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Analysis of osteopontin DNA in patients with urolithiasis

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Abstract We previously reported the importance of osteopontin (OPN) in the formation of urinary calculus. Since OPN protein is present in normal kidneys, we investigated the difference in OPN at the DNA level between normal subjects and urolithiasis patients. There has not been any genetic investigation of OPN in familial urolithiasis previously reported worldwide. To confirm hereditary predisposing factors for urolithiasis, changes in OPN DNA within a family were investigated in relation to the presence or absence of urinary calculus. Leukocyte OPN DNA from two normal subjects and five patients with urinary calculus was investigated by SSCP analysis: OPN DNA nucleotide sequence was determined, based on the result of SSCP analysis. As a result, a mutation of GCC to GCT, encoding amino acid position 250 (Ala-250) was found. To confirm the frequency of mutation at this site, OPN DNA was extracted from peripheral blood in 36 normal subjects (Con group), 25 patients with familial urolithiasis (FSF), and 40 patients with recurrent urinary calculus and who had had two or more previous episodes (RSF). The degree of mutation at Ala-250 was then examined by restriction fragment length polymorphism (RFLP) method. As described above, the nucleotide codon encoding the amino acid sequence position 250, Ala-250, was GCC in two normal subjects. This is the original codon. In five patients with urolithiasis it was GCT, showing a substitution of C with T. On examining the frequency of this mutation, the ratio of normal homozygous GCC was 11/36 in the Con group, 1/25 in FSF and 1/40 in RSF. The ratio of heterozygous GCC/GCT was 16/36 in the Con group, 15/25 in FSF and 26/40 in RSF, and the ratio of homozygous GCT was 9/36 in the Con group, 9/25 in FSF and 13/40 in RSF. Furthermore, the gene frequency of the normal codon GCC was 0.528 in the Con group, 0.3 in FSF and 0.35 in RSF, showing a significantly higher incidence in the Con group ($P < 0.05$). The gene frequency of mutated GCT was 0.472 in Con group, 0.7 in FSF and 0.65 in RSF, showing a significantly higher incidence in urolithiasis patients ($P < 0.05$). On investigating the inheritance of Ala-250 in five families in which both parent and offspring demonstrated urolithiasis, the nucleotide substitution in Ala-250 in parents with urolithiasis was inherited by their offspring. In all five families the offspring developed urinary calculus. This study showed that there is no difference in OPN structure between the Con group and urolithiasis patients. However, it was predicted that due to the frequency of normally coded GCC being high in the Con group a difference in the amount of OPN might be caused by a difference in transcription velocity between the two groups. Furthermore, it was suggested that examining the inheritance of Ala-250 within a family is a diagnostic method for identifying the predisposing hereditary factors for urolithiasis patients.

Key words Osteopontin DNA · Urolithiasis · Mutation

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Introduction

Various protein components have been recently identified as organic components involved in the mechanism of urinary calculus formation, [1, 2]. We have extracted matrix components from calcium oxalate (CaOx) calculus, and identified osteopontin (OPN) from a c-DNA library in human kidney with anti-human OPN polyclonal antibody [3]; we have reported a specific increase in OPN mRNA in distal renal tubular cells after inducing calculus formation in rats [4] and the presence of OPN was noted only in calculi that contained calcium [5]. The OPN protein is known to be essential for the calcification

mechanism in the body [6] because of its strong calcium-binding ability and cell adhesion activity [7]. We previously reported that OPN can be secreted by Madin Darby canine kidney (MDCK) cells and is associated with cell-crystal interaction [8, 9].

Moreover, addition of OPN-antisense oligonucleotide (AS-ODN) to the cell culture medium decreased in OPN accumulation and inhibited calcium oxalate crystal attachment [10]. Thus, the importance of OPN in calculus formation was demonstrated and regarded as an essential protein for calculus formation.

OPN protein is present also in normal kidneys [6]. However, when it strongly influences calculus formation, a quantitative or structural difference in OPN is predicted to be present in urolithiasis patients. Therefore, to confirm the change in the OPN gene structure between urolithiasis patients (familial and recurrent) and normal individuals, OPN DNA was examined by strand conformation polymorphism (SSCP) analysis [11] for the presence of a mutation.

