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The effect of prostate tissue inflammation in benign prostatic hyperplasia on enhancer of zeste homolog 2 ribonucleic acid expression

May Al-Maghrebi,[a] Elijah O. Kehinde,[b] Fahd Al-Mulla,[c] Jehoram T. Anim[c]

From the Departments of [a]Biochemistry, [b]Surgery (Division of Urology), [c]Pathology, Faculty of Medicine, Kuwait University, Kuwait

Correspondence: Dr. May Al-Maghrebi · Department of Biochemistry, Faculty of Medicine, Kuwait University, P.O. Box: 24923, Safat13110, Kuwait · malmaghrebi@hsc.edu.kw

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BACKGROUND AND OBJECTIVES: Enhancer of zeste homolog 2 (*EZH2*) has been recently found to regulate several genes involved in immunoresponse and autocrine inflammation network. The aim of the study was to quantitate *EZH2* messenger ribonucleic acid (mRNA) expression, evaluate its relation to conditions of prostatitis associated with benign prostatic hyperplasia (BPH), and correlate it with the levels of the inflammatory marker interlukin 6 (IL-6).

DESIGN AND SETTING: Cross-sectional study in Middle Eastern men with BPH and prostatitis or BPH only.

PATIENTS AND METHODS: Transrectal ultrasound–guided prostate biopsies were collected from 106 patients suspected of having prostate cancer; however, the histology revealed BPH. Upon further pathological examination, 56 of these cases were identified as BPH with prostatitis and classified as: acute prostatitis (n=13); active chronic prostatitis (n=32); and, chronic inactive prostatitis (n=12). Serum IL-6 levels and *EZH2* mRNA expression were measured and compared between patient groups.

RESULTS: *EZH2* mRNA was overexpressed in BPH with prostatitis patients compared to BPH only patients (*P*<.0001). BPH with active chronic prostatitis had higher *EZH2* expression than BPH with acute or chronic inactive prostatitis compared to BPH only (*P*=.05 and .73, respectively). *EZH2* mRNA expression showed a negative correlation with IL-6 concentrations in BPH with prostatitis patients (r_s=–0.31, *P*=.02). *EZH2* overexpression was associated with an increased risk of having BPH with prostatitis (crude odds ratio 0.20, 95% CI 0.06-0.65, *P*=.0076).

CONCLUSIONS: *EZH2* mRNA expression correlates positively with prostatitis conditions associated with BPH and negatively with serum IL-6 levels. This supports the possible involvement of *EZH2* mRNA overexpression in the development of prostate inflammation, and its new regulatory role in suppressing the expression of some inflammatory network genes.

Benign prostatic hyperplasia (BPH) is one of the most common and progressive diseases affecting aging men, and is histologically defined as an overgrowth of the epithelial and stromal cells of the transition zone and periurethral area. A variety of growth factors associated with epithelial/stromal interaction have been described in the pathophysiology of BPH; however, the cellular and molecular processes underlying the pathogenesis and development of BPH remain poorly understood.[1] It has also been reported that intraprostatic inflammation frequently accompanies BPH, and accelerates the pathogenesis and progression of this condition.[2-6] Acute intraprostatic inflammation causes symptoms that can be easily treated. However, many cases of chronic intraprostatic inflammation may go untreated because the condition has no symptoms and often goes undetected.

Enhancer of zeste homolog 2 (*EZH2*) is a known repressor of gene transcription. *EZH2* is the catalytic subunit of polycomb repressive complex 2 that is a highly conserved histone methyltransferase targeting lysine-27 of histone H3.[7] Several studies showed that *EZH2* is commonly overexpressed in a wide variety of cancerous tissue types, including prostate and breast.[8,9]

