Pin1 and secondary hyperparathyroidism of chronic kidney disease: gene polymorphisms and protein levels

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ABSTRACT

Background: Peptidyl-prolyl cis/trans isomerase NIMA-interacting 1 (Pin1) is a key regulator of PTH mRNA stability. Secondary hyperparathyroidism (SHPT), which is characterized by elevated serum PTH levels, is a common complication of CKD. We investigated the possible associations between CKD with SHPT (CKD SHPT) and single-nucleotide polymorphisms of the Pin1 gene and compared the levels of the Pin1 protein in the CKD SHPT patients with those of the controls.

Methods: The study group included 251 CKD SHPT patients and 61 controls. One putative functional SNP (single nucleotide polymorphism) in the Pin1 promoter (rs2233679C>T: c.-667C>T) is the main object. Genotyping was performed on purified DNA using polymerase chain reaction-restriction (PCR) and restriction fragment length polymorphisms (RFLP). The levels of Pin1 were measured in serum using an enzyme-linked immunosorbent assay.

Results: Genotyping showed that CT+TT in the Pin1 promoter was significantly more common in the CKD SHPT group than in the control group (p<.05). The correlation analysis demonstrated that a significant difference in the C to T transition in the Pin1 promoter contributed to CKD SHPT (x²=12.47, p<.05; Odds ratios (OR)=1.26, 95% confidence (CI) intervals=1.06–1.49). The multivariate logistic regression analysis reported that the OR and 95% CI were 12.693 and 2.029–75.819 (p<.05), respectively, in the Pin1 gene promoter /C0>667T variant genotypes (CT+TT) after adjusting for other factors, and those values in Pin1 were 0.310 and 0.122–0.792 (p<.05).

Conclusion: The /C0>667T genetic variants in the Pin1 promoter contribute to an increased risk of CKD SHPT and may be biomarkers of susceptibility to CKD SHPT.

Introduction

Secondary hyperparathyroidism (SHPT) with elevated serum intact parathyroid hormone (iPTH) is a major complication of chronic kidney disease (CKD). Longstanding SHPT results in bone disease, vascular calcification, and mortality.1 Cardiovascular disease accounts for approximately 45% of the deaths in patients with stage 5 CKD.2 Several observational studies have demonstrated an increased mortality risk with elevated serum iPTH levels in dialysis patients.3-5 Drug therapy with 1, 25-dihydroxyvitamin D3 and calcium salts has been reported to slow the progression of SHPT,6,7 improve bone mineral disease (BMD),8 and reverse abnormal bone histology.7,9,10 However, SHPT may ultimately progress to an advanced stage that is refractory to medical treatment and requires surgical management by parathyroidectomy. Thus, understanding the mechanisms by which PTH synthesis and secretion is very important in developing methods to regulate the overactivity and hyperplasia of the parathyroid gland after the onset of renal insufficiency.

Peptidyl-prolyl cis–trans isomerase NIMA-interacting-1 (Pin1) belongs to the evolutionarily-conserved peptidyl-prolyl isomerase family of proteins.11 A specific catalytic Ser/Thr-pro amino acid motif regulates the cell cycle protein conformation and ultimately affects cell proliferation and differentiation, thereby changing the biological activity, phosphorylation, and turnover of its target proteins.12,13 Pin1-induced conformational changes may function as a critical catalyst that potentiates multiple oncogenic signaling
Recent studies have shown that Pin1 is a key regulator of PTH mRNA stability.\(^{21-23}\) When Pin1 is reduced or its activity is decreased, PTH mRNA stability is increased and degradation is decreased, resulting in elevated levels of iPTH. Nechama et al.\(^{22}\) show that Pin1 activity is decreased in parathyroid extracts from rats with CKD and the stability and levels of PTH mRNA are increased. In transfected cells, the PTH mRNA level was decreased due to Pin1 overexpression, increased by Pin1 knock-down.\(^{22}\) The authors also reported that Pin1\(^{\text{+/+}}\) mice had higher serum PTH and PTH mRNA levels, indicating that Pin1 determines basal PTH expression in vivo. Additionally, Pin1 inhibition alone increases serum PTH and PTH mRNA levels in the rat. Currently, there are no reports on Pin1 gene polymorphisms and Pin1 serum levels in CKD SHPT patients. This study aims to explore the correlation of Pin1 gene single nucleotide polymorphism (SNP) to CKD SHPT in the Chinese Han population in Northwest China.