Recently, etiologic genes have been analyzed in various familial diseases and this analysis has resulted in a diagnostic method [12, 13] being defined. It has also been reported that approximately 30% of urolithiasis cases are familial [14]. Therefore, to investigate predisposing hereditary factors for urolithiasis, OPN DNA inheritance within a family was investigated based on the presence or absence of urinary calculus.

Materials and methods

Analysis of human peripheral blood leukocyte OPN DNA

There were 36 normal subjects without past history of urolithiasis in the control group (20 males and 16 females) with an average age of 54.3 years (Con group). In the patient groups, there were 40 patients having had two or more episodes of calcium-containing calculi, showing 2.7 recurrences on average, (32 males and 8 females) with an average age of 50.4 years in the RSF group, and 25 familial urolithiasis patients having had calcium-containing calculi and relatives within the second degree showing a past history of urolithiasis (14 males and 11 females) FSF group. This group had an average age of 45.4 years and 2.8 recurrences on average in the FSF group (Table 1).

Extraction of human OPN DNA

Leukocytes were selected as extraction samples of OPN DNA [15]. Heparinized peripheral blood (10 ml) was collected and after centrifugation at 2500 rpm for 10 min, serum was removed. The precipitate was repeatedly washed with 0.2% sodium chloride and centrifuged; then white blood cells were collected. Using Sepa Gene, a DNA extraction kit (Wako Pure Chemical Industries Tokyo Japan), total DNA was extracted. Using primers for both regions of an open reading frame, 5′-CCATACCCAGTTAAACAGGCT (forward) and 3′-TTTTAATTGACCTCAGAAGAT (reverse), according to the human OPN sequence [16], 30 cycles of a polymerase chain reaction (PCR) were performed. Approximately 900 bp OPN DNA were extracted from the gel after agarose gel electrophoresis. Since 900 bp is beyond the number of nucleotides appropriate for SSCP analysis, resulting in a reduced accuracy [17], a restriction enzyme Ava II (recognition site: G↓GACC) [16] was added to obtain 200–300 bp fragments; OPN DNA was divided into three fragments, OPN-1, OPN-2 and OPN-3 (Fig. 1).

Analysis of mutation in OPN DNA

A PCR-SSCP was performed following the method reported by Hayashi et al. [11]. Each PCR product was suspended in a sampling buffer (95% formaldehyde, 20 mM EDTA, 0.005% bromophenol blue, 0.05% xylene cyanol, and 0.1% sodium dodecyl sulfate [SDS]) and heated at 95 °C for 5 min. The heat-treated sample underwent electrophoresis on an 8% polyacrylamide gel and then gel was silver-stained (Daiichi Kagaku, Tokyo, Japan) to detect differences in migration; i.e. the presence or absence of a

Table 1 Characteristics of healthy control and stone former. *FSF* familial stone former, *RSF* recurrent stone former

	Control	Stone former	
		FSF	RSF
Cases	36	25	40
Male:female	20:16	14:11	32:8
Mean age	54.3 ± 14.5	45.4 ± 17.7	50.4 ± 15.8
Stone episode		2.8 ± 1.7	2.7 ± 0.6

