REVIEW

Properties and Function of Nephrocalcin: Mechanism of Kidney Stone Inhibition or Promotion

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Abstract. Nephrocalcin (NC), an acidic glycoprotein with molecular weight 14,000, is present in urine and prevents kidney stone formation. Histoimmunochemical staining shows that NC is localized in the proximal tubules in kidneys. Isolated NC from mammalian urine, revealed at least 4 isoforms of NC (we call these isoforms NC-A, NC-B, NC-C, and NC-D in the order of elution) during DEAE cellulose column chromatography with a linear gradient of NaCl elution step. Non-stone forming people excrete more NC-A and NC-B isoforms in urine; however, more NC-C and NC-D isoforms were found in stone formers' urine. When the organic matrix was extracted from surgically removed calcium oxalate kidney stones, we found greater quantities of NC-C and NC-D isoforms than those of NC-A and NC-B isoforms. Amino acid compositions and carbohydrate contents of these 4 isoforms were similar with the exception of the gamma-carboxyglutamic acid (GLA) residues. Only the NC-A and NC-B isoforms contained residues of GLA. There were more phosphate residues present in NC-C and NC-D than in NC-A and NC-B. Upon removal of phosphate residues by alkaline phosphatase, NC-C eluted at the same salt concentration as NC-A eluted. This indicates that the backbone protein could be similar, but the NC-C isoform is modified by excess phosphate residues. Surface tension measurements using a Lauda film balance indicated that NC-A and -B were strongly amphiphilic while NC-C and -D were less amphiphilic. NC-A has an elongated shape, and occupies a smaller area per molecule; whereas NC-C is a bulky molecule. Using NC-A as a model of a "good" inhibitor and NC-C as a model of a "poor" inhibitor, both bound with 4 atoms of Ca^{2+} per molecule as investigated by equilibrium dialysis method, ^{31}P -NMR, and electron spin resonance spectrometry. Isoforms A and B changed their conformation upon Ca^{2+} binding, but C and D did not change their conformation. All these observations suggest that isoforms A and B are strong inhibitors of calcium oxalate monohydrate (COM) crystal growth and aggregation. However, isoforms C and D act as promoters for COM crystal growth-kidney stone formation. Measuring the amount of NC in urine from renal cell carcinoma patients and from NC isolated from a supernatant of a primary renal cell carcinoma cells demonstrated the amount of NC increased with disease progression. (Keio J Med 46 (1): 1-9, March 1997)

Key words: nephrocalcin, kidney stone inhibitor, urolithiasis, kidney stone

Introduction

Kidney stone disease has been well documented in medical history; however, it has been not given a great deal of attention due to fact that it is not a life-threatening disease and affects a minority of the population. Recently this disease has been the focus of special attention,

and meetings and symposiums have been organized in America, Europe, and Japan.

Many hypotheses have been proposed for the formation of calcium kidney stones; kidney stone promoters¹ and inhibitors² which are inorganic and organic compounds. Molecular weights of these promoters and inhibitors range from less than 100 to over 100 kd.

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Known biological minerals, such as bone,³ teeth,⁴ shells,⁵ and kidney stones^{6,7} contain an organic matrix. Many of proteins reported to make up the matrix include: Albumin,⁸ alpha 1-antitrypsin,⁹ chondroitin sulfate,¹⁰ crystal matrix protein,¹¹ prothrombin-like protein,¹² RNA-fragments,¹³ alpha 2-microglobulin (or alpha-1-microglobulin),¹⁴ Apolipoprotein A,¹⁵ Tamm-Horsfall glycoprotein,¹⁶ uropontin.¹⁷ These compounds have been reported to be present in kidney stones, but their function as an inhibitor or a promoter has remained unknown.

We isolated an acidic glycoprotein with a mol. wt. of 14kd in a monomeric form (nephrocalcin, NC) from urine and kidney tissues of 9 mammalian species¹⁸ and from surgically removed kidney stones.⁷ These isolated NCs separated into 4 isoforms.¹⁹ I will discuss the chemical and physicochemical properties of these isoforms and describe their potential functions.

Immunohistochemical staining showed that NC is localized in the proximal tubules of the kidneys. This was further proven by the identification of NC from tissue culture medium of primary proximal tubule cells.

