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Hyaluronan as a predictive biomarker in recurrent renal stone formers

Mayur Danny I. Gohel[1*], H.Y.H. Or[1], M.C.K. Lau[1], and C.F. Ng[2]

[1]School of Medical and Health Sciences, Tung Wah College, Homantin, Kowloon, Hong Kong, SAR China
[2]Dept. of Surgery (Urology), Prince of Wales Hospital, The Chinese University of Hong Kong, Shatin, Hong Kong, SAR China

***Corresponding Author:** Prof. Mayur Danny I. Gohel, School of Medical and Health Sciences, Tung Wah College, 31 Wylie Road, Homantin, Kowloon, Hong Kong, SAR China, Tel: +852 31906680; Email: dannygohel@twc.edu.hk

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Abstract

Recurrence of renal calculi after treatments such as extracorporeal shock wave lithotripsy (ESWL), percutaneous nephrolithotomy (PCNL) and surgeries, remains high. A viable diagnostic biomarker is needed to alert the patient and the clinician to monitor for stone recurrences. This study concentrates on idiopathic stone-formers (SF) and 6 groups of subjects were recruited with Active-SF (pre- and post- treatments), Non-SF (with and without infection) for comparisons. Urine and blood samples were collected from the patients (with inclusion and exclusion criteria) from the hospital clinic and processed at the laboratory with ELISA and biochemical methods (electrophoresis and HPLC). 120 samples were collected amongst the 3 groups. The following demographics were obtained: Age-range (32 – 63 years old); Male: Female ratio (58: 42); Mean urinary pH 6.33 ± 0.23 (though in each group there are differential mean pH) and urinalysis done for all samples to verify the integrity of the samples. The first biomarker studied was the excretion of urinary glycosaminoglycans (GAGs). Chondroitin sulphate A/C (CS), dermatan sulphate (DS), heparin sulphate (HS) and hyaluronan (HA) were extracted and quantified. Active SF (prior treatment) had 70% positive indicator for GAGs and those SF (post treatment) had over 90% compared to the Normals. Other biomarkers (not reported here) under investigations are cytokines including NAG and MIP-1α. This study combines majority of the biomarkers in our study under a case-control investigation to suggest a potential and sensitive marker for recurrent SFs. Hyaluronan is one such candidate biomarker.

Keywords: Biomarkers; Glycosaminoglycans; Hyaluronan; Recurrence; Urolithiasis

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Introduction

Extracorporeal shock wave lithotripsy (ESWL) and percutaneous nephrolithotomy (PCNL) are now widely used to eradicate stones. However, recurrence rates remain high (up to 60%) over the lifetime of certain patients [1]. These recurrent stone-formers are good models for investigations to establish the important biological markers and mediators of inflammation in the blood and urine of renal stone patients. The fragments of stones remaining after ESWL/PCNL, whether they are clinically insignificant or significant, can pose a long-term risk for patients by serving as a nidus for new stone-formation [2]. The main goal of this project is to allow renal stone patients to be monitored with a simple urine test, which will give advanced warning of clinically significant recurrence. This study is designed to study hyaluronan (HA) as a potential biomarker that can be readily tested and validated.

The Objectives are

1. To isolate and quantitate HA (as a result of inflammation) in the urine of patients with renal stones and those treated thereafter;
2. To test for levels of the HA (as identified by Objective 1) and to validate the results from patients in different groups of stone formers (active and post-ESWL/PCNL), compared to the results from non-stone formers.

This prospective study of biomarkers, which will include a panel of cytokines, GAGs, and HA can provide a better understanding of the pathogenesis of urolithiasis and the predictive values of these various factors. Such research can point the way for the identification of populations that are at risk of early recurrence. This study may enable clinical attention to be better directed, with more frequent follow up visits for the high-risk groups and longer intervals for low-risk patients.

Materials and Methods

Subject recruitments and collection of urine samples

Six groups of subjects of stone-formers and normal controls were recruited. These included Normals (non stone-formers), active SF confirmed with the presence of kidney stones by radiographic examination and the post-treated SF which were SF after post-ESWL and declared “stone-free” by radiological and ultrasound assessments. They were recruited from the Department of Surgery (Urology), Prince of Wales Hospital, Hong Kong. Aged-matched normal subjects group with no known urological history were randomly recruited from Hong Kong community as controls. All subjects gave informed consent. Ethics approval was obtained from the Human Subjects Ethics Sub-committee of the Tung Wah College and from the Hospital Authority Hong Kong.

