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ORIGINAL PAPER

# Manganese superoxide dismutase (Mn-SOD) gene polymorphisms in urolithiasis

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**Abstract** Polymorphism in manganese superoxide dismutase gene (Mn-SOD) is a new approach to identify its probable association with urolithiasis. Oxidative stress may be involved in the development of stone formation in the renal system. MnSOD is one of the primary enzymes that directly scavenges potential harmful oxidizing species. A valine (Val) to alanine (Ala) substitution at amino acid 16, occurring in the mitochondrial targeting sequence of the MnSOD gene, has been associated with an increase in urolithiasis risk. This study was conducted to investigate the association of MnSOD gene polymorphism with the risk of urolithiasis.We investigated the MnSOD in 66 stone-forming adults and 72 healthy volunteers. DNA was isolated from peripheral blood and genotyping was performed with PCR-based methods.Then PCR products were cut by *Bsa*W1. Products were run on 3% agarose gel, 246 bp regions were 1-Ala-9, 164 and 82 bp products were determined as 2 Val-9. Chi-square test was used for comparison between patients and controls. In the control group the homozygote Ala allele was significantly higher than in the patient group ($P < 0.01$). The distribution of Ala/Val and homozygote Val alleles in the patient group was significantly higher than in the control group ($P < 0.05$). MnSOD genotype determination may provide a tool to identify individuals who are at risk of urolithiasis. This experiment also provides data about antioxidant status and stone formation.

**Keywords** Gene polymorphisms · Urolithiasis · Manganese superoxide dismutase

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## Introduction

Urolithiasis is a complex disease that is commonly seen in patients with urological disorders. One of the theories of stone formation has to do with fixed particles caused by cellular injury [1, 2]. There is evidence that crystals which interact with the tubular epithelium may lead to retention and accumulation of crystalline material in the kidney, eventually leading to the formation of renal stones [3]. The most common example of this condition is tubular cell injury caused by high oxalate concentration which has been shown in LLC-PK1 and MDCK cell cultures [4]. Furthermore, oxalate increases the production of free radicals which can induce a cell death process, crystal deposition in the renal tubules, and finally, growth of calcium oxalate stones [5].

There is growing evidence in support of the importance of reactive oxygen species (ROS) in both human physiology and pathology and consequently oxidative stress has been assigned a primary or secondary role in a broad spectrum of diseases. Human, animal, explant and in vitro studies indicate that raised oxidative stress is often present in nephrolithiasis and oxalate per se promotes oxidative stress which is substantially retarded by antioxidants [1–3].

Elevated ROS levels and subsequent activation of scavenging enzymes might prevent DNA damage by inducing DNA repair mechanisms [6].

One of the most important key antioxidant enzymes is manganese superoxide dismutase (MnSOD), which is a homotetrameric enzyme that protects mitochondria against ROS by scavenging superoxide anions produced from the electron transport system [6]. This enzyme is the one of several intracellular antioxidant enzymes that cooperate with each other and with dietary antioxidants to protect cells from the damage associated with exposure to ROS. The MnSOD protein is translated in the cytoplasm and transported to mitochondria where it catalyses the dismutation of superoxide anions to hydrogen peroxide ($H_2O_2$) [7, 8].

A common polymorphism exists in the human MnSOD gene. This Ala-9-Val polymorphism is a single-nucleotide substitution of C–T at nucleotide 47, changing the encoded amino acid from Ala (GCT) to Val (GTT) [7]. The amino-acid change occurs within the N-terminal mitochondrial targeting sequence, a 24-amino-acid signal sequence that targets the MnSOD precursor protein for transport into the mitochondria. Mitochondrial localization of MnSOD is required to protect cells from ionizing radiation and other forms of oxidative damage [8–10]. The variant residue is nine amino acids upstream of the cleavage site, hence the polymorphism designation Ala-9-Val. The different polymorphic genotype of MnSOD has previously been associated with various diseases, including diabetus mellitus, tardive dyskinesia, pancreatic, bladder, lung, breast and testicular cancer [9–14]. Variation in the genetic make-up of antioxidant enzymes and environmental exposure to ROS, together may play a role in human stone development. Therefore, in this study, we aimed to investigate the potential role of MnSOD polymorphism as a risk factor in urolithiasis.