The human Pin1 gene (NC_000019.8) spans over \(\sim14\text{ kb}\) on chromosome 19b13, contains four exons, encodes a 163-amino acid protein, and has a promoter region of 1.5 kb. One putative functional SNP in the Pin1 promoter (rs2233679C > T) has been reported\(^{24,25}\) and has been submitted to the Pin1 locus-specific database (www.LOVD.nl/Pin1). Recently, one study investigated the role of Pin1 rs2233679 –667C > T SNP in the etiology of hepatocellular carcinoma (HCC) and found that the –667T allele may contribute to the risk of HCC.\(^{26}\) Therefore, we hypothesized that the –667 SNP in the promoter of Pin1 gene may be associated with CKD SHPT.

### Materials and methods

We used the K/DOQI-Clinical Practice Guidelines for the definition of SHPT. The target range of iPTh was 10–69 pg/mL. The levels of iPTh in the various stages of CKD were greater than the target values. The iPTh levels surpassed 70 pg/mL in stage 3 CKD (eGFR 30–59 mL/min/1.73 m\(^2\)), exceeded 110 pg/mL in stage 4 CKD (eGFR 15–29 mL/min/1.73 m\(^2\)) and crossed 300 pg/mL in stage 5 CKD (eGFR <15 mL/min/1.73 m\(^2\)).\(^{27}\)

The study population comprised 252 non-dialysis CKD patients with SHPT, which was diagnosed by the K/DOQI Guidelines at the Second Hospital Affiliated of Lanzhou University Nephrology Unit from January 2012 to December 2012 and 61 age- and sex-matched healthy volunteers. The average age of the patients and controls, respectively, were 47.48 ± 16.04 and 47.46 ± 13.12. Male and female were 138 and 114 in CKD SHPT group, those in healthy group were 31 and 30. The exclusion criteria were an age < 18 years, presence of acute renal injury, infection, cancer, hepatitis, cirrhosis or other liver dysfunction, primary hyperparathyroidism, pregnancy or childbirth, or oral calcium supplementation, phosphorus binders or any vitamin D analogs. The local ethics committee approved the study, and the patients or their guardians provided informed consent.

Blood samples were drawn by venipuncture in plain tubes. Immediately after the blood draw, each blood sample was placed in an EDTA tube for DNA and protein experiments. Genomic DNA was extracted using UNIQ-10 column blood genomic DNA extraction kits (Solarbio Biotech Inc., Shanghai, China) according to the manufacturer’s instructions. Serum creatinine (Scr), serum calcium (Ca), and serum phosphorus (Pi) were detected using an automatic biochemical analyzer (Beckman Coulter Inc., Brea, CA) from our hospital biochemical room.

### SNP genotyping

The –667C > T genotype was detected by the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method. The polymorphic regions of –667C > T were amplified using the following primer sequences: forward 5’-CGGGCCT GTG ACT CTA TT-3’ and reverse 5’-AAA TTT GGCTCC TCC ATC CT-3’. These primers and the enzyme Sac1 (New England BioLabs, Ipswich, MA) were used to identify the –667C > T genotypes.\(^{24}\) The –667CC genotype produced one band (296 bp); the –667TT genotype produced two bands (213 and 83 bp); and the heterozygotes displayed all three bands (296, 213, and 83 bp). PCR amplification was performed in a 20 μL reaction volume. After the initial denaturation at 94°C for 5 min, there were 37 cycles at 94°C for 45 s, 55°C for 45 s, and 72°C for 45 s, and then a final extension step of 72°C for 7 min. The DNA was substituted with sterile deionized water for the negative control. The PCR products were resolved on 1.5% agarose gel and identified by ethidium bromide staining. For the genotyping of the –667C > T polymorphisms, 10 μL of the PCR products was digested for 16 h at 37°C with 1 μL of Sac1 (New England BioLabs, Beverly, MA) in separate tubes. The cleaved products were separated on 3% agarose gel as previously described. We also randomly selected 60 samples for sequencing, and the results were 100% concordant.
**Determination of the level of Pin1 and iPTH**

The levels of serum Pin1 were determined using specific enzyme-linked immunosorbent assay (ELISA) kits (Cusabio Biotech Inc., Wuhan, China). iPTH was measured using a radioimmunoassay that was completed by the Department of Nuclear Medicine in our hospital.

