

Overexpression of HIF1α in Hunner Lesions of Interstitial Cystitis: Pathophysiological Implications

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Study Need and Importance: Hunner-type interstitial cystitis is an intractable, debilitating chronic inflammatory disease of the urinary bladder extremely deteriorating patients' quality of life by persistent pelvic pain in conjunction with lower urinary tract symptoms such as urinary frequency and urgency. The etiology of Hunner-type interstitial cystitis remains elusive, and that hinders the development of curable treatments or diagnostic markers. There is an urgent need to clarify its pathophysiology to find therapeutic targets and disease biomarkers.

What We Found: We performed the genomic and immunopathological characterization of the Hunner lesion, a characteristic cystoscopic marker of Hunner-type interstitial cystitis, by exploring the whole-transcriptomic landscape and quantitative immunohistochemical and polymerase chain reaction analyses of key inflammatory cells and cytokines in both Hunner lesions and background bladder mucosa (nonlesion areas) of 25 patients with Hunner-type interstitial cystitis (see figure). RNA sequencing identified 109 differentially expressed genes and 30 significantly enriched biological pathways in Hunner lesions, including hypoxiainducible factor (HIF)1a signaling pathway, PI3K-Akt signaling pathway, RAS signaling pathway and MAPK signaling pathway. Quantitative polymerase chain reaction and immunohistochemical analyses confirmed the significant up-regulation of both mRNA and protein levels of HIF1a in Hunner lesions. Otherwise, there were no significant differences between Hunner lesions and nonlesion areas in terms of mRNA levels of inflammatory cytokines or histological features such as lymphoplasmacytic and mast cell infiltration, epithelial denudation and CD4/CD8 T-lymphocyte ratio.



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Figure. Immunohistochemical analysis of HIF1 α . Top: representative images of immunohistochemical analyses of Hunner lesion using anti-HIF1 α antibody. Top left: HIF1 α is highly expressed in mononuclear cells in subepithelial layer (original magnification ×200). Top right: enlarged image of rectangular box in left image (original magnification ×400). Bottom: nonlesion area. Lower left: expression of HIF1 α is rarely observed in infiltrating mononuclear cells (original magnification ×200). Lower right: enlarged image of rectangular box in left image (original magnification ×200).

Limitations: The modest sample size (25) and the lack of validation using independent resources limit interpretation of the results.

Interpretation for Patient Care: Our findings demonstrate significant overexpression of HIF1 α and up-regulation of its related biological pathways in Hunner lesions. The results indicate that ischemia, in conjunction with inflammation, plays a pathophysiological role in Hunner-type interstitial cystitis and HIF1 α could be a potential disease biomarker.

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Overexpression of HIF1α in Hunner Lesions of Interstitial Cystitis: Pathophysiological Implications

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Abbreviations and Acronyms

BPS = bladder pain syndromeDEG = differentially expressedgene EBV = Epstein-Barr virus ESSIC = International Society for the Study of IC/BPS FDR = false discovery rateGO = gene ontologyHIC = Hunner-type interstitial cystitis HIF = hypoxia-inducible factorIC = interstitial cystitis IFN = interferonKEGG = Kyoto Encyclopedia of Genes and Genomes QOL = quality of lifeqPCR = quantitative polymerase chain reaction TGF = transforming growthfactor TNF = tumor necrosis factor

Purpose: The aim of our study was to elucidate biological changes in Hunner lesions, which underlie the pathophysiology of Hunner-type interstitial cystitis, by characterizing their whole transcriptome and immunopathological profiles.

Materials and Methods: Paired bladder mucosal biopsies, 1 sample each from the Hunner lesion and nonlesion area, were obtained from 25 patients with Hunner-type interstitial cystitis. The samples were subjected to whole-transcriptome profiling; immunohistochemical quantification of CD3, CD4, CD8, CD20, CD138, mast cell tryptase, cytokeratin and HIF1 α ; and quantitative polymerase chain reaction for *IFN*- α , *IFN*- β , *IFN*- γ , *TNF*, *TGF*- β 1, *HIF1\alpha*, *IL*-2, *IL*-4, *IL*-6, *IL*-10 and *IL*-12A. The results were compared between the lesion and nonlesion areas.