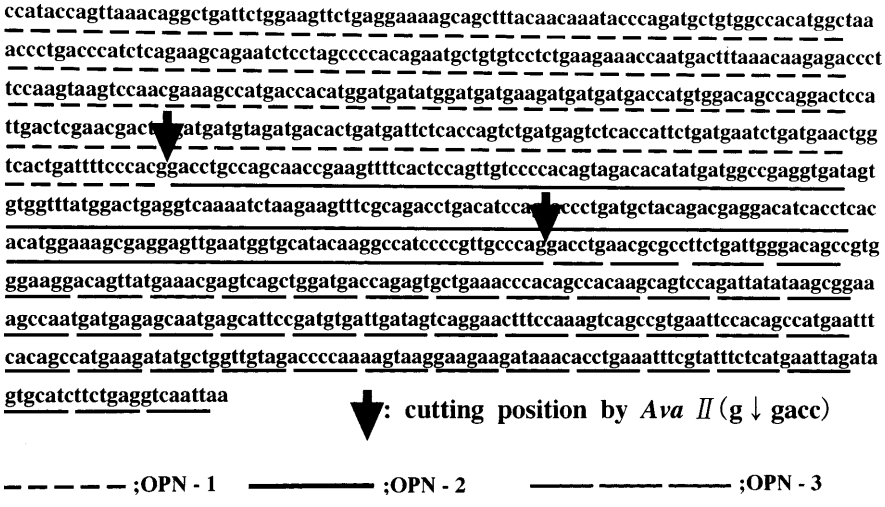

Fig. 1 Human OPN-DNA sequence. OPN-DNA was divided into three fragments by a enzyme Ava II, OPN-1, OPN-2 and OPN-3

mutation. Next, the nucleotide sequence was directly determined (direct sequence) using sequencing high-*plus*-, a DNA sequencing kit (Toyobo, Oasaka, Japan).

Detection of the polymorphic region in OPN DNA

This was done using PCR-restriction fragment length polymorphism (RFLP) analysis [18]. After confirming the presence of DNA mutation by the result described in analysis of mutation in OPN DNA, only three base pairs in the OPN-3 gene were amplified by PCR using both primers for 5′-CACATGGAAAGCGAGGAGTT (forward) and 3′-TTTTAATTGACCTCAGAAGAT (reverse). Then 8 μl PCR product, 2 μl restriction enzyme *Alu* I (recognition site: AG↓CT) [16] and buffer were mixed and reacted at 37 °C for 2 h. Then, after electrophoresis on a 2% agarose gel, the gel was stained with ethidium bromide and DNA bands were detected by visualizing under ultraviolet light.

Statistics

The chi-square test was used to assess the differences between the groups, with $P < 0.05$ considered to indicate significance.

Results

Presence of mutation in OPN DNA detected by SSCP

Results of SSCP analysis in two subjects of the Con group and five subjects of the FSF group are presented. There were no differences detected by SSCP in OPN-1 (Fig. 2a) or OPN-2 (Fig. 2b) sequences among the three fragments dividing the 900 bp OPN sequence.

In contrast, a migration difference between the Con group (C) and the FSF group (S) was found in the OPN-3 sequence at the position indicated by an arrow (Fig. 2c).

Confirmation of mutated sequence by direct sequencing

Since a difference in the OPN-3 DNA sequence between normal subjects and urolithiasis patients was suggested by the result described in presence of mutation in OPN DNA detected by SSCP, OPN-3 was directly sequenced in all cases.

As a result, Ala-250 in the Con group was previously reported to be GCC [16] localized in OPN DNA exon 7. In contrast, the nucleotide substitution from GCC to