**Statistical analysis**

SPSS (version 17.0, SPSS Inc., Chicago, IL) was used to perform the statistical analysis. The chi-square test was used to compare between the groups of categorical variables and test for the Hardy–Weinberg equilibrium of the SNP in the controls. The Mantel–Haenszel was used to determine the correlation between the genotypes or alleles of the −667C>T polymorphisms and CKD SHPT. The Spearman correlation analysis was used to evaluate the main effect of the −667C>T genotypes or alleles in the Pin1 promoter on the serum Pin1 and iPTH. A univariate logistic regression model was used to determine the correlation between the genotype frequencies or alleles in the Pin1 promoter on the serum Pin1 and CKD SHPT. A multivariable logistic regression analysis was performed to determine the association between the genotype frequencies and CKD SHPT with an adjustment for other factors, such as age, gender. The results were considered statistically significant when \( p < 0.05 \).

**Results**

**Characteristics of the study population**

The distributions of demographic variables and risk factors of the two study groups are listed in Table 1.

| Variables               | CKD SHPT | Control |
|-------------------------|----------|---------|
| Cases                   | 252      | 61      |
| Age (years)             | 47.48 ± 16.04 | 47.46 ± 13.12 |
| Male/female             | 138/114  | 31/30   |
| eGFR (mL/min/1.73 m²)   | 51.26 ± 8.91* | 118.79 ± 22.82 |
| Scr (µmol/L)            | 174 (82.25–449.25)* | 61 (59.5–62) |
| Ca (mmol/L)             | 2.06 ± 0.26* | 2.31 ± 0.34 |
| Pi (mmol/L)             | 1.52 ± 0.52* | 1.01 ± 0.35 |
| iPTH (pg/mL)            | 386.67 (256.45–637.75)* | 32.7 (22.55–44.10) |
| Pin1 (ng/mL)            | 16.94 ± 2.37* | 25.76 ± 2.47 |

*\( p < 0.05 \), compared with the controls.

No significant deviation was observed in the distributions for the age and sex between the CKD SHPT group and the controls (\( p > 0.05 \)). There were significant differences in eGFR, Scr, Ca, and Pi between the groups (\( p < 0.05 \) for all).

**Genotyping of the Pin1 promoter −667C>T**

The genotype and allele frequencies of the Pin1 promoter −667C>T SNP are presented in Table 2. The observed genotype frequencies of the Pin1 promoter −667C>T were all in agreement with the Hardy–Weinberg equilibrium in the control subjects (\( p > 0.05 \) for all). The CC genotype was 11.51% of the CKD SHPT group and 29.51% of the controls. Though the CT + TT genotype frequency in the CKD SHPT and control groups was 88.98% and 70.50%, respectively, there were significantly more CC homozygotes in the control group. The correlation analysis showed that a significant difference in the C to T transition in Pin1 promoter contributed to CKD SHPT (\( \chi^2=12.47, p < 0.05; \text{OR} =1.26, 95\%CI =1.06–1.49 \)). In the CKD SHPT patients, the frequency for the T allele was 0.4921 compared with 0.3930 in the control group, and the difference was statistically significant (\( \chi^2=3.83, \text{\,OR} =0.65; 95\%\text{CI} \approx0.39–1.25 \)).

**Comparison of the different genotypes in CKD SHPT group**

For the 252 CKD SHPT patients, the frequencies of the CC genotype, CT genotype, and TT genotype were 29, 198, and 25, respectively. Age, sex, Scr, eGFR, Ca, and Pi were not significantly different between the genotype groups CC and CT + TT, but Pin1 and iPTH were significantly different (\( p < 0.05 \)) between the groups (Table 3).

