Relationship Between the P16 Gene Exon 3 C/G Polymorphism and Hydropyruvate Reductase (GRHPR) Gene Exon 6 G/A Polymorphism in Patients With Calcium Oxalate Stone: No Association

Hsi-Chin Wu, Fuu-Jen Tsai[1,2], Huey-Yi Chen[3], Hsueh-Fu Lu, Cheng-Der Hsu[1], Wen-Chi Chen

Department of Urology, [1]Department of Medical Genetics, [2]Department of Pediatrics, [3]Department of Obstetric and Gynecology, China Medical College Hospital, Taichung, Taiwan, R.O.C.

Background. Serial reports have shown a correlation between genetic polymorphisms and stone disease. Because the p16 protein is related to cell death, it is presumed to be associated with the function of renal cell death in response to oxalate overload. Hydroxypyruvate reductase (HPR) and glyoxylate reducatse (GR) (GRHPR) are enzymes involved in oxalate metabolism and are related to a congenital disorder of calcium oxalate stone formation. We therefore investigated the role these genes play in the formation of calcium oxalate stone disease.

Methods. A normal control group made up of 101 healthy people and 143 patients with recurrent calcium oxalate stone were examined in this study. The polymorphism was detected following the results of polymerase chain reaction based restriction analysis of *Msp* I ("C" allele is excisable) in the p16 gene (exon 3 C/G polymorphism). An exon 6 C/G polymorphism of the GRHPR gene was identified by *Bgl* II endonuclease ("G" allele is excisable).

Results. In the p16 gene *Msp* I polymorphism, the frequency of the C/G heterozygotes was 2.8% in the stone group and 5.9% in the control group. The frequency of the G/G allele was 97.2% and 99.1% in the stone and control groups respectively (p = 0.326, chi-square test). In the GRHPR gene *Bgl* II polymorphism, the frequency of the GG allele was over 98% in both groups (p = 1.0, Fisher's exact test). The frequency of GA heterozygotes was 2.0% in the control group and 1.4% in stone patients. The distribution showed no statistical difference between hypercalciuric and normocalciuric patients.

Conclusions. Neither the p16 gene *Msp* I G/C polymorphism nor the GRHPR *Bgl* II G/A polymorphism is associated with stone disease. **(Mid Taiwan J Med 2002;7:94-100)**

Key words

calcium oxalate urolithiasis, genetic polymorphism, hydroxypyruvate reductase, p16 gene

INTRODUCTION

For a number of years we have been using deoxynucleotide acid (DNA) polymorphisms as a tool for mapping the complex disease genes responsible for urolithiasis [1-3]. Single nucleotide polymorphisms (SNPs) are the most abundant form of DNA polymorphisms and are a powerful tool for the genetic study of complex human diseases [4]. SNPs obtained from a group of patients can be analyzed

Received : February 19, 2002. Revised : April 15, 2002.
Accepted : April 23, 2002.
Address reprint requests to : Wen-Chi Chen, Department of Urology, China Medical College Hospital, No 2, Yuh-Der Road, Taichung 404, Taiwan, R.O.C.

rapidly, allowing for the prediction and prevention of stone disease if found early enough.

The p16 gene is related to cell death [5] and is presumed to be associated with renal cell death in response to oxalate overload. The p16 gene is located at chromosome 9p21 and a C/G polymorphism at the 570th nucleotide of p16 gene (exon 3) has been reported (CGAP-GAI 57619) [6]. Hydroxypyruvate reductase (HPR) and glyoxylate reducatse (GR) (GRHPR) are both enzymes which convert serine to glucose in humans [7]. Lack of this enzyme results in a rare monogenic disorder, primary hyperoxaluria type II, which can cause calcium oxalate stone disease [8]. The genomic structure of the GRHPR gene contains nine exons and eight introns, and it pericentromerically spans ~9kb on chromosome 9. A G/A polymorphism in exon 6 at the third position of codon 193 exists, but does not alter the encoded amino acid (Alanine to Alanine) [9]. The correlation between these genes and stone disease was therefore investigated by studying genetic polymorphisms.

