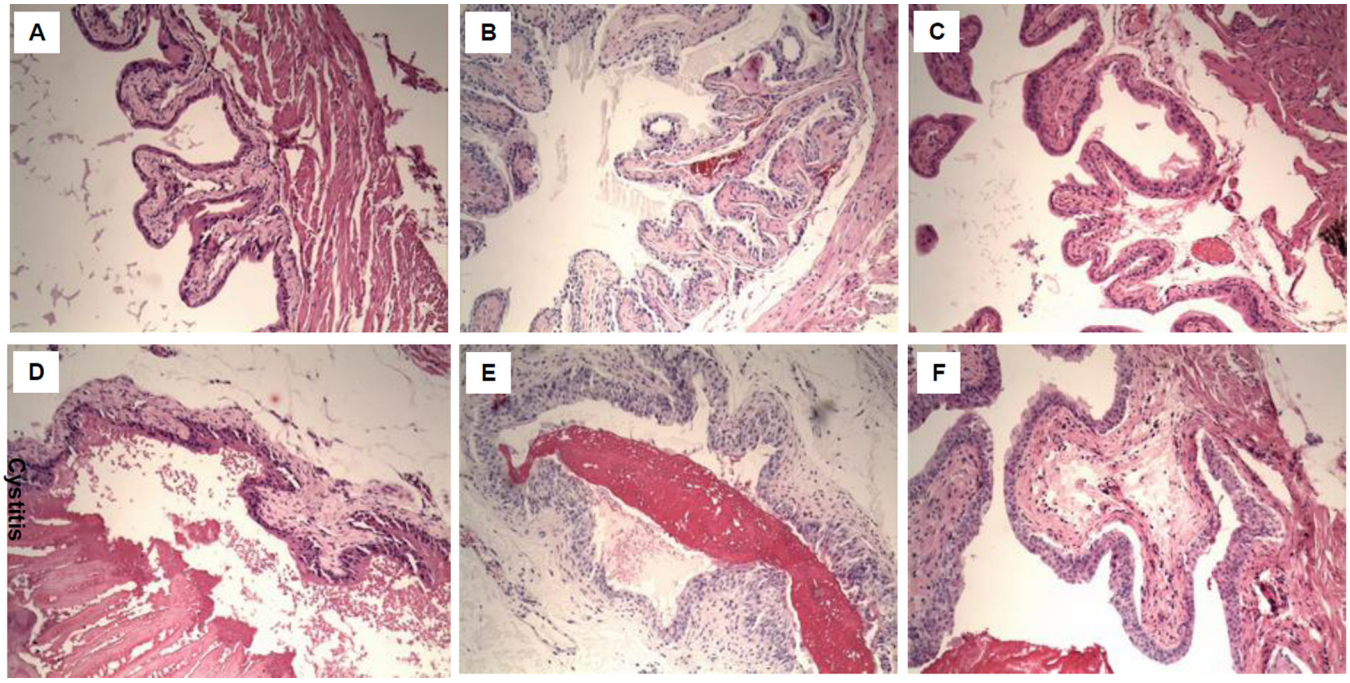

Figure 1. Histopathology of the bladder during chronic cystitis

Figures 1A – 1C: no inflammation is seen in the control bladder specimens at the 1-month, 2-month, and 3-month time points, respectively. Figures 1D – 1F: urothelial edema, necrosis, ulceration, hemorrhage, and leukocyte infiltration are seen in the chronic cystitis bladder specimens at the 1-month, 2-month, and 3-month time points, respectively. The maximum changes with edema, inflammation, and urothelial sloughing was seen at the 1-month time point. (Magnification: 10×)