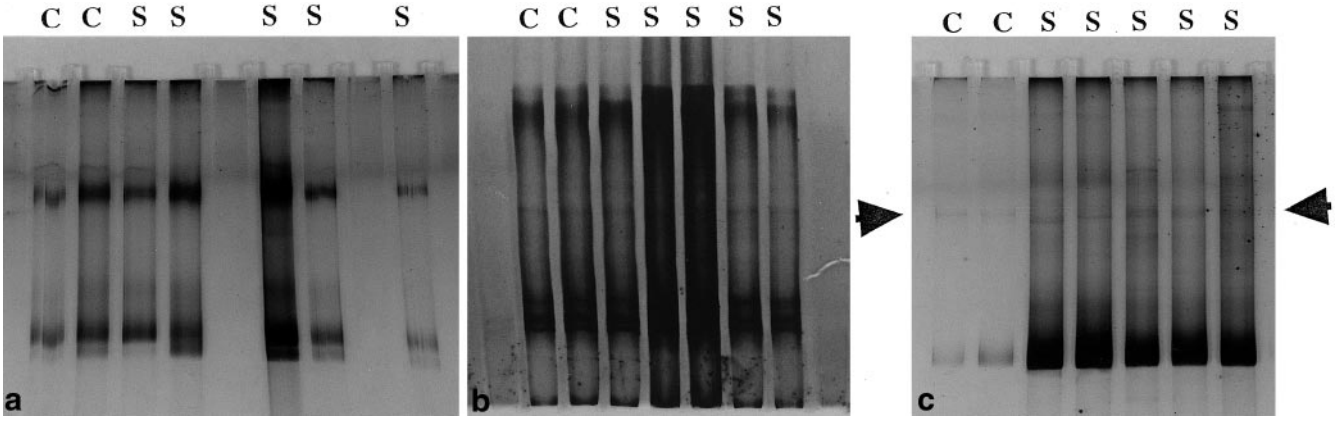

Fig. 2a–c Presence of mutation in OPN-DNA detected by strand conformation polymorphism (SSCP) analysis: **a** results of SSCP in OPN-1 fragments, **b** results of SSCP in OPN-2 fragments, and **c** results of SSCP in OPN-3 fragments. A migration difference between the control group (*C*) and the familial stone former group (*S*) was found in the OPN-3 sequence at the position indicated by an *arrow*

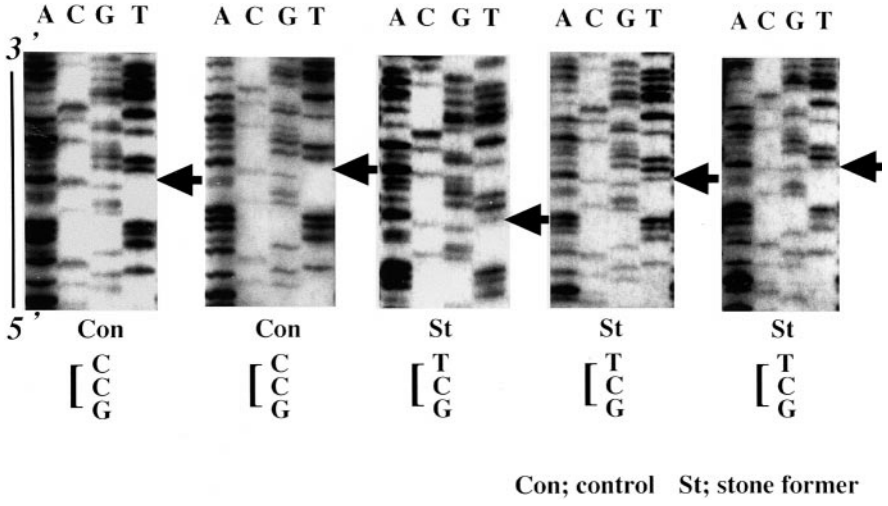

Fig. 3 Confirmation of mutated sequence by direct sequencing. The nucleotide substitution from GCC to GCT (C→T) was found in five patients in the FSF group. However, there was an Ala→Ala synonymous substitution in the amino acid code

GCT (C→T) was found in five patients in the FSF group (Fig. 3). However, there was an Ala→Ala synonymous substitution in the amino acid code.

Detection of the mutation GCC→GCT in Ala-250 in OPN-3 DNA exon 7 by PCR-RFLP analysis

Because of the nucleotide substitution Ala-250/GCC→GCT, the nucleotide sequence at this site was changed from AGCC to AGCT, generating in the recognition sequence of restriction enzyme, *Alu* I (AG↓CT). There is an *Alu* I recognition site (AG↓CT) upstream to this site in the original OPN-3 sequence. Thus, RFLP analysis of the OPN-3 sequence using restriction enzyme *Alu* I was performed in all groups and the presence or absence of GCC→GCT mutation was investigated. Using RFLP analysis, there is one *Alu* I recognition site for homozygous GCC, that generates two bands approximately 100 and 250 bp. In the case of homozygous GCT, the sequence contains two *Alu* I recognition sites, generating three bands of approximately 50, 100 and 200 bp, and in the case of heterozygous GCC/GCT, four bands of approximately 50, 100, 200, 250 bp are generated (Fig. 4).

Results of the RFLP analysis for 36 subjects of the Con group, 25 patients of the FSF group and 40 patients of the RSF group are shown in Table 2. Eleven of 32 subjects in the Con group showed GCC/GCC, 16 subjects showed GCC/GCT and nine subjects showed GCT/GCT. In the FSF group, one of the 25 patients showed GCC/GCC, 15 patients showed GCC/GCT and nine patients showed GCT/GCT. In the RSF group, one of the 41 patients showed GCC/GCC, 26 patients showed GCC/GCT and 13 patients showed GCT/GCT (Table 2).