All subjects were confirmed without diseases (e.g. diabetes mellitus or hypertension with medications) that may affect the kidney functions. Early morning urine (EMU) was collected for each subject after recruitment except subjects from post-ESWL group, which were collected after treatment. Urinalysis were performed on all samples to confirm that they were free of any urinary tract infection and confirm the integrity of the urine specimens.

Recovery and analysis of urinary GAGs

Urinary GAGs were recovered by methylpyridinium chloride and sodium acetate-saturated ethanol precipitation followed by papain (Sigma Chemical Co., USA) digestion as described previously [3,4]. The hexuronate content of the GAGs were measured by carbazole reaction [4]. All samples were done in triplicate. The results of hexuronate in GAGs were standardized against creatinine.

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Urinary creatinine determination

Pre-diluted urine samples (1:50) with MilliQ water were analyzed by the automated chemistry analyzer (Cobas Fara, Roche Diagnostics, USA) with the creatinine regents (Biosystems, Spain) according to the manufacturer's specifications. All samples were performed in triplicate.

Urinary lithogenic ion determination

Levels of calcium, oxalate, citrate and phosphate were determined using appropriate kits obtained from Sigma-Aldrich (USA). The tests were done in triplicate for all samples.

Double enzyme digestions of GAGs for high performance liquid chromatography (HPLC) analysis of HA disaccharides

Individual GAGs extracts and HA from Pig skin (Seikagaku Corporation, Tokyo, Japan) as HA standards were digested sequentially with 0.3 turbidity reducing units of hyaluronidase from *Streptomyces hyalurolyticus* (Seikagaku Corporation, Japan) and 0.1 U of chondroitinase ABC from *Proteus Vulgaris* (Seikagaku Corporation, Japan) to yield the HA disaccharides and analyzed by HPLC as previously described [3].

Statistical analysis

GraphPad Prism version 6.0 for window (GraphPad Software, USA) was used to perform all statistical analysis in this experiment. One-way analysis of variance (ANOVA) was used for the comparison of the mean differences of GAGs and HA between SF, Post-treated SF and normal individuals' groups. All significant ANOVA test results were analyzed by Dunnett's multiple comparison post-test. A *P* value of less than 0.05 was considered significant from the normal and specified with the symbol (*) in the table.

Results

Total glycosaminoglycans (GAGs) excretion in the early morning urine (EMU) of stone-formers (SF), post-treated SF, and normal controls

In table 1, it is shown that normal individuals excreted higher amount of total urinary GAGs than stone-formers (SF). A significant reduction in the urinary GAGs in post-treated SF was found when compared with normal subjects.

Table 1: The total urinary glycosaminoglycans (GAGs) concentration (µg hexuronate/ mmol creatinine), hyaluronan (HA) concentration (ng/ mmol creatinine) and the proportion of HA in total GAGs (%) in normals, stone-formers (SF), and post-treated SF. Results are reported as (mean ± 95% CI). $P < 0.05$ was considered a significant difference from the normals and specified by the symbol *.

	Normals	Stone-formers	Post-treated SF
Total GAGs	233.3 ± 80.8	↓144.7 ± 65.86	↓109.7 ± 44.28 *
Hyaluronan	811 ± 401.5	↑1721 ± 1191	↓681.1 ± 258.8
HA in total GAGs	0.28 ± 0.08	↑0.77 ± 0.37	↑1.09 ± 0.76 *

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Urinary hyaluronan (HA) excretion

SF had an enhanced urinary excretion of HA but no increase of urinary HA in post-treated SF group was found when compared with the normal individuals.

Proportion of HA in total GAGs

SF had a higher proportion of HA in total GAGs while post-treated SF had a significant higher proportion of HA in total GAGs than normals.

Quantitation of Urinary lithogenic ions

Figure 1 indicates the levels of urinary citrate and oxalate and reveals that both citrate and oxalate remain high in stone-formers (after removal of stones) compared to Normals and those stone-formers who have not been treated.