Results: RNA sequencing identified 109 differentially expressed genes and 30 significantly enriched biological pathways in Hunner lesions. Up-regulated pathways (24) included HIF1 α signaling pathway, PI3K-Akt signaling pathway, RAS signaling pathway and MAPK signaling pathway. By contrast, down-regulated pathways (6) included basal cell carcinoma and protein digestion and absorption. The mRNA levels of *HIF1\alpha*, *IFN-\gamma* and *IL-2*, and the HIF1 α protein level were significantly higher in lesion areas. Otherwise, there were no significant differences between the lesion and nonlesion samples in terms of mRNA levels of inflammatory cytokines or histological features such as lymphoplasmacytic and mast cell infiltration, epithelial denudation and CD4/CD8 T-lymphocyte ratio.

Conclusions: Our findings demonstrate significant overexpression of HIF1 α and up-regulation of its related biological pathways in Hunner lesions. The results indicate that ischemia, in conjunction with inflammation, plays a pathophysiological role in this subtype of interstitial cystitis/bladder pain syndrome, particularly in Hunner lesions.

Key Words: cystitis, interstitial; RNA-seq; hypoxia-inducible factor 1, alpha subunit

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Competing interests: The authors have no relevant financial interests to disclose.

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INTERSTITIAL cystitis (IC)/bladder pain syndrome (BPS) is a chronic debilitating disorder characterized by persistent bladder/urethral pain and lower urinary tract symptoms such as urinary frequency and urgency.¹ IC/BPS represents a symptomatic syndrome complex that can be classified into 2 distinct entities, depending on the presence or absence of Hunner lesions.² IC/BPS accompanied by Hunner lesions, hereafter referred to as Hunnertype IC (HIC) according to the East Asian guidelines for IC/BPS,¹ corresponds to BPS type 3 according to the International Society for the Study of IC/BPS (ESSIC) criteria.³ IC/BPS lacking Hunner lesions, hereafter referred to as BPS, corresponds to BPS types 1 and 2 in the ESSIC criteria. A Hunner lesion is a reddish mucosal erosive change usually found at the dome, posterior or posterolateral wall of the urinary bladder.^{4,5} Evidence indicates that HIC is a distinct inflammatory disease, whereas BPS is thought to be a noninflammatory condition.⁶⁻⁸ Histologically, HIC is characterized by epithelial denudation and chronic inflammatory changes including stromal edema, neovascularization and dense inflammatory infiltrates composed predominantly of lymphoplasmacytic and mast cells.⁹⁻¹² Of note, these inflammatory changes and epithelial denudation can be observed not only in Hunner lesions but also in the whole bladder wall including nonlesion bladder mucosa, indicating that HIC is a pancystitic disease.^{10,12} In a previous RNA-seq analysis, we identified a set of biological pathways that are significantly enriched in HIC relative to BPS and normal bladders. However, in that study we detected no significantly altered biological pathways between the Hunner lesion and nonlesion area of HIC.¹³ The similar genomic and histological characteristics of Hunner lesion and nonlesion areas are consistent with the pancystitic nature of HIC. However, the distinctive reddish appearance of Hunner lesions and their predilection for particular bladder sites prompted us to perform a more indepth analysis. The aim of our current study was to identify specific biological changes that may underlie the pathophysiology of Hunner lesions by exploring the whole-transcriptomic landscape using a larger cohort and an updated next-generation sequencing system. In addition, we used quantitative immunohistochemical and polymerase chain reaction (qPCR) analyses to identify key inflammatory cells and cytokines indicative of a paradigm of immune responses in Hunner lesions.

MATERIALS AND METHODS

Ethics Statement

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This study was approved by the Institutional Review Board of The University of Tokyo (approval No. G10046). All participants gave written informed consent before study enrollment.

Participants and Sample Preparation

The participants in the study were 25 consecutive patients (including 23 women) diagnosed with HIC who underwent electrocautery of Hunner lesions with hydrodistension at The University of Tokyo Hospital from 2016 to 2018. Diagnosis of HIC was based on the East Asian clinical guidelines and the ESSIC criteria.13 Patients' demographics were retrieved from medical records, included OSSI (O'Leary and Sant's symptom index) and OSPI (O'Leary and Sant's problem index; OSSI/OSPI), an 11-point numerical rating of pain intensity, with 0 indicating "no pain" and 10 indicating "the worst pain ever;" a 7-grade quality of life (QOL) scale derived from the International Prostate Symptom Score, with 0 indicating "excellent" and 6 indicating "terrible;" urinary frequency; maximum and average voided volume; and bladder capacity measured at hydrodistension under a pressure of 80 cmH₂O. Paired cold-cup biopsy samples, one from Hunner lesion and one from a nonlesion area, were obtained from each patient before hydrodistension (supplementary fig. 1, https://www.jurology.com) and were stored at -80C until analysis. The supplementary material (https://www.jurology.com) describes further details.