Analysis of gene frequency and genotype distribution of OPN Ala-250 in each group

The gene frequency and genotype distribution of OPN Ala-250 in each group are shown in Table 3. The gene frequency of GCC was significantly high in the Con grou ($P < 0.05$), while the frequency of GCT was significantly higher in the FSF and RSF groups than in the Con group ($P < 0.05$). Regarding genotype distribution, the GCC/GCC genotype was significantly high in the Con group ($P < 0.05$), while the GCT/GCT genotype was significantly high in the patient groups ($P < 0.05$). There was no significantly difference in distribution of GCC/GCT genotype among the groups.

Table 2 Results of point mutation GCT from GCC in Ala-250 in human OPN-3 DNA exon 7 by PCR-RFLP analysis. *FSF* familial stone former, *RSF* recurrent stone former

	Control	Stone former	
		FSF	RSF
GCC · GCC	11	1	1
GCC · GCT	16	13	26
GCT · GCT	9	11	13
Totals	36	25	40

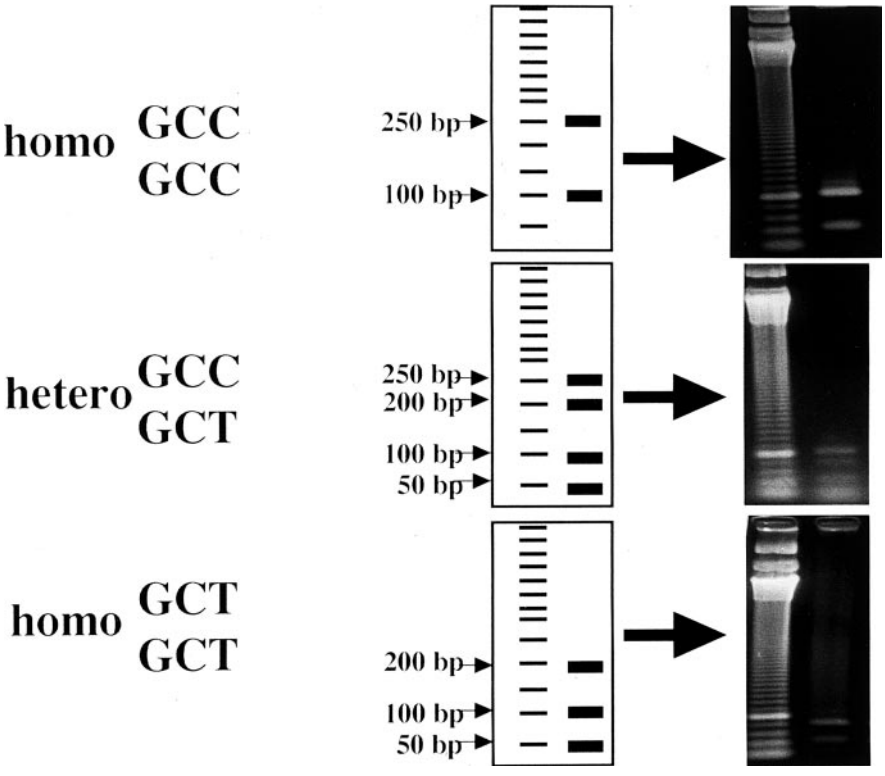

Fig. 4 Restriction fragment length polymorphism (RFLP) analysis of the OPN-3 sequence using restriction enzyme *Alu* I

Table 3 The gene frequency and genotype distribution of OPN Ala-250 in each group. *NSF* non-stone former, *FSF* familial stone former, *RSF* recurrent stone former

Distribution of OPN genotype				Gene frequency			
Genotype	NSF	FSF	RSF	Gene	NSF	FSF	RSF
GCC · GCC	0.279	0.09	0.123	GCC	0.528	0.3	0.35
GCC · GCT	0.4989	0.42	0.455				
GCT · GCT	0.223	0.49	0.423	GCT	0.472	0.7	0.65