PATIENTS AND METHODS

A control group was drawn up of 101 healthy volunteers over the age of 40 with no prior history of familial stone disease, or renal calcification. A total of 143 patients (98 males and 45 females) between the ages of 24 and 73 years (average age: 44.2 ± 12.0 yr) with recurrent calcium oxalate stone were enrolled in this study. Serial blood and urine biochemistry tests were undertaken to exclude possible hypercalcemia, hyperuricemia, and 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 either calcium oxalate monohydrate, dihydrate, or a combination of the two. There were 32 patients with hypercalciuria as defined by 24 hours urine calcium of more than 300 mg in males and 250 mg in females on a random diet (21 male and 11 female patients). The remaining patients revealed normal urine calcium levels and were categorized as normocalciuria (37 male and 34 female patients).

There were 67 males and 34 females between the ages of 40 and 73 (average age: 54.7 yr) in the control group. All patients in the control group underwent renal ultrasonography and assessment for urinary microscopic hematuria to ensure that they were urologically healthy. Informed consent was obtained from both groups that participated in this study. The genomic DNA was prepared from peripheral blood by a DNA Extractor WB kit (Wako, Japan).

The polymorphism was identified from the results of the polymerase chain reactions and restriction analysis by *Msp* I in the p16 gene (exon 3 C/G polymorphism). Polymerase chain reactions (PCRs) were carried out in a total volume of 50 μL, containing genomic DNA; (2-6 pmole of each primer), 1X Taq polymerase buffer (1.5 mM $MgCl_2$); and 0.25 units of AmpliTaq DNA polymerase (Perkin Elmer, Foster City, California, USA). The primers for the p16 gene C/G polymorphism were: forward (5'-ACATCCCCGATTGAA-AGAACCAG-3') and backward (5'-CTAAAT-GAAAACTACGAAAGCGGGG-3'). PCR amplification was performed in a programmable thermal cycler GeneAmp PCR System 2400 (Perkin Elmer, Foster City, California, USA). The cycling conditions for the P16 gene C/G polymorphism were set as follows: one cycle at 94 ℃ for 5 min, 35 cycles at 94 °C for 30 sec, 56 °C for 30 sec, and 72 ℃ for 30 sec, and one final cycle of extension at 72 °C for 7 min.

The PCR 132-bp band product was mixed with 2 units *Msp I* (New England Biolabs, Beverly, USA) and the reaction buffer according to the manufacturer's instructions. The restriction site was located at the allele of the p16 gene +570. Two fragments of 90-bp and 42-bp respectively will be present if the product is excisable. The reaction was

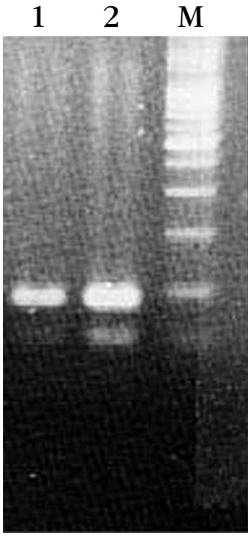

Fig. 1. PCR-base restriction analysis of the p16 gene *Msp* I C/G polymorphism shown on 3% agarose electrophoresis. The polymorphic region was amplified by PCR resulting in a heterozygote in lane 1 (132 bp +90 bp +40 bp) and an undigestable fragment in lane 2 (132 bp). M: marker (lane 3), 100-bp.

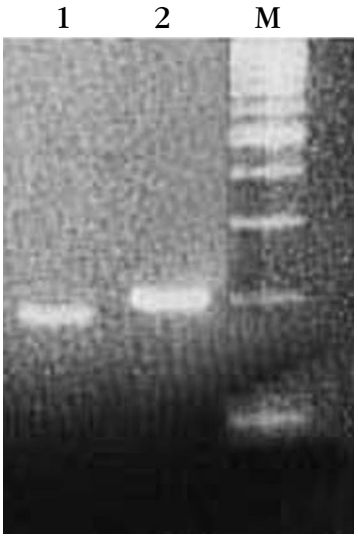

Fig. 2. PCR-base restriction analysis of the GRHPR gene *Bgl* II A/G polymorphism shown on 3% agarose electrophoresis. The polymorphic region was amplified by PCR resulting in a heterozygote in lane 1 (182 bp +160 bp +22 bp) and an undigestable fragment in lane 2 (182 bp). The short segment of 22 bp was obscure during electrophoresis. M: marker (lane 3), 100-bp.

incubated for 3 hours at 37 °C; we then loaded 10 µL of the products into 3% agarose gel containing ethidium bromide for electrophoresis. The polymorphism was divided into three groups: excisable (CC homozygote), unexcisable (GG homozygote) and heterozygote (C/G).