Over 85% of renal carcinoma develops in proximal tubule cells.²⁰ Therefore, we quantitatively analyzed NC levels in the urines of renal cell carcinoma patients by ELISA method and found those levels to be elevated. After renal cell carcinoma patients had a nephrectomy, NC levels were reduced.

Isolation of Nephrocalcin

The first step of isolation was anion exchange column chromatography using DEAE cellulose. In most samples calcium oxalate (CaOx) inhibitory activity appeared in 4 ionic strength regions, A (12–15 mS), B (16–22 mS), C (23–28 mS), and D (29–40 mS). Figure 1 shows typical elution patterns of DEAE cellulose column chromatography of a single urine samples from normal male and female, and stone former male and female. We noticed that normal urine contains more A and B than C and D. However, kidney stone forming patients excretes more C and D than A and B in urine (Fig 2). The same relationship was observed in CaOx kidney stone extracts.

Chemical Compositions of NC Isoforms

Amino acid analyses of the isomers are summarized in Table 1. The amino acid compositions are similar but gamma-carboxyglutamic acid (GLA) contents are different: Isoform A obtained from normal urine contains 3 residues, B and C contain 2 residues, and D does not contain any GLA residues. In contrast, all 4 isoforms obtained from kidney stone patients contained no GLA residue.

Carbohydrate contents are shown in Table 2. No significant differences were observed among normal and

Fig 1 Elution patterns of protein and nephrocalcin from DEAE cellulose column using linear NaCl gradient from 0.1 to 0.5 M in 0.05 M Tris-HCl, pH 7.3 as a solvent. Protein (●—●) and Inhibitor (○—○). Typical elution pattern is demonstrated using a single urine sample obtained from normal male and female, and stone forming male and female patients.

stone former isoforms.

Physicochemical Properties

Dissociation constants against calcium oxalate monohydrate crystals were determined by plotting Langmuir isotherm type plot. The results are shown in Fig 3. Normal isoform A shows a 10-fold stronger binding to CaOx molecule than stone formers' C isoform.

When surface tension of isoforms was measured using a Lauda film balance, normal isoforms, A, B, and C showed high collapse pressure; however, stone formers NC isomers, A, B, C, and D showed low collapse pressure (Fig 4). This indicates that normal NCs are amphiphilic but stone formers' NCs are less amphiphilic,

Fig 2 Distribution ratios of nephrocalcin isoforms in urine of normal subjects and stone formers.

Table 1 Amino Acid Compositions of 4 Nephrocalcin Isoforms Purified from Normal (N) and Stone Formers (P) Urine

	A		B		C		D	
	N	P	N	P	N	P	N	P
Lysine	2.5	1.6	2.4	1.7	2.8	2.3	2.0	2.0
Histidine	2.0	1.4	1.0	2.0	1.8	2.4	2.5	2.5
Arginine	2.4	2.2	1.6	2.6	2.8	2.4	2.3	2.3
Aspartic Acid	12.9	14.2	13.2	11.8	11.4	12.1	11.2	11.2
Threonine	13.9	11.5	8.4	8.0	9.5	10.0	11.5	12.0
Serine	9.8	9.8	10.6	8.8	10.0	11.0	11.4	11.4
Glutamic Acid	13.2	13.1	13.4	14.4	12.6	13.3	12.2	12.2
Proline	8.8	7.5	6.7	7.2	7.5	9.0	7.2	7.2
Glycine	9.5	10.5	9.2	11.8	10.0	12.2	11.0	11.0
Alanine	9.2	7.9	9.4	6.4	9.0	9.7	9.7	8.7
1/2 Cystine	2.0	1.8	1.9	1.3	1.6	1.7	2.4	2.4
Valine	7.2	7.5	7.6	6.4	6.8	7.8	8.9	7.9
Methionine	1.4	0.6	0.7	0.5	0.6	0.8	1.3	1.3
Isoleucine	3.0	3.3	2.0	2.2	2.5	3.1	3.0	3.0
Leucine	9.9	7.5	7.5	5.2	7.6	9.3	7.7	6.7
Tyrosine	1.7	1.2	1.0	1.0	1.8	2.8	2.7	3.5
Phenylalanine	4.0	3.4	2.6	2.8	2.4	2.5	3.5	3.5
Tryptophan	1.4	ND	1.1	ND	0.9	ND	1.2	ND
γ -Carboxyglutamic Acid	3.4	0	2.1	0	2.0	0	0	0