Inheritance of OPN Ala-250 within a family

Using the results for detection of the mutation GCC→GCT in Ala-250 in OPN-3 DNA exon 7 by PCR-RFLP analysis, the inheritance of OPN-3 Ala-250 was investigated. Of the five families in the analysis, the following correlations were found: one of the parents as well as the offspring developed urolithiasis (lineage A), two families in which one of the parents are urolithiasis patients but offspring were normal (lineage B) and four families in which both parents were normal but offspring developed urolithiasis (lineage C) (Fig. 5). In lineage A (Fig. 5a), one of the parents and the offspring were urolithiasis patients. In all five families, offspring with calculus inherited the nucleotide substitution from the parent with urolithiasis. Figure 5a presents RFLP features of lineage A in which the mother and offspring were urolithiasis patients and the genotype of the father was GCC/GCC, while the genotype in mother and offspring was GCC/GCT. In lineage B, one of the parents was a urolithiasis patient (Fig. 5b). The genotype of non-urolithiasis offspring differed from the OPN Ala-250 genotype of the parent with urolithiasis. Figure 5b presents the RFLP features of lineage B in which only the mother was a urolithiasis patient and the genotype of the mother was GCT/GCT; the genotype of the offspring and father who were not urolithiasis patients was GCC/GCT, showing a difference in the OPN Ala-250 genotype between mother and offspring. In lineage C (Fig. 5c), only the offspring were urolithiasis patients. Among four families, the OPN Ala-250 genotype differed between parents without calculi and offspring with calculi in two families, while in the remaining families, both parents and offspring had homologous OPN Ala-250 genotype. Figure 5c presents the RFLP feature of cases in which only the offspring have calculi and the genotype of OPN Ala-250 differed between parents and offspring.

Discussion

We have reported the importance of OPN in various aspects of crystal formation using various methods including inhibition of OPN by an antisense technique [3, 4, 5, 10]. We have also found that OPN protein is present in normal kidneys [6]. However, when it strongly influences calculus formation, a quantitative or structural difference in OPN is predicted to be present in urolithiasis patients. Therefore, to confirm a difference in the OPN gene structure and OPN-DNA level between normal individuals and urolithiasis patients (familial and recurrent) an analysis of their OPN-DNA was performed. Since the conversion from GCC to GCT is synonymous, there was no difference in OPN structure between non-stone formers and stone formers. However, as shown in Table 3, the gene frequency of GCC was clearly higher in the Con group, while the frequency of GCT was higher in the FSF and RSF groups than that in the Con group. Furthermore, GCC/GCC genotype distribution was also high in the Con group, while GCT/GCT genotype was high in the stone former groups. As described above, the gene frequency and distribution of GCC and GCT were clearly different between the Con group and the stone former groups. In the absence of a difference in structure, it is suggested that this may be due to a difference in quantity. Generally, transcription is an important regulatory step in the synthesis of various proteins and transcription proceeds through the binding of RNA polymerase II to the transcription initiation site with other transcription factors [19]. Transcription velocity determines the levels of mRNA and protein. Molecules that regulate transcription velocity, i.e., molecules involved in activation and inactivation of transcription are considered transcription regulator proteins which bind to specific sequences in genes. Considering the clear difference in gene frequency between GCC and GCT, although the synonymous substitution from GCC to GCT at the DNA level produces structurally identical OPN, a difference may be present in the sequence of transcription factor binding site between the gene having GCC and gene having GCT, or among over 100 species of transcription factors present, the transcription factor synthesizing GCC and that synthesizing GCT may differ, suggesting that a difference in transcription step may generate differences in the amount of mRNA and protein. There is no report on measurements of urinary and blood OPN levels to clarify this. However we are now attempting to conduct such a study.