RNA Library Preparation and NovaSeg Sequencing

Purified total RNA (10 ng) was prepared for mRNA amplification by 5' template-switching PCR using the Clontech SMART-Seq® v4 Ultra® Low Input RNA kit (Clontech, Palo Alto, California). Amplified cDNA was fragmented and appended with dual-indexed barcodes using Illumina® Nextera® XT DNA Library Prep kits (Illumina, San Diego, California). Libraries were validated on an Agilent 4200 Tape Station system (Agilent, Santa Clara, California), pooled and sequenced on an Illumina NovaSeq 6000 using a 2×150 paired-end configuration. Raw sequence data (.BCL files) were converted into FASTQ format and de-multiplexed using the Illumina bcl2fastq 2.20 software. The supplementary material (<u>https://www.</u> jurology.com) provides further details.

RNA-Seq Data Processing

Clean paired-end reads were aligned to the UC Santa Cruz human reference genome (hg19) using GeneData Profiler Genome version 11.0.8 (GeneData, Basel, Switzerland). Mapped reads were assembled using STAR version 2.5.3a by referring to gene structures described in National Center for Biotechnology Information RefSeq. To quantify gene expression, FPKM (fragments per kilobase of exon per million mapped fragments) values for each gene model were calculated using GeneData Profiler Genome. The supplementary material (<u>https://www.</u> jurology.com) provides further details.

Differentially Expressed Gene (DEG) Analysis

RNA-seq data were analyzed using GeneData Analyst version 9.1.13 and Agilent Gene Spring software packages version 14.8 (Agilent Technologies). DEGs between lesion and nonlesion were identified based on the log₂-normalized FPKM scores by a paired t-test followed by the Benjamini–Hochberg correction. The statistical significance of differences in gene

| Table 1 | . Demographics | of patients | with H | IC |
|---------|----------------|-------------|--------|----|
|---------|----------------|-------------|--------|----|

| No. pts (No. male/No. female) | 25 | (2/23) | | | | |
|--|------------------|-----------|--|--|--|--|
| Mean \pm SD yrs age at surgery (range) | 66.0±11.8 | (37-80) | | | | |
| Mean±SD yrs from symptom onset to biopsy (range) | 5.2±4.6 | (0-18) | | | | |
| Mean±SD OSSI (range) | 15.4±3.0 | (11-20) | | | | |
| Mean±SD OSPI (range) | 13.3±2.6 | (8—16) | | | | |
| Mean±SD pain intensity (range)* | 8.0±1.7 | (4-10) | | | | |
| Mean±SD QOL score (range)† | 5.7±0.9 | (2—6) | | | | |
| Mean±SD daytime frequency (range) | 14.7±5.2 | (4-27) | | | | |
| Mean±SD nocturia frequency (range) | 3.8±1.8 | (0-7) | | | | |
| Mean±SD mL avg voided volume (range) | 102.9±51.7 | (31-221) | | | | |
| Mean±SD mL max voided vol (range) | 168.0 ± 90.5 | (50-420) | | | | |
| Mean±SD mL max bladder capacity at | 394.0±155.0 | (200-700) | | | | |
| hydrodistension (range) | | | | | | |

* Assessed using an 11-point pain intensity numerical rating scale from 0 ("no pain") to 10 ("the worst pain ever").

t Assessed on a 7-grade QOL scale derived from the International Prostate Symptom Score, with 0 indicating "excellent" and 6 indicating "terrible."

expression were evaluated at a false discovery rate (FDR) ≤ 0.05 and $|log_2[Ratio]| \geq 1$.

Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway Enrichment Analyses

Identified DEGs were subjected to GO/GO slim analyses and KEGG pathway enrichment analysis using DAVID Bioinformatics Resources. The supplementary material (<u>https://www.jurology.com</u>) provides further details.

Histopathology and Immunohistochemistry

Immunohistochemistry was performed using a Ventana BenchMark XT autostainer (Ventana Medical Systems, Tucson, Arizona) and antibodies against CD3, CD4, CD8, CD20, CD138, mast cell tryptase, cytokeratin, and hypoxia-inducible factor- 1α (HIF1 α). Appropriate controls for each antibody were run in parallel (supplementary fig. 2, <u>https://www.jurology.com</u>). The supplemental material (https://www.jurology.com) provides further details.

