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Arginine form of p21 gene codon 31 is less prominent in patients with calcium oxalate stone

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Abstract The formation of urinary stones is associated with cell death in response to various injuries. P21 (WAF1/CIP1) is a downstream protein of P53 and can arrest the cell cycle at G1/S with resulting cell death. We aimed to investigate the polymorphism of p21 gene codon 31 as the genetic marker in searching for the association of urolithiasis. One hundred and nineteen healthy controls and 95 patients with calcium oxalate stone were examined in this study. The polymorphism was seen from the result of polymerase chain reaction-based restriction analysis. The result revealed significant differences between normal individuals and stone patients ($P < 0.05$) and the distribution of arginine homozygote in the control group (31.9%) was higher than in the patient group (16.8%). It is concluded that polymorphisms of p21 codon 31 can be a genetic marker for urinary stone disease. Individuals possessing arginine form of p21 codon 31 have less risk of developing calcium stone disease.

Key words p21 (WAF1/CIP1) codon 31 polymorphism · Urolithiasis · Single nucleotide polymorphisms (SNPs)

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Introduction

Urolithiasis is a multifactorial and complex disease that is commonly seen in urological patients. Although the genetic causes have been studied extensively, no chromosomal mapping has been achieved in stone patients with idiopathic hypercalciuria [3]. The only conclusive comment through genetic studies, made by Resnick and coworkers, is that urolithiasis is a polygenic defect and partially penetrative [13]. Recently, single nucleotide polymorphisms (SNPs) were used as a tool for mapping the complex disease genes and making it possible to search the candidate genes for the causes of stone disease. Furthermore, a hypothetical connection was noted between apoptosis and stone disease [11]. Therefore, a candidate genetic marker can choose the genes related to cell injury or cell death.

P21 (WAF1/CIP1) is a cyclin-dependent kinase inhibitor (CDKI) that regulates the cell cycle [16]. Its ability to inhibit cyclin-cdk complexes resulting in G1 cell cycle arrest is regulated by wild-type tumor-suppressor protein p53. Alterations in p21 could interrupt the p53-mediated pathway of cell cycle arrest and influence the progression of cell death in response to cell injury. However, mutations on p21 are rare and multiple polymorphisms have been reported [1, 7, 10]. The most frequently seen gene polymorphism is codon 31, with a base change from AGC to AGA and amino acid changes from serine to arginine. The allelic frequencies were 91% to 9% for serine to arginine, respectively [7]. There are few reports of the p21 codon 31 polymorphisms related to urolithiasis, and

questions thus arise concerning the distribution of p21 gene codon 31 polymorphism in patients with calcium oxalate stones [5]. We used polymerase chain reaction (PCR)-based restricting analysis to investigate the distribution between the control group and stone patients.

Patients and methods

Patient selection

A total of 95 patients (70 males and 25 females) with calcium oxalate stone were enrolled in this study, aged between 24 and 73 years (average age: 44.2). Serial blood and urine biochemistry tests were undertaken to exclude possible hypercalcemia, hyperuricemia, or hyperuricosuria. Patients who showed symptoms of urinary tract infections during the period of stone treatment were excluded. Stone composition was verified by infrared spectroscopy and revealed calcium oxalate monohydrate or dihydrate, or a combination of the two. A control group was drawn up of 119 healthy volunteers over the age of 40, who had no history of familial stone disease and no renal calcification (following renal ultrasonography tests); routine tests were made in order to exclude any individuals who may have had hematuria. There were 42 males and 77 females in the control group (age range from 42 years to 73 years with an average of 55.5 years). Informed consent was obtained from both groups participating in the study. The genomic DNA was prepared from peripheral blood with the use of a DNA Extractor WB kit (Wako, Japan).