Although *EZH2* is upregulated in advanced and metastatic prostate cancer,[9] its role as a marker or driver of metastasis is yet to be resolved.[10] A new hypothesis has emerged proposing that prostate cancer can be driven by prostate inflammation, which is supported by several epidemiological, histopathological, and molecular pathological studies.[11] This is also partly supported by the fact that almost 20% of cancers are caused by chronic infections and/or inflammation.[12,13] Interestingly, a recent study identified a new regulatory role for *EZH2* in modulating the expression of several genes involved in immunoresponse and autocrine inflammation network. Suppression of *EZH2* expression, by pharmacological inhibition or genetic deletion, resulted in the activation of these immunoresponse genes implicating *EZH2* involvement in determining cancer immunity.[14] Thus, we raised the question of whether *EZH2* expression is modulated during prostatitis, and, if it is correlated with the expression of inflammatory cytokines like interlukin 6 (IL-6).

The aim of the current study was to evaluate the effect of prostate inflammation on the expression of *EZH2*. Thus, we measured the ribonucleic acid (RNA) expression of *EZH2* in patients with BPH only and in BPH with prostatitis. We also investigated the association of *EZH2* RNA expression with IL-6 serum levels and the histological types of prostatitis.

PATIENTS AND METHODS

Transrectal ultrasound (TRUS)-guided prostate biopsies were collected from 106 patients who were suspected of having prostate cancer, but the histology revealed BPH with or without prostatitis. All the men were of Middle Eastern origin. Ten to 12 core biopsies were taken from each patient using 18 fine gauge needles. The prostate tissue was obtained after written consent from each patient. The study was approved by the local ethics committee in compliance with the principles outlined in the Declaration of Helsinki.

Exclusion criteria included a history suggestive of ongoing urinary tract infection or acute prostatitis, the presence of an indwelling urethral catheter, and a history of previous prostate biopsy or previous urological procedures on the prostate gland. We also excluded cases where the histological examination revealed granulomatous prostatitis. We had 5 patients with this diagnosis (2 due to tuberculosis and 3 due to *Schistosoma haematobium* infections of the prostate). All 5 patients had very high prostate-specific antigen (PSA) serum levels (>50 ng/mL). Patients with percentage of free PSA <10% were excluded from the *EZH2* gene expression analysis as they have a very high chance of having prostate cancer.

Tissue samples were fixed in 4% solution of formaldehyde (i.e., 10% formalin), then processed routinely into paraffin blocks, and the sections were then stained with hematoxylin and eosin stains. The extent of prostate inflammation was classified on the basis of the following criteria proposed by Anim et al.[15] Briefly, (1) acute prostatitis (AP): focal grandular disruption with neutrophilic and macrophage cell reaction as well as accumulation in the gland lumen; (2) active chronic prostatitis (ACP): periglandular inflammatory infiltrate consisting predominantly of chronic inflammatory cells, but with some disruption of glandular epithelium as well as neutrophilic and macrophage infiltrate; and (3) chronic inactive prostatitis (CIP): periglandular fibrosis with surrounding chronic inflammatory infiltrate and/or lymphoid aggregate replacing a completely destroyed gland. BPH was diagnosed where there was a glandular and stromal hyperplasia with no significant inflammatory changes.

Serum Markers

IL-6 levels were measured using commercial enzyme-linked immunosorbent assay kits (GE Healthcare Life Sciences, Buckinghamshire, UK). The total PSA levels in blood serum were quantitatively measured by radioimmunoassay using Immulite kits (DPC, Los Angeles California, United States). The serum-free PSA was determined, and the percentage of free PSA was calculated for all patients with total PSA values between 4 and 10 ng/mL. In patients where the PSA levels were greater than 10 ng/mL, but the histology did not show prostate cancer, follow-ups at 3 monthly intervals with PSA estimations were arranged. If PSA normalized within 3 months, the patients were included in the diagnosis of prostatitis. If PSA continued to rise, the patients were subjected to another round of TRUS-guided prostate biopsy to exclude prostate cancer. When three sets of biopsies failed to show cancer, the patients were classified as having chronic prostatitis and followed up at 6 month-interval.[16]