Immunohistochemical Quantification

Digital immunohistochemical quantification was performed using the Tissue Studio software, version 3.5 (Definiens AG, Munich, Germany), as described previously.¹² Analyzed data were expressed as cell density (cells/mm²) or the proportion of the whole-tissue sample area that was cytokeratin-positive, ie the "epithelium/ specimen ratio" (%). Due to slight background staining of the stroma, digital image analysis could not be used to quantify HIF1 α in the immunohistochemistry analyses; therefore, HIF1 α expression was evaluated on the basis of the intensity of nuclear immunoreactivity and was scored as negative, mild, moderate or strong, as per our previous study.¹⁴ The supplementary material (<u>https://www.</u> jurology.com) provides further details.

qPCR

Levels of mRNA encoding $HIF1\alpha$, interferon $(IFN)-\alpha$, $IFN-\beta$, $IFN-\gamma$, interleukin (IL)-2, IL-4, IL-6, IL-10 and IL-12A, transforming growth factor $(TGF)-\beta 1$ and tumor necrois factor (TNF) were analyzed by qPCR using TaqManTM Gene Expression Assays (Thermo Fisher Scientific, San Jose, California; supplementary table 1, https://www.jurology.com). The

 $supplementary\ material\ (\underline{https://www.jurology.com})\ provides\ further\ details.$

Statistical Analysis

Statistical analyses (except for RNA-seq data processing) were performed using JMP® Pro version 13 (SAS Institute, Cary, North Carolina) and GraphPad Prism version 8 (GraphPad Software, San Diego, California). Comparisons of continuous variables between lesion and nonlesion samples were conducted in a pairwise manner. The supplementary material (https://www.jurology.com) provides further details.

RESULTS

Characteristics of the HIC Patients

Patient demographics are shown in table 1. The study cohort comprised 23 women and 2 men, all of whom were diagnosed with HIC. Mean age at surgery and duration of illness were 66.0 ± 11.8 and 5.2 ± 4.6 years, respectively. Average voided volume, maximum voided volume and maximum bladder capacity at hydrodistension were 102.9 ± 51.7 , 168.0 ± 90.5 and 394.0 ± 155.0 mL, respectively. OSSI/OSPI, pain intensity and QOL scores were $15.4\pm3.0/13.3\pm2.6$, 8.0 ± 1.7 and 5.7 ± 0.9 , respectively. Hunner lesions were mostly located at the dome, posterior and posterolateral walls. Accordingly, biopsies of nonlesion area were taken from anterolateral walls (table 2).

RNA-Seq

Total and sample-by-sample read counts are shown in supplementary table 2 (<u>https://www.jurology.com</u>).

Analysis of DEGs

Of 13,951 genes detected in all samples, 109 were differentially expressed between Hunner lesion and nonlesion areas. Of the 109 DEGs, 74 were upregulated and 35 were down-regulated in Hunner lesions (fig. 1 and supplementary table 3, <u>https://</u>www.jurology.com).

GO and KEGG Enrichment Analysis

The results of GO and GO slim analyses for Hunner lesion-specific 109 DEGs are shown in supplementary tables 4-6 (<u>https://www.jurology.com</u>) and figure 2. GO slim analysis revealed that Hunner

| | No. Location of | No. Biopsied Area | | |
|----------------|-----------------|-------------------|-----------|--|
| | Hunner Lesions* | Hunner Lesion | Nonlesion | |
| Neck | 0 | 0 | 0 | |
| Trigone | 0 | 0 | 0 | |
| Posterior | 22 | 15 | 0 | |
| Dome | 12 | 3 | 0 | |
| Lateral: | | | | |
| Posterolateral | 17 | 7 | 0 | |
| Anterolateral | 1 | 0 | 25 | |
| Anterior | 3 | 0 | 0 | |

* Counted twice when multiple lesions were present.