Table 2 Carbohydrate Compositions of 4 Nephrocalcin Isoforms

Carbohydrate	A		B		C		D	
	N	P	N	P	N	P	N	P
Fucose	0.3	0.3	0.3	0.1	0.3	0.1	0.1	0.4
Galactose	3.2	3.2	2.5	3.7	2.2	1.3	3.7	0.7
Mannose	0.8	0.9	1.2	0.2	1.1	0.4	0.2	0.6
Glucose	0.6	0.6	1.2	0.3	0.1	0.2	0.3	0.9
Galactosamine	0.8	0.8	1.5	2.7	0.8	1.4	2.7	1.2
Glucosamine	0.4	0.4	1.0	0.4	0.8	0.1	0.4	1.2
<i>N</i> -Acetylneuraminic Acid	+	+	+	+	+	+	+	+
Total	6.1	6.2	10.3	7.4	5.3	3.5	7.7	5.0

A–D refer to fractions eluted from DEAE-cellulose; see Methods and legend to Table 1. N; normal subjects. P; patients. All values are weight percentage.

Fig 3 Langmuir adsorption coefficients. Values $\pm$ SEM for normals (N, crosshatch) and patients (P, open bars) for A – D isoforms.

Fig 4 Force area curves for A – D isoforms (Figs 4 A – D, respectively) from normals (●) and patients (○). Surface films of 135 μ g protein were compressed and expanded between 600 and 20 cm^2 at 2.2 cm^2/sec . Collapse pressures of normal A–C exceeded corresponding values for patient materials: 33.2 vs 27.8, 41.4 vs 17.6, and 36 vs 20.4; values for D were nearly the same: 34.2 vs 32.

that is normal NCs are less water soluble than stone formers NCs.

^{32}P -NMR spectrometer indicates that phosphate residues are present as phosphoserine, and a chemical shift indicates 4 Ca^{2+} atoms saturate one molecule of NC (Fig 5).

Circular dichroism (CD) spectrometer was used for investigation of conformational changes of these NCs by titrated with Ca^{2+} . Isomer A changed its conformation when titrating with Ca^{2+} , causing decreased alpha-helical structure and increased beta-sheet. Isomer C, an example of stone former's inhibitor, did not change its conformation upon adding Ca^{2+} (Fig 6).

We used electron paramagnetic resonance spectroscopy (EPR) and electron nuclear double resonance spectroscopy (ENDOR) to investigate metal binding mode of these NCs.²¹ Since Ca^{2+} is spectroscopically silent, we used vanadyl ion (VO^{2+}). Figure 7 shows the EPR spectra of VO^{2+} -NC-A complexes. An increase in signal amplitude was observed up to a VO^{2+} -NC-A molar ratio of 4:1. The $-3/2$ perpendicular resonance feature was plotted as a function of the VO^{2+} :NC-A ratio. The signal intensity increased up to a VO^{2+} :NC-A ratio of 4:1, and then remained constant. This indicates that the stoichiometry of VO^{2+} -binding to NC-A is 4:1 (Fig 8). A similar conclusion was observed by ^{32}P -NMR and equi-

librium dialysis of NC-A with ^{45}Ca . When VO^{2+} was added to Ca^{2+} saturated NC-A, no VO^{2+} bound to the protein. Thus we concluded that Ca^{2+} and VO^{2+} compete for the same calcium binding sites. We also analyzed EPR linewidths, and found that the linewidth of solvated vanadyl ion decreased 6 G (Gauss), but VO^{2+} -NC-A complexes remained unchanged. This indicates that in the VO^{2+} -NC-A complex no solvent molecule is directly coordinated to the VO^{2+} ion. A model of a poor inhibitor, NC-C, was used in the same manner as described above for NC-A. The binding of VO^{2+} to NC-C was competitive with Ca^{2+} , and metal protein binding stoichiometry was 4:1. Contrary to NC-A, the decrease in linewidth of the $-3/2$ perpendicular EPR upon introduction of D_2O was 2.37 G. This suggests that in the VO^{2+} -NC-C complex at least 1 or 2 water molecules are directly involved with the VO^{2+} ion as an equatorial ligand.

Localization of NC in Kidney Tissues

Polyclonal antibody against human NC was raised in rabbits using Freund's adjuvant, and boosted twice before

Fig 5 Effects of Ca^{2+} concentration on the ^{31}P NMR chemical shift.