Polymerase chain reaction

Polymerase chain reactions (PCRs) were carried out to a total volume of 50 μl, containing genomic DNA, 2–6 *p*mol of each primer, 1× Taq polymerase buffer (1.5 mM $MgCl_2$), and 0.25 units of AmpliTaq DNA polymerase (Perkin Elmer, Foster City, USA). The primer for p21 codon 31 was designed from codon 1 start (5′-GTCAGAACCGGCTGGGGATG-3′) to codon 91 (5′-CTCCTCCCAACTCATCCC GG-3′), according to the procedure described by Li et al. [10]. PCR amplification was performed in a programmable thermal cycler GeneAmp PCR System 2400 (Perkin Elmer). The cycling condition for codon 31 was set as follows: one cycle at 94 °C for 5 min, 35 cycles at 94 °C for 30 s, 57 °C for 40 s, and 72 °C for 40 s, and one final cycle of extension at 72 °C for 7 min.

The PCR product of 272-bp was mixed with 2 units *Blp I* (New England Biolabs, Beverly, USA) and the reaction buffer, according to the manufacturer's instructions. The restriction site was designed to be located at the frequently seen allele of codon 31 (AGC) to form a site suitable for cutting. Two fragments of 89 bp and 183 bp will be present if the product can be cut. The reaction was incubated for 3 h at 37 °C. Then, 10 μl of the products was loaded into 3% agarose gel containing ethidium bromide for electrophoresis. The polymorphism was divided into sites suitable for cutting (CC homozygote), unsuitable for cutting (AA homozygote) and half-cut (C/A heterozygote). Statistical analysis of the allelic frequency distribution in this polymorphism in the control and stone patient groups was compared using the chi-square test. Results are considered statistically significant when the probability of findings occurring by chance is less than 5% ($P < 0.05$).

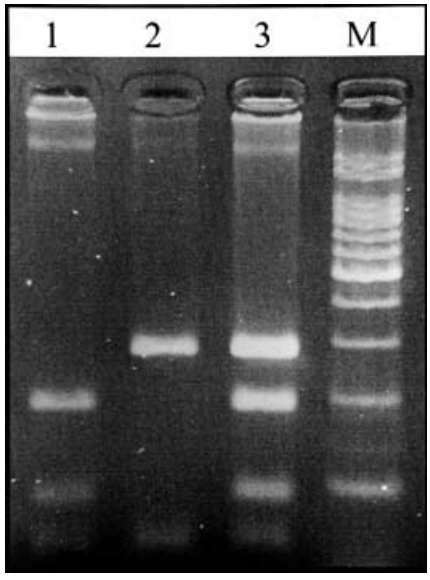

Fig. 1 PCR-base analysis of p21 codon 31 polymorphism. The polymorphic region was amplified by PCR which resulted in a cut fragment at lane 1 (89-bp and 183-bp), an uncut fragment at lane 2 (272 bp), and a heterozygous form at lane 3. M: marker (*lane 4*), 100-bp ladder

Results

Those bands showed on the gel revealing suitable for cutting (CC) or unsuitable for cutting (AA) homozygotes and heterozygotes (C/A) (Fig. 1). The frequencies of the genotype in the stone group and control group are shown in Table 1.

Using the chi-square test, the distribution of p21 codon 31 polymorphism was compared, showing significant differences between the healthy control group and the stone patient group ($P < 0.05$). The distribution in the control group was 18.6% serine homozygote (CC), 50.0% heterozygote (C/A) and 31.9% arginine homozygote (AA). The frequency of arginine allele in the patient group (16.8%) was lower than in the control group. The allelic distribution of C/A polymorphism at codon31 of p21 gene in normal healthy subjects shows C allele: 0.43 and A allele: 0.57. Whereas the stone patient shows C allele: 51.1 and A allele: 48.9. The odds ratio is 0.719 (95% confidence interval = 0.490 ~ 1.055). There was no association of p21 codon 31 polymorphism with

Table 1 Distribution of p21 codon 31 polymorphism between the healthy control subjects and the calcium oxalate stone patients by chi-square test

	CC	C/A	AA	Total	*P*-value
Control	21 (17.6%)	60 (50.4%)	38 (31.9%)	119 (100.0%)	0.03701
Stone patient	18 (18.9%)	61 (64.2%)	16 (16.8%)	95 (100.0%)	

$df = 2$, $\chi^2 = 6.59334$

calcium oxalate stone disease when comparing the distribution in both groups by sex (Tables 2, 3).