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Figure 1. Volcano plot of DEGs. Volcano plot of DEGs between Hunner lesions and nonlesion areas. Red dots indicate FDR \leq 0.05 and $|log_2Ratio| \geq$ 1. Blue dots indicate FDR \leq 0.05 but $|log_2Ratio| <$ 1. Green dots indicate $|log_2Ratio| \geq$ 1 but FDR >0.05.

lesion-specific up-regulated DEGs were associated with the biological process terms "anatomical structure development" and "signal transduction," the cellular component terms "plasma membrane" and "extracellular region," and the molecular function terms "ion binding" and "enzyme binding" (fig. 2, *A*). The down-regulated DEGs were associated with the biological process terms "anatomical structure development" and "signal transduction," the cellular "plasma component terms membrane" and "extracellular region," and the molecular function terms "ion binding" and "structural molecular activity" (fig. 2, B). KEGG pathway analysis identified 24 pathways significantly up-regulated in Hunner lesions, including PI3K-Akt signaling, HIF1a signaling, EGFR tyrosine kinase inhibitor resistance, RAS signaling, PPAR signaling, calcium signaling, focal adhesion, neuroactive ligand-receptor interaction, ECM-receptor interaction and MAPK signaling. The KEGG analysis also identified 6 pathways downregulated in Hunner lesions including basal cell carcinoma, protein digestion and absorption, and TNF signaling (table 3).

qPCR

A qPCR analysis revealed that the relative expressions of $HIF1\alpha$, $IFN-\gamma$ and IL-2 were significantly higher in Hunner lesions than in nonlesion areas (fig. 3).

Immunohistochemical Quantification

Digital immunohistochemical quantification revealed that lymphoplasmacytic (sum of CD3, CD20 and CD138-postive cells) and mast cell densities, CD4 and CD8-positive T-cell densities, the CD4/CD8 ratio and the epithelial/specimen ratio were comparable between the Hunner lesions and nonlesion areas (fig. 4). Extensive HIF1 α immunoreactivity



Figure 2. GO slim analysis for DEGs. A, GO slim analyses of up-regulated DEGs in Hunner lesions. B, GO slim analyses of down-regulated DEGs in Hunner lesions.

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| Pathway ID | KEGG Pathway Name (No. of genes) | No. of Enriched Genes | p Value | |
|------------|--|-----------------------|---------|--|
| | Un-regulated pathways at Hunner lesion | | | |
| hsa04151 | PI3K—Akt signaling pathway (354) | 9 | < 0.01 | |
| hsa04510 | Focal adhesion (199) | 6 | | |
| hsa04080 | Neuroactive ligand-receptor interaction (340) | 7 | | |
| hsa05165 | ECM-receptor interaction (88) | 4 | | |
| map09020 | Pathways in cancer (526) | 8 | | |
| hsa04014 | Ras signaling pathway (232) | 5 | | |
| hsa04610 | Complement $+$ coagulation cascades (79) | 3 | | |
| hsa01521 | EGFR tyrosine kinase inhibitor resistance (79) | 3 | | |
| hsa04012 | ErbB signaling pathway (85) | 3 | | |
| hsa04010 | MAPK signaling pathway (295) | 5 | | |
| hsa04722 | Neurotrophin signaling pathway (119) | 3 | < 0.05 | |
| hsa04926 | Relaxin signaling pathway (129) | 3 | | |
| hsa04270 | Vascular smooth muscle contraction (132) | 3 | | |
| hsa05144 | Malaria (49) | 2 | | |
| hsa04514 | Cell adhesion molecules (CAMs) (146) | 3 | | |
| hsa04072 | Phospholipase D signaling pathway (148) | 3 | | |
| hsa05226 | Gastric cancer (149) | 3 | | |
| hsa04066 | HIF-1 signaling pathway (100) | 4 | | |
| hsa04060 | Cytokine-cytokine receptor interaction (294) | 4 | | |
| hsa05218 | Melanoma (72) | 2 | | |
| hsa03320 | PPAR signaling pathway (76) | 2 | | |
| hsa05412 | Arrhythmogenic rt ventricular cardiomyopathy (ARVC) (77) | 2 | | |
| hsa04020 | Calcium signaling pathway (193) | 3 | | |
| hsa05165 | Human papillomavirus infection (330) | 4 | | |
| | Down-regulated pathways at Hunner lesion | | | |
| hsa05217 | Basal cell carcinoma (63) | 2 | < 0.01 | |
| hsa04974 | Protein digestion $+$ absorption (95) | 2 | < 0.05 | |
| hsa04668 | TNF signaling pathway (112) | 2 | | |
| hsa04550 | Signaling pathways regulating pluripotency of stem cells (140) | 2 | | |
| hsa04310 | Wnt signaling pathway (160) | 2 | | |
| hsa05202 | Transcriptional misregulation in cancer (186) | 2 | | |

| Tahla 3 | Significantly | anrichad | hiological | nathwave i | n Hunner | lecione | rolativo t | n nonlesion | aroad |
|-----------|---------------|----------|------------|-------------|----------|---------|-------------|-----------------|-------|
| i able 5. | Significantiy | enncheu | Dibiogical | patrivays i | n numer | resions | relative to | <i>nomesion</i> | areas |

was detected in subepithelial mononuclear cells. Nuclear immunoreactivity of HIF1 α was significantly higher in Hunner lesions than in nonlesion areas (fig. 5 and table 4).