The PCR primers for the GRHPR gene exon 6 polymorphism (Genebank AF146689-exon 6 sequence) were: forward 5'-GCT-GTTCCGGAAATGCTGGG-3' and backward 5'-ACCAAACTCTGCCTGGAGATC-3'. The 5939-5940th nucleotide sequence was changed from "AT" to "TC" as a mismatched primer (underlined backward primer sequence). The PCR conditions were the same as for the p16 gene, except that the annealing temperature was set at 60 °C X 30S. The PCR product of 182 bp was digested by *Bgl* II and the polymorphisms were divided into three groups: excisable (A/A homozygote, 160-bp and 22-bp), unexcisable (G/G homozygote, 182-bp) and heterozygote (A/G).

The allelic frequency distribution of polymorphisms between the control and patient groups was compared using the chi-square test. When the assumption of the chi-square test was violated (i.e. when 1 cell had an expected count of < 1, or greater than 20% of the cells had an expected count of less than 5), the Fisher's exact test was used. Results were considered statistically significant when the probability of findings occurring by chance was < 5 % ($p < 0.05$).

RESULTS

The bands on the gel revealed both undigested (GG) homozygotes and heterozygotes (C/G) of the p16 gene *Msp* I polymorphism (Fig. 1). This polymorphism revealed 4 C/G heterozygotes (2.8%) and 139 G/G homozygotes (97.2%) in the stone patient group and 6 C/G heterozygotes (5.9%) and 95 G/G (94.1%) homozygotes in the control group (Table 1, p = 0.326, chi-square test). The frequency of the G/C allele revealed no significant differences between the two groups (p = 0.331, chi-square test).

Both undigested (GG) homozygotes and heterozygotes (A/G) of the GRHPR gene *Bgl* II polymorphism were revealed (Fig. 2). The

Table 1. Distribution of the p16 gene *Msp* I polymorphism between the healthy control group and the calcium oxalate stone patient group by chi-square test

	GG	CG	Total	*p* value
Control group	95 (94.1%)	6 (5.9%)	101 (100%)	
Stone patient group	139 (97.2%)	4 (2.8%)	143 (100%)	0.326*
Hypercalciuria	31 (96.9%)	1 (3.1%)	32 (100%)	
Normocalciuria	108 (97.3%)	3 (2.7%)	111 (100%)	0.315**

*Compared with control group; ** Compared with hypercalciuric group. GG = gel revealed both undigested; CG = heterozygote.

Table 2. Distribution of the GRHPR gene *Bgl* II polymorphism between the healthy control group and the calcium oxalate stone patient group by Fisher's exact test

	GG	AG	Total	*p* value
Control group	99 (98.0%)	2 (2.0%)	101 (100%)	
Stone patient group	141 (98.6%)	2 (1.4%)	143 (100%)	1.0*
Hypercalciuria	32 (100%)	0 (0.0%)	32 (100%)	
Normocalciuria	109 (98.2%)	2 (1.8%)	111 (100%)	1.0**

*Compared with control group; ** Compared with hypercalciuric group. GG = gel revealed both undigested; AG = heterozygote.

frequency of the GG homozygotes of the GRHPR gene *Bgl* II polymorphism was over 98% in both groups (p = 1.0, Fisher's exact test). There were 2 GA heterozygotes (2.0%) in the control group and 2 (0.7%) in the stone group. No statistical differences between the hypercalciuric and normocalciuric patients and the control group were found (Table 2).

DISCUSSION

The data revealed that the p16 gene +570 C/G polymorphism is not an appropriate genetic marker of stone disease. The distribution of the frequency of the C/G polymorphism in the patient group and control group was similar. Also, there was no apparent association between the GRHPR gene *Bgl* II exon 6 G/A polymorphism and stone disease. Due to the lack of published reports regarding the association between this polymorphism and stone disease, other polymorphisms in this gene should be investigated.