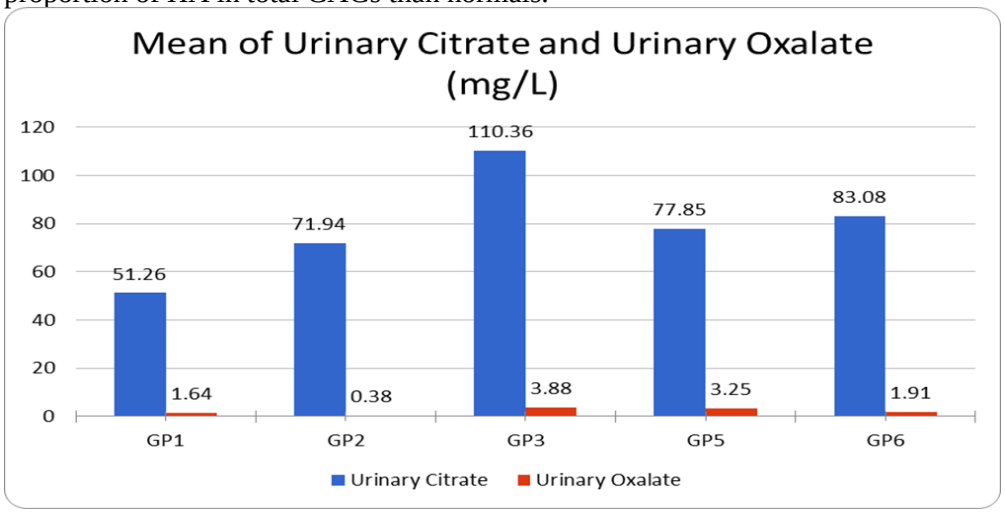

Figure 1: Levels of urinary citrate and oxalate in 6 groups of subjects. Groups 1-3, Active stone formers without UTI (1), with UTI (2) and following ESWL/PCNL (3). Groups 4-6, Non stone-formers with UTI (4), bladder cancer with BCG instillation (5) and Normal Controls (6).

Discussion

Increased supersaturation of urine and/or enhancement of urinary crystallization promoters and reduction of crystallization inhibitors leads to renal stone formation [5]. Urinary glycosaminoglycans (GAGs) are one of the major macromolecular modifiers for the pathogenesis of renal stones. Our results correlated with previous studies [6,7] that normal individuals excreted higher number of urinary GAGs than stoner-formers (SF). We also found that there is a significant reduction in the urinary GAGs in stone-former after extracorporeal shock wave lithotripsy (ESWL) (post-treated SF) than normal subjects even after standardization of GAGs with creatinine to eliminate the dilution effect of increase intake of water which is usually recommended by urologists after treatment.

GAGs are highly negatively charged polysaccharides with repeated disaccharides units and presence of carboxyl or sulphate groups lead to the stretched or extended conformation of polysaccharide chains of GAGs [8] as an expanded random coil structure [9]. Addition of GAGs can significantly inhibit the adherence of CaOx monohydrate crystals to renal epithelial cells [10-11] by coating onto the crystals instead of coating onto the cells [11]. The increase production of GAGs by tubular epithelial cells may protect cells from toxic effects of calcium oxalate crystals and oxalate ions [6]. Therefore, GAGs can prevent the

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binding of CaOx crystals onto the negatively charged urinary epithelial cells surface due to the charge repulsion and the steric hindrance by itself being covered on the surface of the crystals and thus prevents crystals to be retained and becoming a potential stone.

GAGs can also enhance the urinary supersaturation by forming complex with calcium salts [12] so that higher concentration of calcium can be tolerated in the urine without precipitation of CaOx crystals. The higher GAG content in normal population can be protective against kidney stone disease by preventing CaOx crystals binding to the renal epithelial cells surface as well as reducing the CaOx crystals being formed by raised calcium holding capacity in urine. Reduced excretion of GAGs in SF and the lower GAGs amount in the post-treated SF group is a risk factor for the occurrence and recurrence of stones.

Hyaluronan (HA) is a non-sulfated GAGs involved in several fundamental cell biological processes such as regulation of cell-cell adhesion, development, proliferation, migration, differentiation, metastasis, inflammation and wound healing [13]. HA fragments are released into urinary tract [14] as a consequence of active turnover of renal tissue in the diseased state. Studies showed that migrating cells produce large amounts of HA during repair of damaged renal epithelial cells [15-16]. Up-regulation of HA was observed in human kidney proximal epithelial (HK-2) cells during CaOx crystals induced cell injury [16] for mediating repair of an injured epithelium.

SF had an enhanced urinary excretion of HA but no increase of urinary HA was found in post-treated SF group when compared with the normal individuals. This can be explained that SF had CaOx induced cell injury occurring and in the post-treated SF, the enhanced excretion of HA is absent as there is no cell injury to trigger the excretion of HA at 14 days after the stone removal by ESWL because the wound caused by stone and ESWL had healed already. (Earlier measurements of HA were not possible due to presence of blood).

However, when the proportion of HA in total GAGs is investigated, it was found that SF had higher proportion of HA that correlated with our previous study [3]. It was also interesting that post-treated SF had a significantly higher proportion of HA in total GAGs than normals although the HA concentrations in post-treated SF was no different from normal individuals. The higher proportion of HA in post-treated SF was suggested to be due to the lower total GAG amount.