Discussion

The data indicate that p21 gene codon31 polymorphism might be a candidate for the genetic marker to screen the causes of calcium oxalate stone. An uncut band of the PCR product representing encoding amino acid of arginine was prominent in the control group. This distribution was different from that in the report by Koopmann et al.on brain tumor tissue, and 91% was serine in amino acid form [7]. The differences in distribution may be due to the ethnicity of the subjects. Owing to the lack of any previous reports on this polymorphism and a significantly lower percentage of arginine form, we suggest that it should be further investigated. However, the human p21 gene is localized to the chromosome 6p21.2 and not to a sex chromosome [7]; this may be the reason why there is no significant difference when considering gender differences.

Single nucleotide polymorphisms (SNPs) are the most abundant type of DNA sequence variation in the human genome [9]. SNP is a single base pair on the DNA which varies from person to person. It has gained increasing popularity as the genetic marker of choice in recent years for the study of complex genetic traits [2]. Based on SNPs, a population genetic approach provides a new way of identifying the genes associated with disease. A set of markers evenly distributed throughout the genome is needed to make an association study. Therefore, a subset of SNPs is functionally important in complex disease traits, such as stone disease.

Traditionally, mutation analysis was used in the monogenic disease. Urolithiasis is a complex disease and many genes were proposed for the possible cause of the disease. The use of linkage and association studies in the candidate gene have also played some role in searching for the causes of disease, but their ability to predict genetic variants is limited [6]. The SNP marker possesses the properties that fulfill the requirements of genetic approaches and provides the marker to be typed quickly, accurately, and inexpensively. SNPs analysis result in the definition of genomic regions linked to disease.

Urolithiasis is a multifactorial disease that involves the process of crystallization. Evidence shows that crystals interact with the tubular epithelium, which may lead to the retention and accumulation of crystalline material in the kidney and eventually to the formation of a renal stone. The most frequently associated phenomenon was tubular cell injury from a high oxalate load in cell culture [14]. Oxalate induces an increase in the production of free radicals and cell death that leads to crystal deposition in the renal tubules, and finally to the growth of calcium oxalate stones [8, 15]. As p21 is closely associated with cell death in the response to the p53 [4, 6], it was chosen as the SNPs marker and activation of the p21 gene results in the inhibition of cell cycle progression from the G1 to the S phase. The p21 gene encodes a 21 kDa protein with two protein binding domains, an N-terminal domain which binds and inhibits cyclin-cdk complexes, and a short sequence near the C-terminus (between amino acids 144–151) which binds to proliferating cell nuclear antigen (PCNA), resulting in the inhibition of DNA replication [1, 10]. This cell-cycle checkpoint prevents the replication of a damaged DNA template and permits time for repair. If damaged cells leak to the S phase, apoptosis of the cells is initiated. Although p21 may not be directly linked to stone disease, it is strongly associated with the cell death.

Searches for mutations within p21 sequence have been made in colorectal cancer, brain tumors, and esophageal cancers. However, instead of mutations, two different DNA variants were identified at codon31 and 91 [1, 7, 10]. As the polymorphism of condon 31 in p21 is more frequently distributed than codon 91 and the haplotype frequency is 18–8.5% from serial reports, this polymorphism is more suitable as the genetic marker for studying possible causes of urolithiasis through SNPs.