Urolithiasis is a multifactorial disease that involves crystallization. Evidence shows that crystals interact with the tubular epithelium which leads to retention and accumulation of crystalline material in the kidney and, eventually, to the formation of renal stones [10]. The most frequently associated phenomenon is tubular cell injury from a high oxalate load. Oxalate overload induces an increase in the production of free radicals and cell death that leads to crystal deposition in the renal tubules and finally to growth of calcium oxalate stones [11]. Although p16 may not be directly linked to stone disease, it is associated with cell death [5]. Activation of the p16 gene results in the inhibition of cell cycle G1 and S phase progression. This cell cycle checkpoint prevents the replication of damaged DNA templates and permits time for repair. If damaged cells leak to the S phase, apoptosis of the cells is initiated. Therefore, p16 was chosen as an SNP marker. However, we did not find an association between stone disease and the p16 gene *Msp* I G/C polymorphism in this study.

The GRHPR gene encodes a 328 amino acid protein with a mass of 35,563 Daltons. The GR activity reduces glyoxylate to glycolate, and may be important in regulating the amount of glyoxylate that is converted to oxalate [12]. The enzyme also has D-glycerate dehydrogenase (DGDH) activity. HPR and DGDH enzymes perform reciprocal reduction/dehydrogenation reactions, with

equilibrium favoring the reduction reaction [13]. GRHPR uses NADPH as a coenzyme, generating NADP+, which is required for a functional pentose pathway. Absence of an active GRHPR enzyme results in over production of urinary oxalate and finally calcium oxalate stone formation. Because this gene is related to the production of oxalate and because no previous study has reported on this polymorphism, it was chosen as a potential genetic marker of stone disease.

SNPs are based on the differences between individuals, and are usually either part of a gene or close to a gene making them more effective than conventional methods for locating disease genes [14]. Many diseases can be diagnosed or predicted in some groups of individuals using SNPs. The large volume of data produced by high-throughput sequencing projects is a rich and largely untapped source of SNPs. Data can be calculated for susceptible genes, resistant genes, and interaction with environmental factors for many diseases [15]. We have studied many genes using SNPs [1-3]. However, we have been unable to find any significant association between the p16 gene +570 polymorphism and stone disease, although the possibility of a type II error must be kept in mind in view of the small patient sample size in our study.

Although DNA polymorphisms are useful when searching for genetic markers of complex diseases [15], more than a million SNPs have been reported, making the choice of SNP difficult. A rapid, economic, and accurate method is essential for further research of genetic markers. Genotyping large numbers of SNPs in linkage and association studies will shed light on stone disease in the future. Perhaps, one may expect that the DNA microarrays technique will issue a high-density SNP map, allowing urologists to expand their capabilities of treating their patients by focusing not only on symptoms [16], but also on their patients' genetic background. However, the cost of the gene-chips technique is one of the major problems. Therefore, more effective and cheaper techniques are needed and are currently underdevelopment in our laboratory.

In conclusion, there was no association between the P16 gene *Msp* I and the GRHPR gene *Bgl* II polymorphisms and stone disease found in this study. Other polymorphic sites of these genes should be studied. Further development of accurate, rapid and economic techniques in SNPs is also needed.

ACKNOWLEDGMENT

This study was supported by a grant from China Medical College Hospital (DMR-91-064).

REFERENCES

1. Chen WC, Chen HY, Lu HF, et al. Association of vitamin D receptor gene start codon Fok I polymorphism with calcium oxalate stone disease. *BJU Int* 2001;87: 168-71.
2. Chen WC, Wu HC, Lin WC, et al. The association of androgen- and estrogen-receptor gene polymorphisms with urolithiasis in men. *BJU Int* 2001; 88:432-6.
3. Chen WC, Lu HF, Chen HY, et al. Arginine form of p21 gene codon 31 is less prominent in patients with calcium oxalate stone. *Urol Res* 2001;29:94-7.
4. Melton L. From DNA to drugs. Novartis Foundation Symposium. From genome to therapy: integrating new technologies with drug development. *Mol Med Today* 1999;5:468-9.
5. Liggett Jr WH, Sidransky D. Role of the p16 tumor suppressor gene in cancer. [Review] *J Clin Oncol* 1998;16:1197-206.
6. Jaffrain-Rea ML, Ferretti E, Toniato E, et al. p16 (INK4^a, MTS-1) gene polymorphism and methylation status in human pituitary tumors. *Clin Endocrinol (Oxf)* 1999;51:317-25.
7. Williams H, Smith L. L-glyceric aciduria. A new genetic variant of primary hyperoxaluria. *N Eng J* Med 1968;278:233-8.
8. Chlebeck PT, Milliner DS, Smith LH. Long-term prognosis in primary hyperoxaluria type II (L-glyceric aciduria). [Review] *Am J Kid Dis* 1994;23: 255-9.
9. Cramer SD, Ferree PM, Lin K, et al. The gene encoding hydroxypyruvate reducatse (GRHPR) is mutated in patients with primary hyperoxaluria type II. *Hum Mol Genet* 1999;8:2063-9.
10. Scheid C, Koul H, Hill WA, et al. Oxalate toxicity in LLC-PK1 cells: role of free radicals. *Kid Int* 1996; 49:413-9.
11. Verkoelen CF, van der Boom BG, Houtsmuller AB, et