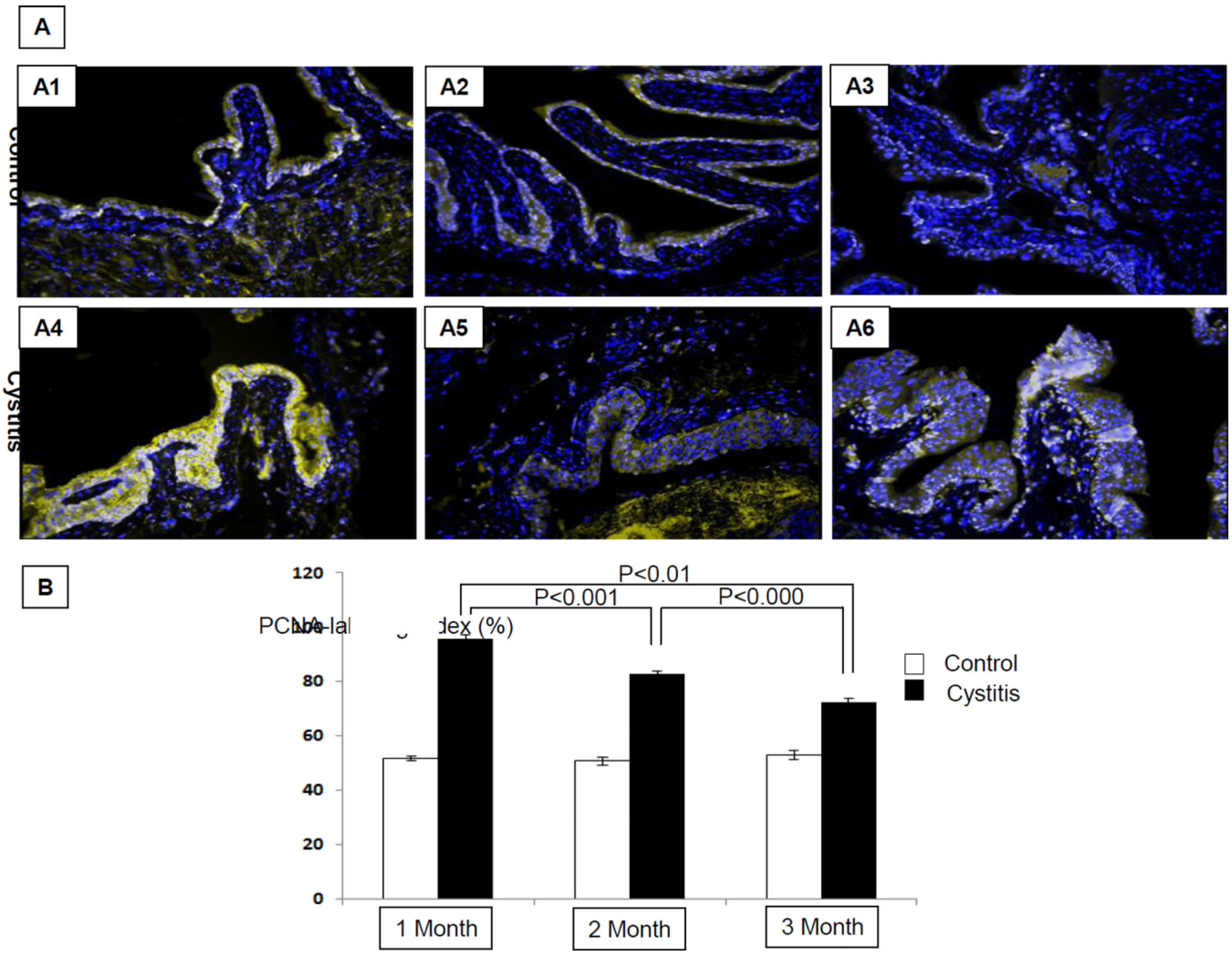

Figure 2. Pcna Immunohistochemical analysis during chronic cystitis

Figure 2A: Pcna staining in the control mice at the 1-month, 2-month, and 3-month time points, respectively (A1 – A3), and Pcna staining in the chronic cystitis mice at the 1-month, 2-month, and 3-month time points, respectively (A4 – A6). The most active cell proliferation was seen in the chronic cystitis urothelium at the 1-month time point. (Magnification: 20×) Figure 2B: The Pcna-labeling index, as the percentage number of positive cells stained for Pcna in each group, was significantly higher in the chronic cystitis mice as compared to the control mice.