Fig 6 Circular dichroic spectra of nephrocalcin in the absence and in the presence of Ca^{2+} . A (upper panel) shows NC-A: Free NC-A (---), and 4 Ca^{2+} atoms bound per 1 Na-A molecule (—). B (lower panel) shows NC-C: The same as above, Ca^{2+} free (---) and Ca^{2+} bound (—).

Fig 7 First derivative EPR spectra of VO^{2+} -NC complexes in frozen aqueous solution. The solutions were buffered to pH 5.8 with 0.03 M Pipes. The spectra were recorded under identical spectrometer settings with different NC: VO^{2+} molar ratios as indicated. The final NC concentration was 1 mM.

blood collection. The titer was 1:15,000. Using this antibody, slice of kidney tissue were double stained immuno-histochemically using Tamm-Horsfall glycoprotein as a marker. Darker staining patterns were observed along proximal tubules.

Proximal tubules were isolated from female mice kidneys by density gradient centrifugation using 45% Percoll, and cultured to confluency in serum-free culture

Fig 8 EPR spectrometric titration of the VO^{1+} ion complexed to NC-A isoform. The EPR signal intensities of $-3/2$ perpendicular line of VO^{2+} are plotted as a function of VO^{2+} :NC-A ratio.

medium for 7–9 days. The tissue culture media was collected every 3 days and stored for isolation of NC. Purified NC, 636 μg , was obtained from 1 g of protein in the tissue culture media. Although carbohydrate contents were higher than those found in human and rat NCs, amino acid compositions and dissociation constants were very similar to NCs isolated from human and other vertebrates (Table 3).

Nephrocalcin in Human Renal Carcinoma Cells

Since 85% of renal cell carcinoma develop in proximal tubule cells, renal cell carcinoma kidney tissue cells were immuno-histochemically stained. The amount of NC in urine of renal cell carcinoma patients (9 men and 10 women, age 40 to 71 years old, mean age 55 years) was increased as shown in Fig 9. Tumors were of the clear cell and granular cell varieties, moderately differentiated and of pathological stage $\text{T}_2\text{N}_0\text{M}_0$ with exception of 4 patients with stage T_3 disease but without local or distant metastases (Table 4). Urine was collected the day before and 5 to 90 days (mean 14 days) after radical nephrectomy. Four healthy adult biologically related kidney donors submitted a urine sample 1 day before and 5 days after

Table 3 Amino Acid Compositions of Nephrocalcins Isolated from Proximal Tubule Cell Cultured Medium

Amino Acid	DEAE Fraction, wt%				Human Urine NC (12)	Rat NC (11)	
	A	B	C	D		Kidney	Urine
Lysine	3.6	4.3	4.5	5.7	4	3	4
Histidine	3.5	2.1	3.1	4.0	2	2	2
Arginine	4.9	3.9	2.6	3.2	5	3	4
Aspartic Acid	12.3	12.1	12.1	11.8	12	12	12
Threonine	7.1	10.8	5.6	6.5	10	9	8
Serine	8.1	8.1	9.0	7.6	11	12	13
Glutamic Acid	15.4	13.0	12.0	12.6	13	16	18
Proline	8.1	8.5	4.7	6.7	6	7	6
Glycine	15.4	12.8	9.7	11.9	12	9	9
Alanine	14.6	13.1	14.0	11.0	8	6	7
Valine	7.9	6.6	5.8	6.8	7	7	7
Isoleucine	4.1	3.7	4.1	4.5	3	4	4
Leucine	9.7	5.9	6.1	7.9	7	8	8
Tyrosine	1.3	0.6	0.8	1.7	1	2	2
Phenylalanine	3.4	2.4	3.6	4.5	3	4	3
γ -Glutamic Acid	1.7	0.3	0.03	0	2	2	1