**Correlation analysis**

As shown in Table 1, the level of Pin1 was significantly lower in the CKD SHPT group compared with the control group, but the level of iPTH was higher than the controls. The Spearman correlation analysis yielded a negative correlation between the levels of Pin1 and

| Group        | Number | CC        | CT + TT   | Allele frequency |
|--------------|--------|-----------|-----------|------------------|
| CKD SHPT     | 252    | 29 (11.51)| 223 (88.49)| \( \chi^2 \)     |
| Healthy      | 61     | 18 (29.51)| 43 (70.50)| \( p \) \text{ Value} |
| p Value      | –      | –         | 12.47     | 3.83              |
| OR Value     | –      | 0.000     | 0.84      | 0.050             |
| 95%CI        | –      | 0.23–0.65 | 0.06–1.49 | 0.71–0.99         |

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\( \chi^2 \) and \( p \) values calculated using the chi-square test and Fisher’s exact test, respectively.
iPTH in the patients \((r = -0.862, \ p < .05)\) but not in the controls \((r = -0.126, \ p > .05)\) in Table 4.

A significant negative correlation between Pin1 and the \(-667T\) variant genotypes \((CT + TT)\) was observed in the CKD SHPT patients \((r = -0.212, \ p < .05)\) but not in the controls \((r = -0.105, \ p > .05)\). However, there was a significant positive correlation between iPTH and the \(-667T\) variant genotypes in the CKD SHPT patients \((r = 0.143, \ p < .05)\) but not in the control group \((r = -0.047, \ p > .05)\) (Table 4).

**Analyzing risk factors for CKD SHPT**

CKD SHPT was the dependent variable, whereas genotype, age, gender, Scr, Ca, Pi, iPTH, and Pin1 were the independent variables for the univariate non-conditional logistic binary regression analysis. This analysis demonstrated that the genotype, eGFR, Ca, Pi, iPTH, and Pin1 were the dependent variables. The multivariate logistic regression analysis yielded an OR and 95% CI of 12.693 and 2.029 \((p < .05)\), respectively, in the Pin1 gene promoter \((-667T\) variant genotypes \((CT + TT)\) after an adjustment for other factors. The OR and 95% CI for Pin1 were 0.310 and 0.122–0.792 \((p < .05)\), respectively.

**Discussion**

In this case-control study, we found that the \(-667T\) variant genotypes \((CT + TT)\) were associated with CKD SHPT of the Chinese Han population in Northwest China. We found that the \(-667T\) variant genotypes \((CT + TT)\) in the Pin1 gene promoter may be a risk factor of CKD SHPT.

Pin1 is the only mammalian enzyme known to specifically catalyze the cis–trans isomerization of Ser/Thr-Pro peptide bonds.\(^{28,29}\) The fulfillment of the Pin1 functions depends on the conserved WW domains in Pin1, which are classified into five distinct groups.\(^{30–32}\) The WW domain on the Pin1 protein is classified as a class IV domain.\(^{31}\) It is the domain that specifically recognizes Ser/Thr-Pro motifs and regulates the conformation of pre-directed phosphorylation sites\(^{30}\) and the turnover rate of ARE-containing mRNAs.\(^{33}\) The effects of Pin1 are dependent on the PTH mRNA 3′-UTR ARE.\(^{34}\) The ARE in the 3′-UTR of mRNA-encoding PTH binds two proteins, K-homology splicing regulatory protein (KSRP) and AU-rich element-binding protein 1 (AUF1).\(^{35,36}\) Pin1 interacts with KSRP, and Pin1 overexpression leads to KSRP dephosphorylation, which determines KSRP-mediated PTH mRNA decay.\(^{22,23}\) Pin1 interacts with AUF1 which increases mRNA half-life and stabilizes mRNA-encoding.\(^{33,37}\) Based on this role, Pin1 was confirmed as a key protein for the PTH mRNA-regulating protein. Here, we demonstrated that the \(-667C > T\) SNP can influence the expression of Pin1 and thus increase the risk of CKD SHPT.