DISCUSSION

In this study, using the updated NovaSeq sequencing system we found that HIF1 α regulatory biological pathways, including PI3K—Akt signaling, Ras signaling, MAPK signaling and EGFR tyrosine kinase inhibitor resistance were significantly enriched in Hunner lesions. In addition, immunohistochemistry revealed that HIF1 α protein was up-regulated in Hunner lesions relative to nonlesion areas. By contrast, the degree of epithelial denudation and chronic inflammation were almost equivalent between Hunner lesion and nonlesion areas.

In a previous study, we performed RNA-seq analysis showing that the HIF1 α signaling pathway was significantly more active in HIC than in BPS and normal controls.¹³ The mean FPKM scores of HIF1 α mRNA in that study were 35.6 ± 13.8 , 23.5 ± 7.3 , 9.5 ± 6.0 and 10.4 ± 2.7 in Hunner lesions, nonlesion areas of HIC, BPS and normal controls, respectively. However, because we did not identify a sufficient number of DEGs, we were unable to detect biological pathways that were significantly altered between Hunner lesion and nonlesion areas. In this present study, we identified a modest number of DEGs and discovered the significant up-regulation of HIF1 α -related biological pathways in Hunner lesions. The overexpression of HIF1 α protein in Hunner lesions, as demonstrated by immunohistochemistry, emphasizes the importance of HIF1 α in the pathophysiology of HIC.

HIF1 α is the oxygen-sensitive subunit of HIF1, a heterodimeric transcription factor that plays a pivotal role in the response to hypoxia/ischemia and inflammation.¹⁵ HIF1 α is up-regulated in response to tissue hypoxia/ischemia or inflammatory cytokines such as IL-1 β , TNF α or TGF β via intracellular signaling pathways like EGFR/PI3K/Akt/mTOR signaling pathways and the NF- κ B signaling pathway.¹⁶

HIF1 α and ischemia have been implicated as potential etiologies of IC/BPS.^{17–19} HIF1 α is expressed at higher levels in IC/BPS bladders than in normal control bladders.²⁰ In addition, internal iliac arterial flow and resistive indices are significantly lower in IC/BPS patients than in normal healthy controls.¹⁷ Irwin et al used laser Doppler flowmetry to analyze bladder blood flow at the trigone and posterolateral walls during hydrodistension under a pressure of 80 cmH₂O; they observed significantly impaired bladder blood flow amplification in IC bladders relative to control bladders.¹⁸ Another study using endoscopic laser Doppler flowmetry confirmed significantly impaired blood



Figure 3. Quantitative real-time polymerase chain reaction of $HIF1\alpha$, $IFN-\alpha$, $IFN-\beta$, $IFN-\gamma$, IL-2, IL-4, IL-6, IL-10, IL-12A, $TGF-\beta 1$ and TNF mRNAs. Expression levels of each gene relative to *IPO8*. Asterisk indicates p <0.05, statistically significant difference by Wilcoxon signed –rank test.

perfusion at the trigone and posterior walls of IC bladders relative to control bladders.¹⁹ Notably, in both studies bladder perfusion was impaired more severely at the posterolateral or posterior walls than

at the trigone wall. This observation coincides with the finding that Hunner lesions are observed most often at the posterolateral and posterior walls, and are rarely seen at the trigone or bladder neck.^{5,18}



Figure 4. Immunohistochemical quantification. Densities of lymphoplasmacytic cells (the sum of CD3, CD20 and CD138-positive cells), mast cells, and CD4 and CD8-positive cells, as well as ratios of epithelium to specimen and CD4-positivity to CD8-positivity. Values are expressed as median (interquartile range). p Values were determined using Wilcoxon signed—rank test.