The high levels of oxalate and citrate, even after treatment in stone-formers, suggests that they are still at risk of forming stones, though sustained high levels of citrate could counteract the high oxalate by preventing calcium to bind to the oxalate and keeping it soluble to pass it out.

Conclusions

Our previous study found that HA secreted by the injured HK-2 cells leads to the adherence of CaOx crystals to the injured epithelial cells and results in the internalization of crystals [16]. This suggested that increased HA production during inflammation of renal epithelial cells in SF do enhance the risk of renal stone formation and an higher HA proportion in total GAGs of both SF and Post-SF indicated that they have a higher risk for the occurrence and recurrence of kidney stone disease due to the crystallization-promoting property of HA. It is suggested that HA becomes an accidental participant of the pathogenesis of stone disease and patients who have undergone procedures or treatments, may have compromised integrity of the urothelium lining with subsequent HA being released as an inflammatory response molecule and a potential diagnostic marker.

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References

1. Chung VY, Benjamin W. 2016. The success of shock wave lithotripsy (SWL) in treating moderate-sized (10-20 mm) renal stones. The success of shock wave lithotripsy (SWL) in treating moderate-sized (10-20 mm) renal stones, Urolithiasis. 44: 441-444. Ref.: https://bit.ly/35S7ifx
2. Miller NL, Gillen DL, Williams JC, et al. 2009. A formal test of the hypothesis that idiopathic calcium oxalate stones grow on Randall's plaque., 103: 966-971. Ref.: https://bit.ly/33E4roX
3. Shum DK, Gohel MD, Tam PC. 1999. Hyaluronans: crystallization-promoting activity and HPLC analysis of urinary excretion. 14: S397-S403. Ref.: https://bit.ly/2LcDEK9
4. Gohel MD, Shum DK, Tam PC. 2007. Electrophoretic separation and characterization of urinary glycosaminoglycans and their roles in urolithiasis. 342: 79-86. Ref.: https://bit.ly/2P3ftil
5. Tiselius HG. 2011. A hypothesis of calcium stone formation: an interpretation of stone research during the past decades. 39: 231-243. Ref.: https://bit.ly/37SUVBM
6. Borges FT, Michelacci YM, Aguiar JA, et al. 2005. Characterization of glycosaminoglycans in tubular epithelial cells: calcium oxalate and oxalate ions effects. 68: 1630-1642. Ref.: https://bit.ly/2L8ckN9
7. Shum DK, Gohel MD. 1993. Separate effects of urinary chondroitin sulphate and heparan sulphate on the crystallization of urinary calcium oxalate: differences between stone formers and normal control subjects. 85: 33-39.
8. Marszalek PE, Oberhauser AF, Li H, et al. 2003. The force-driven conformations of heparin studied with single molecule force microscopy. 85: 2696-2704. Ref.: https://bit.ly/2LslwfB
9. Verkoelen CF. 2006. Crystal retention in renal stone disease: a crucial role for the glycosaminoglycan hyaluronan? 17: 1673-1687. Ref.: https://bit.ly/2ODcfTG
10. Ebisuno S, Kohjimoto Y, Tamura M, et al. 1995. Adhesion of calcium oxalate crystals to Madin-Darby canine kidney cells and some effects of glycosaminoglycans or cell injuries. 28: 68-73. Ref.: https://bit.ly/34G0Xnm
11. Lieske JC, Leonard R, Toback FG. 1995. Adhesion of calcium oxalate monohydrate crystals to renal epithelial cells is inhibited by specific anions. 268: F604-F612. Ref.: https://bit.ly/2Y5uDYu
12. Grases F, Costa-Bauza A, March JG, et al. 1991. Glycosaminoglycans, uric acid and calcium oxalate urolithiasis. 19: 375-380. Ref.: https://bit.ly/2rLOBLE
13. Laurent TC, Fraser JR .1992. Hyaluronan. FASEB J 6: 2397-2404.
14. Yeung KS, Chong YC, Petillo PA (2001) Chapter 12. Synthesis of glycosaminoglycans. In: Wang PG, Bertozzi CR, eds. Glycochemistry: principles, synthesis and applications. Vol. New York: Marcel Dekker. 425-492. Ref.: https://bit.ly/2DDcwzP
15. Chen WY, Abatangelo G. 1999. Functions of hyaluronan in wound repair. Wound Repair Regen 7: 79-89. Ref.: https://bit.ly/34Fd3x1
16. Yuen JW, Gohel MD, Poon NW, et al. 2010. The initial and subsequent inflammatory events during calcium oxalate lithiasis. 411: 1018-1026. Ref.: https://bit.ly/33Fo0xg