This study used an integrated approach to explore the contribution of the Pin1 promoter \(-667C > T\) SNP to the development of CKD SHPT in the Chinese Han population in Northwest China (genotyping and determination of protein level). We investigated SNPs in the Pin1 promoter \(-667\) site, the level of serum Pin1, and the correlation of both to the development of CKD SHPT.

**Table 3.** Comparison of the differences of genotypes in the CKD SHPT group \([x \pm s\text{ or } M\text{ (interquartile range)}\).]

| Parameter | CKD SHPT group |
|-----------|----------------|
|           | CC genotype | CT + TT genotype |
| Cases     | 29           | 223              |
| Age (years) | 43.83 ± 15.46 | 47.96 ± 16.09 |
| Male/female | 13/16       | 125/98          |
| eGFR (mL/min/1.73 m\(^2\)) | 52.34 ± 49.23 | 47.96 ± 40.71 |
| Scr (μmol/L) | 75 (62–548) | 185 (89–427) |
| Ca (mmol/L) | 2.05 ± 0.23 | 2.06 ± 0.26    |
| Pi (mmol/L) | 1.53 ± 0.65 | 1.51 ± 0.50    |
| iPTH (pg/mL) | 260.09 (110–397.76) | 385 (264–613.5) |
| Pin1 (ng/mL) | 22.35 ± 2.57 | 14.19 ± 2.49 |

\(*p < .05\), comparing with the CC genotype.

**Table 4.** Spearman correlation analysis between Pin1, iPTH, and \(-667T\) variant genotypes in the CKD SHPT patients.

| Pin1 | iPTH | \(-667T\) variant genotypes (CT + TT) |
|------|------|-------------------------------------|
|      |      | \(r = -0.862, \ p < .05\)          |
|      |      | \(r = -0.212, \ p < .05\)          |
|      |      | \(r = 0.143, \ p < .05\)           |

**Table 5.** Logistic binary regression analysis of risk factors in CKD SHPT.

| Independent variables | \(B\text{ value}\) | \(Wald\text{ value}\) | OR value | 95% CI  |
|-----------------------|------------------|---------------------|----------|--------|
| \(-667T\) variant genotypes (CT + TT) | 1.169 | 11.604 | 3.219 | 1.643–6.307 |
| eGFR (mL/min/1.73 m\(^2\)) | -0.071 | 38.108 | 0.932 | 0.911–0.953 |
| Scr (μmol/L) | -5.127 | 32.517 | 0.006 | 0.001–0.035 |
| Ca (mmol/L) | 4.044  | 30.790 | 57.030 | 13.67–237.9 |
| Pi (mmol/L) | 0.013  | 24.703 | 1.013 | 1.008–1.019 |
| iPTH (pg/mL) | -0.405 | 48.184 | 0.667 | 0.595–0.748 |
SHPT. Polymorphisms in the Pin1 promoter −667C>T may contribute to increased susceptibility to CKD SHPT and could influence the expression of the gene product.

One significant finding was an association between the pathogenesis of CKD SHPT and the Pin1 promoter region −667C>T polymorphism. There are two alleles C and T and three genotypes (CC, CT, and TT) in the Pin1 gene promoter region −667 locus in the Chinese Han population in Northwest China. Our study found that the heterozygous CT genotype is more common in the CKD SHPT patients and healthy controls whom we studied, with frequencies of 78.75% and 62.30%, respectively. The frequencies of the homozygous CC genotype are 11.51% and 29.5% for CKD SHPT patients and healthy controls, respectively. The frequencies of the homozygous CC genotype accounted for only 9.92% and 8.2% in the CKD SHPT patients and the controls, respectively. However, an Italian study investigated the association between the Pin1 promoter SNPs and the risk of HCC in 228 patients and 250 controls. In the controls, the genotype frequencies were 13% for CC, 45% for CT, and 42% for TT. In another study on Pin1 polymorphisms and the risk for Alzheimer’s disease in a French population, the −667C>T genotype frequencies in 655 healthy control subjects were 11% for CC, 42% for CT, and 47% for TT. The discrepancy between these studies in the genotype frequency distribution may be due to the different alleles that are involved in different diseases, study sizes or ethnic admixtures.