## Materials and methods

### Study population

In this prospective study, a total of 66 Turkish patients (33 men and 33 women) with recurrent stone disease (age range 17–52, average 33.2 ± 13.2 years) were enrolled regardless of family history. Serial blood and urine biochemistry tests were performed in order to exclude patients with hypercalcemia, hyperuricemia, hyperoxaluria or hyperuricosuria. Patients who showed symptoms of urinary tract infections during the period of stone treatment were excluded. The stones were analysed with X-ray crystallography at the Tübitak Marmara Research Center (MRC) using a Shimadzu XRD-6000 device with a Cu X-ray tube. A control group was drawn up of 72 healthy volunteers who had no family history of stone disease; renal ultrasonography and routine tests for urinary microscopic hematuria were performed in order to exclude individuals who may have had renal calcification. There were 61 men and 11 women in the control group (age range 19–55, average 34 ± 12.3 years). Informed consent was obtained from all individuals in both groups. The Helsinki Declaration was strictly observed regarding the use of human samples. Also, all studies were undertaken with the approval and institutional oversight of the Institutional Review Board for Ethics of Human Subjects.

We evaluated the subjects in both groups for weight, height and body mass index (BMI). The patients with diseases which could affect calcium metabolism or hyperthyroidism, hyperparathyroidism, hypercorticolism, some metabolic diseases, renal diseases and malignant diseases were excluded from the study. In the selection process of the study population, we eliminated patients who were using drugs such as estrogens, progesterone, glycocorticoids, diuretics, anticonvulsants, vitamin D, antiacid drugs, heparin, prostaglandin preparations, etc., menopausal women and patients with other urinary system diseases. In the control group, immobilization for more than 2 months during the last 5 years, prolonged corticosteroid therapy (>3 months), and alcohol consumption, vitamin D insufficiency and secondary hyperparathyroidism, metabolic acidosis, steroid and anticonvulsant drug usage were exclusion criteria. Donors with a history of genitourinary infection, symptoms or instrumentation were also excluded from the study. Five of 66 patients (5.57 %) had given up smoking in the last 5 years and the rest had never smoked, with 27 of 72 subjects (37.5 %) smoking in control group. The case and control groups were not using antioxidant pills regularly (Table 1).

### DNA isolation and PCR reaction

Genomic DNA was extracted from blood samples using a commercialized kit (QIAamp®, QIAGEN Inc., Valencia, CA). MnSOD polymorphism was then analyzed with polymerase chain reaction (PCR) amplification followed by

**Table 1** Patient characteristics

| | Control | Patients |
|---|---|---|
| Gender (*n*) | | |
| Female | 11 | 33 |
| Male | 61 | 33 |
| Age (mean, range) | 34.0 ± 12.3 | 33.2 ± 13.2 |
| BMI (kg/m$^2$) | 25.1 | 24.8 |
| Smoking (*n*) | 27 | 5 |
| Recurrence (*n*) | 0 | 66 |
| Familial history of urolithiasis (*n*) | 0 | 47 |

restriction fragment length polymorphism (RFLP). For all genotyping, PCR condition containing 25 µM of each primer, 2 mM dNTP, 2.5U Taq DNA polymerase was used. The PCR conditions were as follows; 94°C for 15 min, then 40 cycles of 94°C for 1 min, 58°C for 1 min, 72°C for 1 min, and finally 72°C for 10 min. The PCR products were digested with *Bsa*W1 (New Biological Laboratories, UK), then separated by electrophoresis on a 3% agarose gel. PCR products of MnSOD was 260 bp which digested with *Bsa*W1 at 60°C for 16 h (Fig. 1).

### Statistical analysis

The laboratory researchers were unaware of the case–control status. Differences in MnSOD allele frequencies between cases and controls were determined using a $\chi^2$ test. Departures from Hardy–Weinberg equilibrium were determined by comparing the observed genotype frequencies with expected genotype frequencies calculated using observed allele frequencies. The effects of genetic polymorphism on the risk of stone patients were estimated using odds ratio and its 95% confidence interval, which were derived from unconditional multivariate logistic regression analysis. Deviations from Hardy–Weinberg frequencies in cases and controls were compared using the one sided *P*-value determination test by SPSS version 11.0. The 0.05 level was selected as the point of minimal statistical significance.