Table 4 Status of 19 Patients with Renal Cell Car Inoma

Patient	Age/Sex	Stage	Grade	Cell Type (Clear, Granular)	ug NC/mg Creatinine	
					pre-op	post-op
	58 F		2	Cl	.077	.108
	41 F	T _{3a} N ₀ M ₀	2	Gr	.464	.243
	45 F	T ₂ N ₀ M ₀	3	Gr	1.432	.255
	68 F	T _{3b} N ₀ M ₀	1	Cl	.158	.031
	49 F	T ₂ N _x M ₀	2	Cl	.258	.187
	62 M	T _{3b} N _x M ₀	2	Cl	.153	.176
	66 M	T ₂ N _x M ₀	2	Gr	.065	.013
	42 F	T ₂ N ₀ M ₀	2	Cl	.068	.064
	40 M	T ₂ N _x M ₀	2	Cl	.066	.030
	67 F	T ₂ N ₀ M ₀	2	Gr	.012	.079
	65 M	T ₂ N _x M ₀	1	Cl	.030	.030
	61 M	T ₂ N ₀ M ₀	2	Gr	.060	.037
	63 M	T ₂ N _x M ₀	2	Cl	.049	.039
	70 F	T ₂ N ₀ M ₀	1	Cl	.454	.320
	60 M	T ₂ N ₀ M ₀	2	Cl	.086	.038
	41 M	T ₁ N ₀ M ₀	2	Gr	.026	.118
	43 M	T ₃ N _x M ₀	2	Cl	.125	.173
	62 F	T ₂ N _x M ₀	1	Gr	.681	.372
	71 F	T ₂ N ₀ M ₀	2	Cl	.288	.082
		T ₂ N ₀ M ₀				

transplant nephrectomy to determine the effect of non-tumor nephrectomy on excretion of nephrocalcins. The mean level of NC in 19 renal cell carcinoma patients was $0.241 \pm 0.341 \mu\text{g}$ per mg creatinine compared to $0.022 \pm 0.012 \mu\text{g}$ per mg creatinine for normal controls

($p = 0.0112$). Mean NC after tumor nephrectomy was (0.097 ± 0.087) μg per mg creatinine. Chemical and physicochemical properties of purified NC isolated from urine of these patients were similar to those of previously described NCs.

Fig 9 Changes of NC levels of 19 renal cell carcinoma patients before and after nephrectomy.

Discussion

I have described chemical and physico-chemical properties between 4 isoforms of NC molecules. Good inhibitors, isoforms A and B, have strong amphiphilicities, stronger binding capacities toward CaOx, change conformation upon binding with Ca^{2+} , and bind metal-proteins without the involvement of water molecules. In contrary, poor inhibitors, isoforms C and D, are less amphiphilic, have no conformational change upon binding with CaOx,

demonstrate less potential binding toward CaOx, and require water molecules for met binding. We have yet determined the primary sequence of NC, and currently it is impossible to demonstrate an exact inhibition mechanism. However, a model can be hypothesized: A good inhibitor, isoform A or B, changes its conformation upon binding Ca^{2+} and becomes ellipsoidal or rod-shaped with one side being hydrophobic and the other hydrophilic. The hydrophilic side of the inhibitor attaches at the crystal growth point of the CaOx crystal, and the hydrophobic side of the molecule faces outward which prevents further crystal growth. However, a poor inhibitor, isoform C or D, does not change its conformation upon binding to Ca^{2+} atoms, thus the molecule remains bulky, possibly globular with hydrophilic and hydrophobic sites scattered over the surface of the molecule. Although this molecule attaches at the crystal growth point of CaOx, it is not able to prevent crystal growth, since hydrophilic sites are on the surface of the molecule. Additional CaOx molecules can attach to the surface of the poor inhibitor causing crystal growth to continue.

Crystal growth inhibitors and promoters have been previously suggested; however, we propose that crystal growth inhibitors and promoters could essentially have the same origin. During evolutionary processes, genetic mutations resulted in the replacement of certain amino acid residues, producing a "good" inhibitor and a "poor" inhibitor which could in fact be a promoter.

NC is produced in proximal tubule cells, and it has been found that NC production increases with disease progression, and that the majority of patients return to normal NC levels after nephrectomy. This supports the idea that proximal tubule cells are responsible for production of NC molecules.

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References

1. Grover PK, Marshall VR, Ryall RL: The promotion of calcium oxalate crystallization by urate: can it be that epitaxy is not the cause after all? In: Ryall RL, ed, *Urolithiasis 2*, New York, Plenum Press, 1992; 201-203
2. Nancollas GH, Smesko SA, Campbell AA, Coyl-Rees M, Ebrahimpour A, Binette M, Binette JP: Mineralization inhibitors and promoters. In: Walker VR, ed, *Urolithiasis*, New York, Plenum Press, 1989; 83-90
3. Sela J, Schwartz Z, Swain LD, Boyan BD: Role of matrix vesicles in calcification. In: Bonucci E, ed, *Calcification in Biological Systems*, Chapter 5, Boca Raton, CRC Press, 1992; 73-105
4. Linde A: Dentin matrix proteins: composition and possible functions in calcification. *Anat Rec* 1989; 224: 154-166
5. Addadi L, Weiner S: Interactions between acidic proteins and crystals: stereochemical requirements in biomineralization. *Proc Natl Acad Sci USA* 1985; 82: 4110-4114