Recently, genes responsible for various familial diseases have been identified, showing that nucleotide substitution in these genes are causes of disease. Subsequently examination of gene sequence has become a diagnostic method; for example in APRT deficiency [20] and cystinuria [21], diseases causing calculosis, and where the gene responsible for the disease has been identified. Iguchi et al. [14] reported that approximately 30% of urolithiasis is inherited within a family. There is no report on the genes generally considered responsible for calcium-containing calculus. We focused on the importance of the OPN gene and investigated the presence or absence of mutation in OPN DNA. As a result,

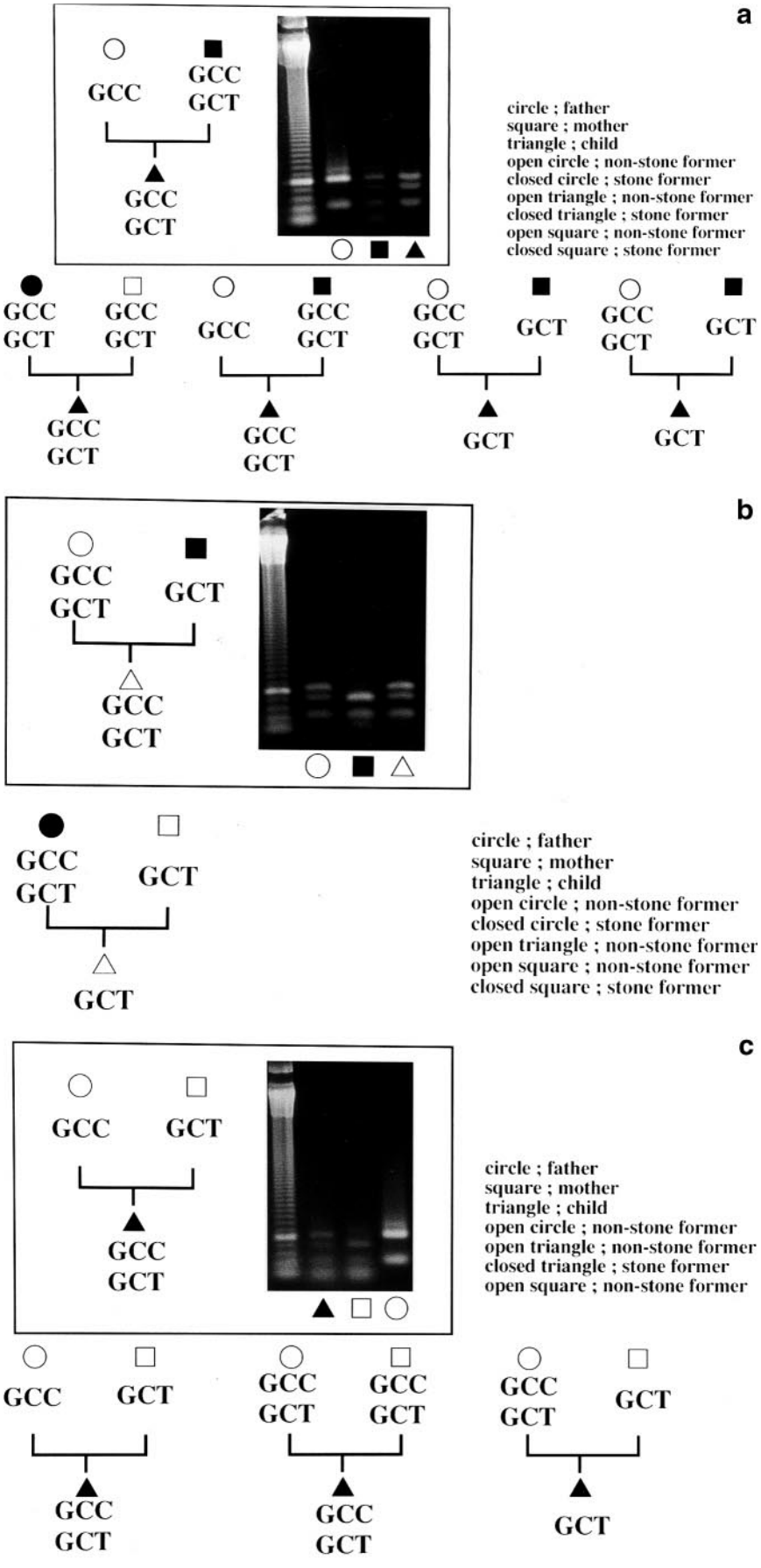

Fig. 5 Inheritance of OPN Ala-250 within a family: **a** one of the parents and the offspring were urolithiasis patients, **b** one of the parents was a urolithiasis patient and **c** only the offspring were urolithiasis patients