## Results

MnSOD genotypes and allele frequencies among stone patients and the disease-free control group were evaluated in this study. In the patient group, 7 (10.6%), 48 (72.7%) and 11 (16.7%) of 66 patients had Ala, Ala/Val and Val genotypes, respectively. These ratios were estimated as 29 (40.2), 38 (52.8%) and 5 (7%) of 72 subjects in the control group with Ala, Ala/Val and Val genotypes, respectively (Table 2).

**Table 2** Observed allelic and expected allelic distribution

| | Ala/Ala | Ala/Val | Val/Val | Ala[a] | Val[a] | $P^b$ value | $P^c$ value |
|---|---|---|---|---|---|---|---|
| Control | 29 | 38 | 5 | 0.67 | 0.33 | 0.28 | 0.045 |
| Patient | 7 | 48 | 11 | 0.47 | 0.53 | 0.001 | |

[a] Allele frequency for Ala, alanine; Val, valine

[b] *P*-value based on $\chi^2$ test for actual versus expected

[c] *P*-value based on *t* test for control versus patients

The genotype distribution was statistically significantly different between stone patients and control subjects, where Val/Val was observed in more stone patients and Ala/Ala was observed in more control subjects ($P < 0.05$). (OR = 8.800 [95% Cl = 2.297 to 33.707]). When genotypic comparison of the patient and control group was made, the Ala genotype was statistically significantly higher in the control group, but Ala/Val alleles were lower in this group (OR = 5.333 [95% Cl = 2.095 to 13.574]). The distribution of Ala/Val and homozygote Val alleles in the patient group was significantly higher than in the control group ($P < 0,05$) (OR = 3.650 [95% Cl = 0.527 to 5.170]).

The frequencies of the genotype in the stone group and control group are shown in Table 3. The frequency of the Ala allele in the control group was statistically higher than that in the patient group and the frequency of Val alleles was determined to be statistically significantly higher in the patient group than in the control group (OR = 2.25 [95% Cl = 1.126 to 4.528]). When MnSOD gene polymorphisms of the patients according to the stone types were compared, no statistically meaningful difference was found ($P > 0.05$) (Table 3).

According to our results, the patient and control groups were carrying 0.47 and 0.67 Ala allele frequency in all the study population. The rest of these groups' Val frequencies were 0.53 and 0.33, respectively. When we calculated expected genotype distribution (Table 2), the control group was closer than the patient group to a population distributed according to Hardy–Weinberg equilibrium ($\chi^2$ test, $P = 0.28$ and 0.001). However when we compared the

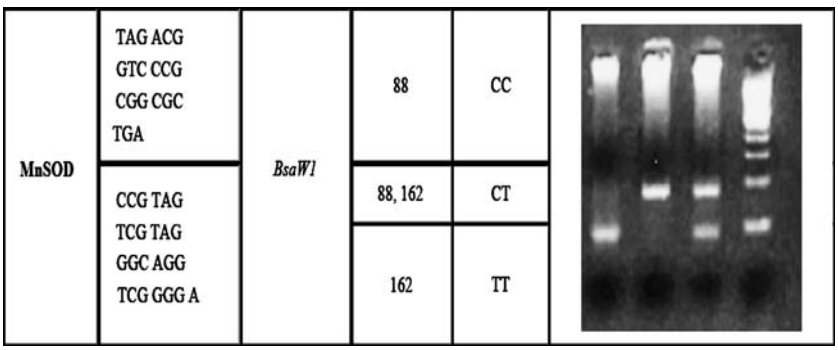


**Fig. 1** This figure represents the 3% agarose gel electrophoresis results for *MnSOD* polymorphism analysis. Homozygote dominant and recessive characters were determined as *CC*, *CT* and *TT* using *Bsa*W1 restriction enzyme