HIF1 α can also be up-regulated via infectioninduced inflammation. Lipopolysaccharides, compounds derived from pathogenic microorganisms that are potentially related to IC/BPS etiologies,²¹ increase the levels of mRNA expression of HIF1a by activating NF-KB signaling pathway through Tolllike receptor 4.¹⁵ Epstein-Barr virus (EBV) infection, which is also implicated in IC/BPS pathophysiology,²² induces the up-regulation of HIF1 α protein synthesis by activating the MAPK signaling pathway in lymphoid cells.¹⁵ Activation of HIF1a cascades leads to the up-regulation of VEGF (vascular endothelial growth factor), erythropoietin, iNOS (inducible nitric oxide synthase) and/or glucose transporters, which protect cells from lethal damage or apoptosis induced by ischemia or inflammation.¹⁶ Previous studies have reported overexpression of VEGF and iNOS in HIC bladders.^{13,23} Although we did not observe up-regulation of these genes in Hunner lesions in our current study, it is possible that they are also regulated by other biological events that occur downstream of HIF1a cascades. $^{\rm 24,25}$ Overall, our present findings suggest that HIF1*a*-related biological pathways are involved in the pathophysiology of HIC, and potentially that of Hunner lesions.

Presumably, the initial events in developing HIC involve inflammation of unknown origins; this inflammation may be pronounced at anatomically ischemic areas, resulting in formation of Hunner lesions at the posterior and posterolateral walls. We found that the degree of epithelial denudation was comparable between Hunner lesions and nonlesion areas; however, there is a slight difference between the denudation manner of the two areas, ie not represented by the epithelium/specimen ratio. Specifically, full layers of epithelium are frequently denuded at Hunner lesions where the subepithelial layer is exposed to the bladder lumen, while a couple of basal layers remain attached at nonlesion areas, protecting the subepithelial layer against exposure to urine.¹² The completely denudated epithelium at Hunner lesions is likely to provoke permeation of urinary contents into the subepithelial layer of the bladder, leading to a vicious inflammatory cycle and intractable symptoms. The elevated mRNA levels of *IFN-\gamma* and *IL-2* at Hunner lesions may reflect the relative intensification of inflammatory reactions at these sites.

This study had some limitations. First, the number of analyzed samples was modest (25). Second, it is not clear whether up-regulation of HIF1 α



С



Figure 5. Immunohistochemical analysis of HIF1 α . *A*, representative images of immunohistochemical analyses of Hunner lesion using anti-HIF1 α antibody. Left: HIF1 α is highly expressed in mononuclear cells in subepithelial layer (original magnification ×200). Right: enlarged image of rectangular box in left image (original magnification ×400). *B*, nonlesion area. Left: expression of HIF1 α is rarely observed in infiltrating mononuclear cells (original magnification ×200). Right: enlarged image of rectangular box in left image (original magnification ×200). Right: enlarged image of rectangular box in left image (original magnification ×200). Right: enlarged image of rectangular box in left image (original magnification ×200). Right: enlarged image of rectangular box in left image (original magnification ×200). Right: enlarged image of rectangular box in left image (original magnification ×200). Right: enlarged image of rectangular box in left image (original magnification ×200). Right: enlarged image of rectangular box in left image (original magnification ×200). Right: enlarged image of rectangular box in left image (original magnification ×400). *C*, HIF1 α intensity in HIC cases. HIF1 α intensity in Hunner lesion and nonlesion areas was determined for each case.

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Table 4. *HIF1* α *intensity*

| | No. Neg | No. Mild | No. Moderate | No. Strong | p Value |
|---------------|---------|----------|--------------|------------|---------|
| Hunner lesion | 2 | 4 | 5 | 14 | <0.05* |
| Nonlesion | 4 | 9 | 8 | 4 | |

Assessed based on the intensity of nuclear immunoreactivity: negative, mild, moderate, strong.

* p <0.05, statistically significant (Fisher's exact test)

reflects an etiological cause of Hunner lesion or a consequence of inflammatory or ischemic processes. Third, we did not validate the results of this study using independent resources. Further experiments are required to confirm the overexpression of HIF1 α in Hunner lesions.

CONCLUSIONS

Our findings reveal significant overexpression of HIF1 α and up-regulation of related biological pathways in Hunner lesions. These observations are consistent with a pathophysiological role of ischemia, in conjunction with inflammation, in the formation of Hunner lesions and the development of HIC. Further studies of HIF1 α overexpression should elucidate the underlying pathophysiology of HIC and improve management of IC/BPS.