although it was synonymous, we found a nucleotide substitution in the encoding Ala-250. Based on this result, we increased the number of cases analyzed genetically. Based on the clear difference in gene frequency between stone formers and non-stone formers, we investigated the inheritance of Ala-250 focusing on the predisposing hereditary factors observed in urolithiasis patients. Families of patients with familial urolithiasis (predisposing genetic factor) having a relative with urolithiasis within the second degree and families of non-hereditary urolithiasis patients were classified into three types and interesting results were obtained from the investigation of Ala-250 inheritance. Although there were not many families investigated, the nucleotide substitution in Ala-250 was inherited in 100% of families in which parent and offspring had urinary calculus (Fig. 5a); in families in which only the parent was a stone former, Ala-250 in non-stone forming offspring differed from that in the urolithiasis parent (Fig. 5b). These results showed the inheritance of OPN DNA Ala-250, suggesting that the probability of developing urolithiasis is increased in offspring with the same nucleotide substitution as that found in the parent with urolithiasis. Conversely, when Ala-250 shows a different sequence from that in the parent with urolithiasis, the probability of developing urolithiasis may be low. That is, it is suggested that in order to predict whether or not offspring of a parent with urolithiasis are likely to develop urolithiasis, examination of DNA Ala-250 in parent and offspring could be an appropriate method for doing this. This is a very interesting possibility and considered an important subject to investigate as a genetic diagnostic method for calcium-containing calculi. The fact that families in which only the offspring are stone formers, the nucleotide sequence differed between parent and offspring having calculus in two of four families, while in the remaining two families, the sequence was the same (Fig. 5c), showing that not all patients with urolithiasis have predisposing hereditary factors, which may be supported by the fact that approximately 30% of patients with urolithiasis have predisposing hereditary factors [14].

This is the first study on mutation of OPN DNA in urolithiasis patients. Although it was a synonymous substitution, we found a nucleotide substitution in Ala-250. There was no structural difference between stone formers and non-stone formers because of synonymous substitution, but as a result of investigating peripheral leukocyte OPN DNA in 101 individuals in total, gene frequency of GCC, the nomad code, was high among non-stone formers, while the gene frequency of GCT, the substituted sequence, was high among stone formers, and Ala-250 was inherited from a stone-former parent by stone-former offspring. These results suggested that there was a difference in the amount of OPN between stone formers and non-stone formers and that analysis of OPN DNA Ala-250 in patients with calcium-containing calculus, the most common form of calculi, may be a method of diagnosing familial urolithiasis.

To confirm the results from this study, it is necessary to measure the amounts of OPN in urine and blood, to investigate a larger number of cases and to analyze OPN DNA at the transcription level that determines the codons of GCC and GCT.

Conclusion

We investigated the difference in OPN at the DNA level between normal subjects and urolithiasis patients. To confirm hereditary predisposing factors for urolithiasis, changes in OPN DNA within a family were investigated in relation to the presence or absence of urinary calculus. The nucleotide codon encoding the amino acid sequence position 250, Ala-250, was GCC in normal subjects, which is the original codon, while it was GCT in five patients with urolithiasis, showing a substitution of C to T. On examining the frequency of this mutation, the ratio of normal homozygous GCC was 11/36 in Con group, 1/25 in FSF group and 1/40 in RSF group. On investigating the inheritance of Ala-250 in five families in which both parent and offspring demonstrated urolithiasis, the nucleotide substitution in Ala-250 in parents with urolithiasis was inherited by their offspring who developed urinary calculus in all five families.

This study showed that there is no difference in OPN structure between normal subjects and urolithiasis patients. However, it was predicted from the fact that the frequency of normally coded GCC was high in normal subjects that a difference in the amount of OPN may be caused by a difference in transcription velocity between the two groups. Further, it was suggested that examining the inheritance of Ala-250 within a family is available as a diagnostic method for urolithiasis patients with predisposing hereditary factors.

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