Ribonucleic acid isolation, reverse transcription, and quantitative real-time polymerase chain reaction

Of the 10 to 12 core biopsies taken per patient, a representative core biopsy specimen was selected for *EZH2* RNA isolation. Either ends of the selected core biopsy specimens were sent for histological analysis, and the central piece of the core was used for *EZH2*A isolation. Total RNA was purified using TRIzol reagent (Invitrogen, Carlsbad, California, United States) following the manufacturer's instructions. RNA concen-

tration and purity were determined spectrophotometrically at 260 and 280 nm. Total RNA (1 μg) was reverse transcribed into first-strand complementary deoxyribonucleic acid (cDNA) in a 20 μL reaction volume, using M-MuLV reverse transcriptase RNase H (Invitrogen). Standardized specific primers and the fluorescent taqman probe for the *EZH2* gene were purchased from Applied Biosystems (Darmstadt, Germany). Real-time polymerase chain reaction (PCR) was carried using ABI 7000 thermal cycler (Applied Biosystems). The detection of PCR products was accomplished by measuring the emitting fluorescence (Rn) at the end of each reaction cycle. Threshold cycle (CT) corresponds with the cycle number required to detect a fluorescence signal above the baseline. We analyzed the gene expression data using the comparative (2-DDCT) CT method provided by the Sequence Detection System 1.2.3 Software from Applied Biosystems. Based on this method, the copy numbers of *EZH2* mRNA were normalized to those of the endogenous control gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and relatively to a standard human normal prostate, using total RNA as a calibrator (Ambion, Austin, Texas, United States).

Table 1. Descriptive statistics of variables in benign prostatic hyperplasia (BPH) only and benign prostatic hyperplasia with prostatitis patients.

Characteristic	BPH with prostatitis (n=56)	BPH (n=50)	*P*
Age, year			
Median	67	62	.011
Range	47-84	42-79	
PSA, ng/mL			
Median	11.75	5.9	<.0001
Range	0.75-68.7	0.1-26	
Serum IL-6, pg/mL			
Median	6.07	4.1	<.0001
Range	2-48	1.1-6.6	
Histological types of prostatitis	n=56 (%)		
Acute prostatitis	12 (21.4)		
Chronic active prostatitis	32 (57.1)		
Chronic active prostatitis	12 (21.4)		
[a]Mann-Whitney U test			

BPH: Benign prostatic hyperplasia; IL-6 = interleukin 6; PSA: prostate-specific antigen.

Since the distribution of *EZH2* expression in BPH and BPH with prostatitis patients was not Gaussian, the differences among two groups were assessed by the nonparametric Mann-Whitney U test. Any possible correlations between *EZH2* expression and other continuous variables were assessed by the Spearman correlation coefficient (r_s). We used univariate and multivariate unconditional logistic regression analysis to study the ability of the variables to predict the presence of prostatitis. The relationship between *EZH2* mRNA expression and types of histological prostatitis was determined using chi-square and Fisher exact test where appropriate. A *P* value <.05 was considered statistically significant.

RESULTS

The clinical and pathological data of the patients investigated in this study are listed in **Table 1**. Based on histological analysis, the patient population was classified into the following categories: BPH only (n=50) and BPH with prostatitis (n=56). The BPH with prostatitis patients were further divided into the following groups: AP (n=12), ACP (n=32) and CIP (n=12). High levels of total PSA were detected in our patient cohort as compared with whites in the United States and Europe. This finding was previously reported for men of Middle Eastern origin; however, no clear reason was reported for these high PSA levels in BPH and BPH with prostatitis conditions in these men.[16]

Enhancer of zeste homolog 2 expression in prostatic diseases

EZH2 mRNA expression in the BPH group had a range of 0.03 to 3.9 (*EZH2* mRNA copy number/GAPDH mRNA copy number) with a mean (standard error, SE) of 1.3 (0.13). In BPH with the prostatitis group, *EZH2* mRNA expression varied from 0.51 to 17.58 with a mean (SE) of 5.7 (0.58). Compared to the BPH group, the data were indicative of a significant increase ($P \leq .0001$) in the expression level of *EZH2* mRNA in BPH with prostatitis specimens mainly AP and ACP, but not CIP ($P=.413$) (**Table 2**). The distribution of *EZH2* mRNA expression in the two groups is shown in **Figure 1**.