6. Iwata H, Kamei O, Abe Y, Nishio S, Wakatsuki A, Ochi K, Takeuchi M: The organic matrix of urinary uric acid crystals. *J Urol* 1988; 139: 607-610
7. Nakagawa Y, Ahmed M, Hall SL, Deganello S, Coe FL: Isolation from human calcium oxalate renal stones of nephrocalcin, a glycoprotein inhibitor of calcium oxalate crystal growth. Evidence that nephrocalcin from patients with calcium oxalate nephrolithiasis is deficient in gamma-carboxyglutamic acid. *J Clin Invest* 1987; 79: 1782-1787
8. Maxfield M: Urinary mucopolysaccharides. *Ann Rev Med* 1995; 14: 99-110
9. Umekawa T, Kohri K, Amasaki N, Yamate T, Yoshida K, Yamamoto K, Suzuki Y, Sinohara H, Kurita T: Sequencing of a urinary stone protein, identical to alpha-one antitrypsin, which lacks 22 amino acids. *Biochem Biophys Res Commun* 1993; 193: 1049-1053
10. Rodgers A: Nucleation and growth inhibitor of calcium oxalate crystallization. In: Walker VR, eds, *Urolithiasis*, New York, Plenum Press, 1989, 189-191
11. Doyle IR, Ryall RL, Marshall VR: Inclusion of proteins into calcium oxalate crystals precipitated from human urine: a highly selective phenomenon. *Clin Chem* 1991; 37: 1589-1594
12. Stapleton AM, Ryall RL: Blood coagulation proteins and urolithiasis are linked: crystal matrix protein is the F_I activation peptide of human prothrombin. *Br J Urol* 1995; 75: 712-719
13. Ito H, Coe FL: Acidic peptides and polyribonucleotides crystal growth inhibitors in human urine. *Am J Physiol* 1977; 233: F455-F463
14. Morse RM, Resnick MI: A new approach to the study of urinary macromolecules as a participant in calcium oxalate crystallization. *J Urol* 1988; 139: 869-873
15. Dussol B, Geider S, Lilova A, Leonetti F, Dupuy P, Daudon M, Berland Y, Dagorn JC, Verdier JM: Analysis of the soluble organic matrix of five morphologically different kidney stones. Evidence for a specific role of albumin in the constitution of the stone protein matrix. *Urol Res* 1995; 23: 45-51
16. Fraij BM: Separation and identification of urinary proteins and stone-matrix proteins by mini-slab sodium dodecyl sulfate-polyacrylamide gel electrophoresis. *Clin Chem* 1989; 35: 658-662
17. Shiraga H, Min W, VanDusen WJ, Clayman MD, Miner D, Terrell CH, Sherbotie JR, Foreman JW, Przysiecki C, Neilson EG, Hoyer JR: Inhibition of calcium oxalate crystal growth *in vitro* by uropontin: another member of the aspartic acid-rich protein superfamily. *Proc Natl Acad Sci USA* 1992; 89: 426-430
18. Nakagawa Y, Renz CL, Ahmed M, Coe FL: Isolation of nephrocalcin from kidney tissue of nine vertebrate species. *Am J Physiol* 1991; 260(Pt 2): F243-F248
19. Nakagawa Y, Otsuki T, Coe FL: Elucidation of multiple forms of nephrocalcin by ³¹P-NMR spectrometer. *FEBS Lett* 1989; 250: 187-190
20. deKernion JB, Beldegrun A: Renal tumors. In: Walsh PC, Retik AB, Stamey TA, Vaughan ED Jr, eds, *Campbell's Urology*, 6th Ed, Vol 2, Chapter 27, Philadelphia, W. B. Saunders, 1992; 1053-1093
21. Mustafi D, Nakagawa Y: Characterization of calcium-binding sites in the kidney stone inhibitor glycoprotein nephrocalcin with vanadyl ions: electron paramagnetic resonance and electron nuclear double resonance spectroscopy. *Proc Natl Acad Sci USA* 1994; 91: 11323-11327