Table 2 Distribution of p21 codon 31 polymorphism between the healthy control subjects and the calcium oxalate stone patients by chi-squared test in female

	CC	C/A	AA	Total	*P*-value
Control	13 (16.9%)	40 (51.9%)	24 (31.2%)	77 (100.0%)	0.754
Stone patient	4 (16.0%)	15 (60.0%)	6 (24.0%)	25 (100.0%)	

$df = 2$, $\chi^2 = 0.56$

Table 3 Distribution of p21 codon 31 polymorphism between the healthy control subjects and the calcium oxalate stone patients by chi-square test in male

	CC	C/A	AA	Total	*P*-value
Control	8 (19.0%)	20 (47.6%)	14 (33.3%)	42 (100.0%)	0.052
Stone patient	14 (20.0%)	46 (65.7%)	10 (14.3%)	70 (100.0%)	

$df = 2$, $\chi^2 = 5.92$

This study might provide a method for the further study of disease genes in urolithiasis, with SNPs providing a future method for defining patients at risk from disease, assisting in the exact prognosis of a patient with a common disease, and leading on to the selection of a specific therapy based on specific genetic variations [12]. With the accumulation of mapped genes, the likelihood that these regions will contain a candidate gene is growing. In conclusion, polymorphism of p21 gene codon 31 is associated with calcium oxalate stone disease. The candidate genes might provide further analysis for the tissue expression or the clinical presentations in a variety of groups.

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References

1. Bahl R, Arora S, Nath N, Mathur M, Shukla NK, Ralhan R (2000) Novel polymorphism in p21 waf1/cip1 cyclin dependent kinase inhibitor gene: association with human esophageal cancer. Oncogene 19: 323
2. Collins FS, Guyer MS, Chakravarti A (1997) Variation on a theme: cataloging human DNA sequence variation. Science 278: 1580
3. Danpure CJ (2000) Genetic disorders and Urolithiasis. Urol Clin N Am 27: 287
4. el-Deiry WS, Tokino T, Waldman T, Oliner JD, Velculescu VE, Burrell M, Hill DE, Healy E, Rees JL, Hamilton SR (1995) Topological control of p21WAF1/CIP1 expression in normal and neoplastic tissues Cancer Res 55: 2910
5. Goodman HO, Brommage R, Assimos DG, Holmes RP (1997) Genes in idiopathic calcium oxalate stone disease. World J Urol 15: 186
6. Gujuluva CN, Baek JH, Shin KH, Cherrick HM, Park NH (1994) Effect of UV-irradiation on cell cycle, viability and the expression of p53, gadd153 and gadd45 genes in normal and HPV-immortalized human oral keratinocytes. Oncogene 9: 1819
7. Koopmann J, Maintz D, Schild S, Schramm J, Louis DN, Wiestler OD, von Deimling A (1995) Multiple polymorphisms, but no mutations, in the WAF1/CIP1 gene in human brain tumours. Br J Cancer 72: 1230
8. Kumar S, Sigmon D, Miller T, Carpenter B, Khan SR, Malhotra R, Scheid C, Menon M (1991) A new model nephrolithiasis involving tubular dysfunction injury. J Urol 146: 1384
9. Kwok PY, Gu Z (1999) Single nucleotide polymorphism libraries: why and how are we building them? Mol Med Today 5: 538
10. Li YJ, Laurent-Puig P, Salmon RJ, Thomas G, Hamelin R (1995) Polymorphisms and probable lack of mutation in the WAF1-CIP1 gene in colorectal cancer. Oncogene 10: 599
11. Lorder N (1999) Genetic variations can point the way to disease gene. Nature 401: 734
12. Pratt RE, Dzau VJ (1999) Genomics and hypertension. Concepts, potentials, and opportunities. Hypertension 33(part II): 238
13. Resnick MI, Pridgen DB, Goodman HO (1968) Genetic predisposition to formation of calcium oxalate renal calculi. N Engl J Med 2778: 131
14. Scheid C, Koul H, Hill WA, Luberz-Narod J, Kennington L, Honeman T, Fonassen J, Menon M (1996) Oxalate toxicity in LLC-Pk1 cells: role of free radicals. Kidney Int 49: 413
15. ThamilSelvan S, Hackett R, Khan SR (1997) Lipid peroxidation in ethylene glycol induced hyperoxaluria and calcium oxalate nephrolithiasis. J Urol 157: 1059
16. Xiong Y, Hannon GJ, Zhang H, Casso D, Kobayashi R, Beach D (1993) p21 is a universal inhibitor of cyclin kinases. Nature 366: 701