Correlation between enhancer of zeste homolog 2 messenger ribonucleic acid expression and other variables in benign prostatic hyperplasia with prostatitis

In BPH with prostatitis, we found a statistically significant negative correlation between *EZH2* expression and serum IL-6 levels using these parameters as continuous variables ($r_s=-0.31$, $P=.02$) (**Figure 2**). A

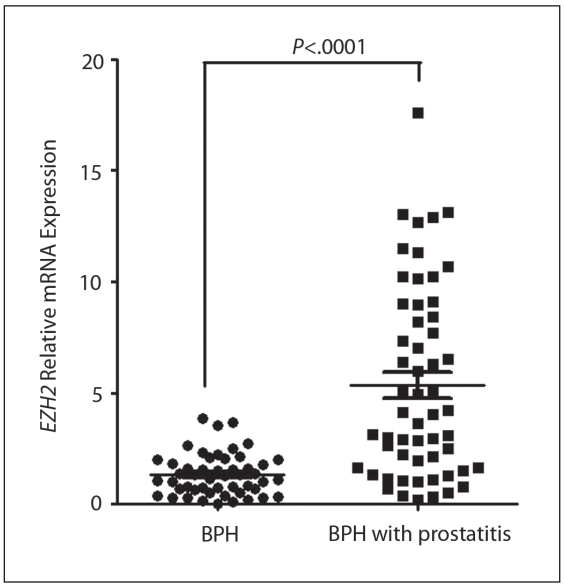

Figure 1. Distribution of *EZH2* mRNA expression levels in BPH and BPH with prostatitis patients. The difference between categories was tested by the Mann-Whitney U test.

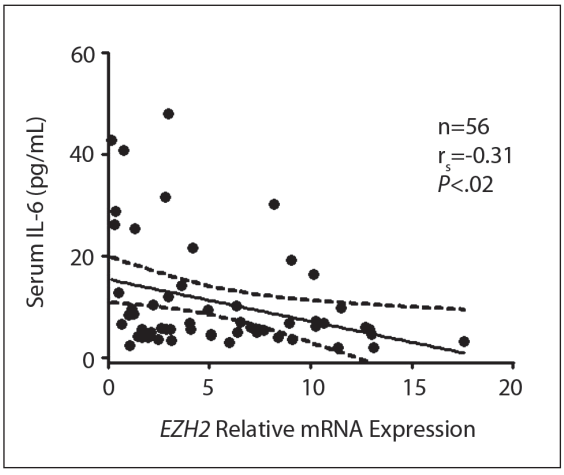

Figure 2. Correlation between EZH2 mRNA relative expression and serum IL-6 concentration in BPH and BPH with prostatitis patients. rs, Spearman correlation coefficient.

significant correlation between *EZH2* expression and patient age (r_s=-0.099, *P*=.466) and PSA levels (r_s= 0.18, *P*=.184) was not observed. The data were then analyzed using a univariate logistic regression model to investigate whether *EZH2* mRNA expression levels are associated with conditions of BPH with prostatitis (**Table 3**). High levels of *EZH2* expression were found to be associated with an increased risk of having BPH with prostatitis (crude odds ratio 0.20, 95% CI 0.06-0.65, *P*=.0076). Similarly, total serum PSA levels proved to be an important parameter for distinguishing simple BPH from BPH with prostatitis (crude odds ratio 0.19, 95% CI 0.06- 0.56, *P*=.0028). Furthermore, the multivariate analysis showed that *EZH2* mRNA expression was an independent predictor of the development of BPH with prostatitis with crude odds ratio of 0.02 (95% CI 0.003-0.92, *P*=.044), improving the diagnostic significance of PSA.