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EDITORIAL COMMENTS

In this study, Akiyama et al have demonstrated significant overexpression of hypoxia-induced factor 1- α (HIF-1 α) and up-regulation of related biological pathways in interstitial cystitis/bladder pain syndrome (IC/BPS) with Hunner's lesion (HIC), indicating that ischemia in conjunction with inflammation plays an important role of pathophysiology of HIC. Increased expression of HIF-1a in the IC/BPS bladders have been reported and postulated to associate with glomerulations during hydrodistention in IC/BPS (reference 20 in article). The authors found that bladder tissues from HIC areas had several more up-regulated biological pathways than nonHIC areas, but similar expressions of inflammatory cytokines and histology. In an immunohistochemistry study we found diminished expression of proliferation proteins tumor protein 63 and mature urothelium marker CK20 in both HIC and grade 3 glomerulation nonHIC bladders, suggesting the urothelial dysfunction in IC/BPS might be in a state of persistent or chronic injury that related to the limited expression of cell proliferation proteins.¹ Notably, we also found HIC patients had a higher proportion of diffused (86.7%) and focal (30.6%) bladder wall thickening compared to those with

nonHIC in computerized tomography. Interestingly, some patients with nonHIC also exhibited focal (32.9%) and even diffused (2.6%) bladder wall thickening.² Bladder Epstein-Barr virus (EBV) infection in T cells had been found to link to the pathogenesis of IC/BPS. EBV infection was present in 87.5% of HIC and 17.4% of nonHIC bladder specimens, and a total of 46.2% of bladders with IC/BPS (reference 22 in article). In chronic inflammation, poor oxygenation could activate survival signaling pathways, including HIF-a. Expression of HIF-1a induced EBV lyticgene expression in cells harboring wild-type EBV have been reported.³ Put together, these findings point out that HIC could be an eruption of the bladder urothelium from the underlying diffused inflammation, and infection in the inflammatory environment might be the fundamental cause of IC/BPS.

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In this issue of *The Journal of Urology*®, Akiyama and coworkers demonstrate overexpression of hypoxia induced factor 1 alpha (HIF1 α) as well as an up-regulation of its related biological pathways in Hunner lesions. Their own interpretation of their results was that ischemia and inflammation play pathophysiological roles in just this subtype of interstitial cystitis. The very same research team reported a couple of years ago that lymphoplasmocytic infiltration was significantly more severe in Hunner type interstitial cystitis specimens than in nonHunner type interstitial cystitis specimens. In that paper, the conclusion was that Hunner type interstitial cystitis and nonHunner type interstitial cystitis were different pathological entities, the latter being characterized by pancystitis, B-cell expansion and loss of

epithelium. Since the pivotal paper by Fall and coworkers more than thirty years ago,¹ it has become progressively more clear that the diverse forms of interstitial cystitis indeed represent entirely different pathological entities, though sharing similar symptomatology and frequently also the same long-lasting course. Reports on excessive production of intraluminal nitric oxide, an inflammation marker, in Hunner type interstitial cystitis lend further support to this notion.² Nevertheless, there are in fact still considerable discrepancies in explanations on how to define interstitial cystitis and, ever more importantly, to discriminate between Hunner type interstitial cystitis (ESSIC Type 3C) and nonHunner type interstitial cystitis (reference 7 in article). These discrepancies certainly call for a solution, principally

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due to that the correct subtyping of patients with bladder pain syndrome/interstitial cystitis is of paramount importance, allowing not only for the option to adequately compare scientific materials but also for selecting a rewarding remedy for the specific patient.

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REPLY BY AUTHORS

As commented by Dr. Peeker, Hunner type interstitial cystitis (HIC; ESSIC BPS Type 3C) is a distinct entity to be separated from other conditions under the umbrella of interstitial cystitis/bladder pain syndrome (IC/BPS). It is worth praise that Dr. Fall and coworkers had implied this notion more than thirty years ago (reference 9 in article). We have found the immune-mediated inflammation properties of HIC that is characterized by clonal expansion of infiltrating B cells and pancystitis (references 12 and 13 in article). The present study has added another biological feature of HIC, that is, local ischemia as demonstrated by overexpression of hypoxia induced factor 1 alpha and its related biological pathways. Such accumulated evidence enables us to revise the terminology of interstitial cystitis. The currently used term of "interstitial cystitis" might refer to the overall bladder pain syndrome complex as equivalent to IC/BPS, regardless of the presence or absence of histological inflammation in the bladder. This ambiguity should be resolved by designating the term "interstitial cystitis" to denote HIC alone, leaving other clinically mimicking noninflammatory conditions as BPS. Otherwise, the current HIC may be renamed, for example, as "Hunner disease," so as to be discerned from other chronic cystitis that is not associated with Hunner lesions.