The frequency of the −667T variant genotype (CT + TT) in the CKD SHPT group and the controls were 88.49% and 70.5%, respectively. The correlation analysis showed a significant difference between the two groups (OR = 1.26, p < .05) (Table 2). The T allele frequency was higher in the CKD SHPT patients than in the healthy controls (49.21% and 39.30%, respectively), and the Mantel–Haenszel test demonstrated an OR of 1.25 (p ≤ .05) (Table 2). In addition, the C allele frequency (50.79/60.66) was higher than T allele (49.21/39.34) for the CKD SHPT patients and controls. This result is in line with Lu et al., who observed that the Chinese population C allele frequency was higher than that of the T allele. These data indicate that the Pin1 gene promoter −667C>T polymorphism may be associated with the susceptibility to CKD SHPT.

Although it has been reported that Pin1 activity is reduced in rats with CKD, there have been no studies on the Pin1 levels in the human CKD population. This paper is first to show the possible ranges of Pin1 levels in CKD SHPT patients and healthy controls. We detected the Pin1 concentrations in CKD SHPT patients and healthy controls by ELISA and found that the Pin1 levels in patients with CKD SHPT (16.94 ± 2.3 ng/mL) were significantly lower than those of the healthy controls (25.76 ± 2.47 ng/mL) (p < .05), which coincides with the levels found in the animal study. The levels of Pin1 were significantly lower in the −667T variant genotype (CT + TT) group than in the CC genotype group among the CKD SHPT patients (p < .05) (Table 3), indicating that this site may affect the expression of Pin1. This lower Pin1 level may be the governing factor in promoting CKD SHPT in individuals carrying the T allele. Studies in a large population are essential to confirm this hypothesis.

In contrast with Pin1, the level of ipPTH was higher in the CKD SHPT patients and the −667T variant genotype (CT + TT) group of the CKD SHPT patients than in the controls and the CC genotype group of the CKD SHPT patients. The Spearman correlation analysis demonstrated a significant negative correlation between the levels of Pin1 and iPTH in the patients (r = −0.862, p < .05) but no significant correlation in the controls (r = −0.126, p > .05) (Table 4). Using the Spearman correlation coefficient, a negative correlation was obtained between the level of Pin1 with the −667T variant genotypes (CT + TT) in the CKD SHPT patients (r = −0.212, p < .05) but not in the controls (r = −0.105, p > .05). However, the level of iPTH was positively associated with the −667T variant genotypes in CKD SHPT patients (r = 0.143, p < .05) but was not in the control group (r = −0.047, p > .05). Therefore, the observed correlation between the levels of Pin1 and iPTH with the −667T variant genotypes (CT + TT) adds to the validity of the results of this study.

In addition to the association analyses, the logistic regression analysis further confirmed that the −667T variant genotypes (CT + TT) may be a risk factor (OR = 12.693, p < .05) for CKD SHPT and Pin1 may be a protective factor (OR = 0.310, p < .05) for CKD SHPT in the Chinese Han population in Northwest China.

Although our finding of an association between CKD SHPT susceptibility and the −667T variant of the Pin1 promoter region is novel and this is the first study in which serum Pin1 has been evaluated, this study had some limitations. First, this is a case-controlled study which serum Pin1 has been evaluated, this study had some limitations. Second, we did not have the opportunity to examine Pin1 mRNA and PTH mRNA levels in study subjects with the different −667C>T genotypes, and we did not know the exact mechanism of the −667C>T variant regulation of the Pin1 transcript activity. Third, because we did not have reported values for the range of human serum Pin1 levels in CKD SHPT and control
patients, the ELISA method may not have been the correct assay to use.

In conclusion, our data suggest that the −667T genetic variants in the Pin1 promoter contribute to an increased risk of CKD SHPT and that these variants may be biomarkers for susceptibility to CKD SHPT. Serum Pin1 may be a protective factor for CKD SHPT. Validation with larger population-based studies in different ethnic groups is warranted to validate our findings.

Disclosure statement

The authors have declared that no competing interests exist.

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