Relationship between enhancer of zeste homolog 2 messenger ribonucleic acid expression and histological types of prostatitis

The relationship between *EZH2* mRNA expression and the different types of prostatitis is outlined in **Table 4**. Overall, 92.5% (52/56) of BPH with prostatitis had detectable *EZH2* mRNA expression compared with 72% (36/50) of simple BPH (c2=16, *P*=.008). Except for the CIP that showed 66.7% *EZH2* expression, AP and ACP had detectable *EZH2* expression (100%). A trend was observed toward differential *EZH2* mRNA expression between ACP and simple BPH specimens (*P*=.0006) but not CIP (*P*=.73).

DISCUSSION

EZH2 is a polycomb group protein that acts as a transcriptional repressor controlling cellular memory and helps in maintaining cell type identity.[17] Thus, deregulation of its expression and subsequently that of the transcriptional machinery can result in cell identity loss and oncogenic transformation. Upregulation of its gene expression has been linked to cell growth promotion and prostate cancer aggressiveness.[9] In breast cancer, down regulation of *EZH2* expression, chemically or genetically, caused reactivation of 58 *EZH2*-repressed immune response genes like interleukin 8 (IL-8), tumor necrosis factor (TNF), chemokine (C-C motif) ligand 2 (CCL2), chemokine (C-X-C motif) ligand 2 (CXCl2), and many others.[14] Our study showed that although *EZH2* expression is detected in both types of BPH, overexpression of *EZH2* mRNA in BPH with acute or chronic active prostatitis was more frequent than in BPH only conditions (*P*<.0001). The logistic regression analysis showed that *EZH2* may be an independent factor in the discrimination of the two prostatic conditions. This is further supported by the parallel increase in PSA levels in BPH with the prostatitis group, and the additive effect of *EZH2* to the predictive power of the multivariate model, despite the lack of correlation between the two factors (r_s= 0.18, *P*=.184). We also investigated the relationship of *EZH2* expression positivity with the different types of histological prostatitis (**Table 4**). *EZH2* expression had a remarkable low de-

Table 2. Relative *EZH2* mRNA expression in benign prostatic hyperplasia (n=50) and benign prostatic hyperplasia with prostatitis (n=56) patients.

Pathological specimen	Mean (SE)	Median	Range	*P* [a]
Benign prostatic hyperplasia	1.3 (0.13)	1.2	0.03-3.9	
Benign prostatic hyperplasia with prostatitis	5.7 (0.58)	4.58	0.51-17.58	<.0001
Acute prostatitis	1.1 (0.22)	0.9	0.19-2.6	<.0001
Chronic active prostatitis	7.6 (0.74)	8	1.1-18	.0001
Chronic active prostatitis	3.6 (0.5)	3.4	1.1-7	.413

EZH2:Enhancer of zeste homolog 2; mRNA:messenger ribonucleic acid; SE: standard error.

[a]Mann-Whitney U test: Comparison between EZH2 mRNA expression in the different types of prostatitis versus benign prostatic hyperplasia only samples.

Table 3. Logistic regression analysis for predicting the presence of benign prostatic hyperplasia with prostatitis.

Covariant	Crude odds ratio	95% CI	*P*
Univariate analysis			
EZH2	0.20	0.06-0.65	.0076
PSA	0.19	0.06-0.56	.0028
Multivariate analysis			
	0.05	0.003-0.92	.044

EZH2:Enhancer of zeste homolog 2; PSA:prostate-specific antigen.

Table 4. Relationship between *EZH2*, mRNA expression, benign prostatic hyperplasia, and different types of prostatitis.