al. Increased calcium oxalate monohydrate crystal binding to injured renal tubular epithelial cells in culture. *Am J Physiol* 1998;274:958-65.
12. Webster KE, Cramer SD. Genetic basis of primary hyperoxaluria type II. *Mol Urol* 2000;4:355-64.
13. Cregeen DP, Rumsby G. Recent developments in our understanding of primary hyperoxaluria type 2. *J Am Soc Nephrol* 1999;10(Suppl 14):348-50.
14. Collins A, Ennis S, Taillon-Miller P, et al. Allelic association with SNPs: metrics, populations, and the linkage disequilibrium map. *Hum Mutat* 2001;17:255-62.
15. Collins A, Lonjou C, Morton NE. Genetic epidemiology of single-nucleotide polymorphisms. *Proc Natl Acad Sci USA* 1999;96:15173-7.
16. Tillib SV, Mirzabekov AD. Advances in the analysis of DNA sequence variations using oligonucleotide microchip technology. [Review] *Curr Opin Biotechnol* 2001;12:53-8.

P16基因第三表現序列C/G多形性及氫氧焦葡萄糖酸鹽還原酶(GRHPR)基因第六表現序列G/A多形性與草酸鈣結石之相關性：無關聯

吳錫金　蔡輔仁[1,2]　陳慧毅[3]　呂學甫　許正德[1]　陳汶吉

中國醫藥學院附設醫院　泌尿科部　優生保建部[1]　小兒科部[2]　婦產科部[3]

目的　基因多形性與結石疾病的關聯性已多次被報告。由於p16與細胞死亡相關，我們假定它與腎細胞對草酸負荷造成的細胞死亡反應並進而產生結石有關，氫氧焦葡萄糖酸鹽還原酶與乙二醛還原酶（GRHPR）是與草酸代謝且與先天性草酸鈣結石的形成有關的酵素，我們也就此討論這些基因與一般草酸鈣結石的角色做探討。

方法　本研究收集101個健康正常人與143個復發的草酸鈣結石病人的去氧核醣核酸，p16基因多形性的偵測方法是以多聚合酶鏈反應爲基礎的限制酶分析法，*Msp* I可分辨（C對偶基因可被酵素切割）位於p16基因的第三表現序列(C/G多形性)。GRHPR基因則是第六表現序列上*Bgl* II內切酶（G對偶基因可被酵素切割）G/A的多形性。

結果　在p16基因*Msp* I多形性的分佈上，結石病人的C/G異形合子是2.8%而正常對照組是5.9%，結石病人的GG同源合子的對偶基因頻率是97.2%且正常人是99.1%（p = 0.326，以卡方試驗）。在GRHPR的*Bgl* II基因多形性方面，兩組的GG同源合子頻率均是98%（p = 1.0），對照組的異形合子是2.0%，而結石病人是1.4%。高尿鈣症與正常尿鈣的結石病人其分布頻率並無統計學上的差異。

結論　p16基因的*Msp* I G/C多形性與氫氧焦葡萄糖酸鹽還原酶*Bgl* II多形性均與結石疾病沒有關聯。（中台灣醫誌 **2002;7:94-100**）

關鍵詞

草酸鈣結石，基因多形性，氫氧焦葡萄糖酸鹽還原酶，p16

聯絡作者：陳汶吉

地　　址：404台中市北區育德路2號

中國醫藥學院附設醫院　泌尿部

收文日期：2/19/2002　　修改日期：4/15/2002

接受日期：4/23/2002