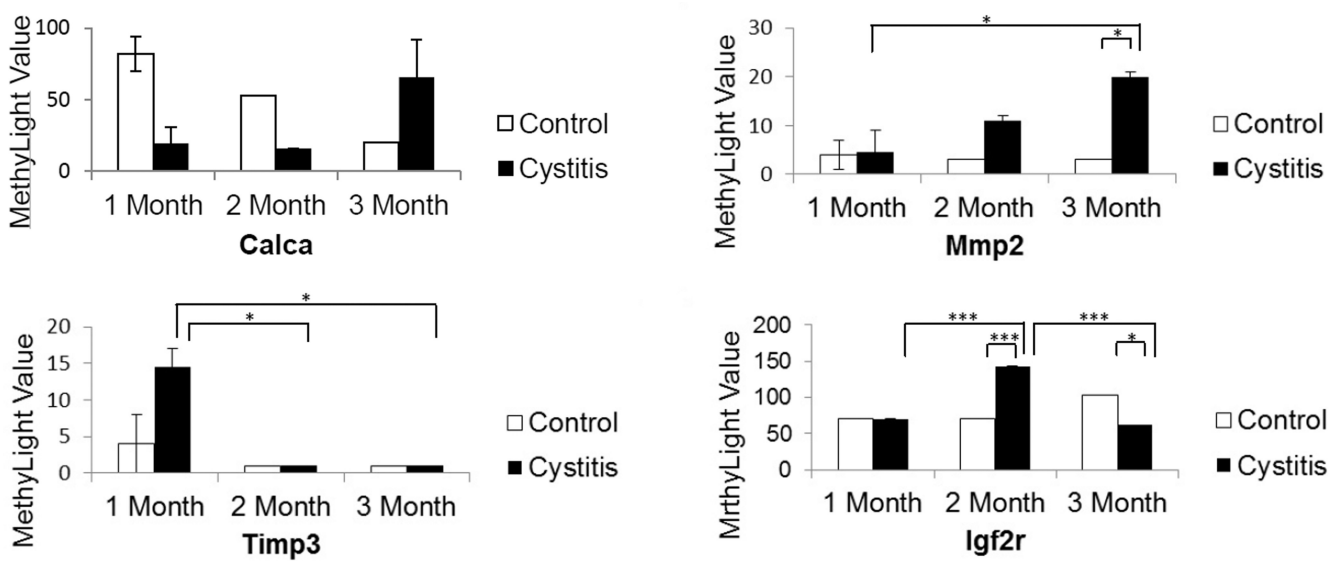

Figure 3.

MethyLight analysis for the *Calca, Timp3, Mmp2,* and *Igf2r* loci during chronic cystitis. Quantitative DNA methylation-sensitive real-time polymerase chain reaction (MethyLight) analyses were performed on bladder samples from control bladders and chronic cystitis bladders. Columns display average of two replicates; error bars display S.D. *p<0.05, **p<0.01, ***p<0.001.

Table 1

MethyLight primer and probe sequences

Genes	Reaction Number	Reaction ID	Forward primer sequence (5'→3')	Reverse primer sequence (5'→3')	Probe primer sequence (5'→3')
Calca	MB-051	M-Calca-M1B	TTTTCGTTGTTAGTTTTTGGTTTGTT	AAACTCTACGAACCAATTCTATAAAAACCT	6FAM-TCTAAATCGAAATCTAACACGAACCACGCTAAAACT–BHQ-1
Timp3	MB-025	M-Timp3-M1B	GAGAGGCGGTGGGCGTAG	CGAAAATATAAACTAAACGCGTCCT	6FAM-CGATATACGCTACAACGACGTCCCACGA–BHQ-1
Mmp2	MB-088	M-Mmp2-M1B	GTTTTAGGGGCGATATCGTCG	CGAATAACCTAAAAAACGCTAACCG	6FAM-AACTATTAAACCGCGCCATCGCTACACCAT–BHQ-1
Igf2r	MB-099	M-Igf2r-M1B	CGTGCGATGTTTATGTGATTCG	CCCGCCTCCTACTCACGTAA	6FAM-TCCTCGTTCAAATACTCTCCGCCCAA-BHQ-1