Pathology of specimen	No. specimen	Number expressing *EZH2* (%)	*P* [a]
Total	106		
Benign prostatic hyperplasia	50	36 (72)	
Benign prostatic hyperplasia with prostatitis	56	52 (92.8)	.008
Acute prostatitis	12	12 (100)	.05
Chronic active prostatitis	32	32 (100)	.0006
Chronic inactive prostatitis	12	8 (66.7)	.73

EZH2:Enhancer of zeste homolog 2; mRNA:messenger ribonucleic acid.

[a]Fisher exact test: Comparison between *EZH2* mRNA expressions in the different types of prostatitis versus benign prostatic hyperplasia only samples. Chi square test (χ^2=16, *P*=.0014) was used to compare the expression of *EZH2* across the different types of benign prostatic hyperplasia with prostatitis with that of benign prostatic hyperplasia only.

tectability level in simple BPH (72%) when compared to BPH with prostatitis (92.5%) (*P*=.008). Moreover, patients with chronic active prostatitis displayed a stronger relation with *EZH2* expression compared to acute and chronic inactive prostatitis (*P*=.05 and .73, respectively).

It is commonly known that inflammatory cascades are crucial in the development of simple BPH; however, the primary event in the activation of this cascade is still unclear.[18] The importance of these inflammatory reactions in the pathogenesis of BPH symptoms were confirmed by the positive correlation between the young-onset prostatitis and later development of lower urinary tract symptoms.[19] IL-6 is a cytokine expressed in the stromal and luminal epithelial BPH cells, and characterized by its proliferative action, which suggests its role during the IL-17-dependent BPH-associated inflammatory processes.[20] We report elevated serum levels of IL-6 in BPH with prostatitis patients, as compared to BPH only. A strong negative correlation was also reported between IL-6 and *EZH2* levels in BPH with prostatitis (*P*=.011), reinforcing the potential novel role of *EZH2* in suppressing the expression of some immune response genes. Several studies have reported on the expression pattern of many cytokines and shed a light on their role in prostate tissue inflammation. IL-8 is a direct mediator of neutrophil accumulation and activation at inflammatory sites.[21] Recently, IL-8 has been suggested as a reliable marker of BPH with chronic prostatitis.[22] IL-15 plays an important role in the generation and maintenance of intraprostatic infiltrates.[23] IL-17 is the principal player in the pathogenesis and maintenance of the immune responses because of its role in the activation of the proinflammatory network.[24-26] Thus, it is not surprising that a cytokine-like IL-6 is overexpressed in BPH with prostatitis conditions and

found to be inversely correlated with the new regulatory role of *EZH2*.

The probable use of *EZH2* expression may be used in deciding which patients may develop prostate cancer in the future by checking for the presence of its overexpression. In this study, 5 patients with high grade prostatic intraepithelial neoplasia (HGPIN) in addition to BPH + chronic prostatitis had higher levels of expression of *EZH2* compared to those without HGPIN or those with BPH. Furthermore, patients with prostate cancer had the highest expression levels of *EZH2* compared to patients with BPH only, BPH + prostatitis, or BPH + prostatitis + high-grade prostatic intraepithelial neoplasia. From basic principles, it would appear that patients with high PSA and overexpression of *EZH2* may subsequently develop prostate cancer. Unfortunately, our follow-up period was just about 4 years and no such cases were documented by us. It could be argued that such patients require a closer follow-up and repeat prostate biopsy.

Our data shows that *EZH2* mRNA expression is positively associated with prostatitis conditions associated with BPH. Also, its inverse correlation with serum IL-6 expression supports the new proposed regulatory role of *EZH2*. Future studies should aim at the evaluation of *EZH2* protein concentrations in BPH and prostatitis prostate tissues using immunohistochemistry and immunochemical assays. Our findings would be certainly strengthened if other inflammatory factors (cytokines and chemokines) were tested for their expression, including IL-8, IL-15, IL-17, and TNF and their relationships with *EZH2* expression levels in such inflammatory conditions of the prostate are explored.

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