**Table 3** Distribution of stone types in patient group

| Stone type | Ala | Ala/Val | Val | Ala | Val |
|---|---|---|---|---|---|
| L-Cysteine | 1 | 2 | 0 | 0.67 | 0.33 |
| Whewellite | 2 | 28 | 8 | 0.42 | 0.58 |
| Whewellite-urate | 0 | 2 | 0 | 0.50 | 0.50 |
| Uricite | 1 | 1 | 0 | 0.75 | 0.25 |
| Struvite | 0 | 0 | 1 | 0.00 | 1.00 |
| Whewellite-weddelite | 3 | 9 | 1 | 0.58 | 0.42 |
| Hidroxiapatite + whewellite | 0 | 1 | 0 | 0.50 | 0.50 |
| Whewellite-uricite | 0 | 2 | 0 | 0.50 | 0.50 |
| Whewellite-calcium phosphate | 0 | 1 | 0 | 0.50 | 0.50 |
| Calcium phosphate-struvit | 0 | 1 | 0 | 0.50 | 0.50 |
| Calcium phosphate-whewellite | 0 | 0 | 1 | 0.00 | 1.00 |
| Uricite/xantine | 0 | 1 | 0 | 0.50 | 0.50 |
| Control | 29 | 38 | 5 | 0.67 | 0.33 |

genotype distribution ratio for patients versus control groups, these groups were significantly different from each other ($t$ test, $P = 0.045$).

## Discussion

Earlier studies indicated that ROS formation may cause a large number of physiological disorders. Induced oxidative stress may be triggered by nephrolithiasis [1–3]. Hyperoxaluria is a major risk factor for calcium oxalate (CaOx) urolithiasis and is augmented and promoted when combined with cellular degradation products derived from renal tubular injury [15]. Earlier studies have shown that acute and chronic production of CaOx crystal deposition induces lipid peroxidation and therefore the process may play an important role in CaOx stone formation [16]. Lipid peroxidation usually refers to the functional impairment of cellular components by ROS such as superoxide radicals, hydroxyl free radicals and hydrogen peroxide.

Elevated ROS levels have no effect or minor deleterious effects on the physiological process if there is balance between antioxidants or nutritional supplements and ROS. However, if there is an excessive production of ROS because of exposure to toxic agents or depending on the aging process, any pathological inducer or insufficient in vivo defense mechanisms, oxidative stress easily may occur and disturb very important sites of the cell including damage in DNA, induced lipid peroxidation, membrane disruption, and mitochondrial damage [17–20].

In vivo experimental studies in rats showed that green tea supplement decreased urinary oxalate excretion and CaOx deposit formation. Green tea supplement in the daily diet also increased SOD activity compared to stone carrying experimental rats [21]. Induced SOD activity would be a benefit for preventing superoxide radical ($O_2^{\bullet}$) and the concomitant exposure to exogenous antioxidants attenuates the adverse effects of oxalate [4].

Numerous in vitro studies have demonstrated that oxalate toxicity is accompanied by the generation of ROS in renal epithelial cells. Although the antioxidant capacity of the renal cortex of Sprague–Dawley rats was unaffected by hyperoxaluria per se, their renal antioxidant enzymes were attenuated when the oxalate load had induced a limited effect on renal insufficiency. In the same study Wistar rats had diminished levels of SOD only after 3 weeks of study. The important outcome was shown as diminished antioxidant capacity or elevated free radicals which may have a role in the pathogenesis of kidney stone disease. However, the complex redox balance in the whole organism because of metabolic processes and bioavailability is not an easily understandable mechanism [4, 5].

Thamilselvan et al. [4] showed that renal epithelial cells exposed to oxalate or oxalate+COM crystals significantly decreased cellular glutathione peroxidase and catalase activities. The observed changes in renal antioxidant enzymes suggested that the renal epithelial cells are influenced by oxalate-induced free radical stress, and that the antioxidant defense system tries to dispose off the enhanced influx of reactive species in order to reduce oxalate-induced free radical injury. Vitamin E significantly restored these antioxidant enzymes (glutathione peroxidase and catalase) towards the control level, suggesting that the antioxidant defense system of the renal epithelial cells reacts positively to combat oxalate toxicity giving the tissue more resistance against free radical attack [3–5]. It is generally accepted that vitamin E in eukaryotic cells functions as an inhibitor of lipid peroxidation. Vitamin E donates a hydrogen atom to the chain propagating lipid peroxyl radicals giving rise to phenoxyl radicals of the antioxidants [1, 2, 4].

The substitution of T to C in the mitochondrial targeting sequence of the MnSOD gene leads to an amino acid exchange in signal peptide, resulting in a conformational change of the protein [22, 23]. This conformational change in protein structure has a direct effect on the manganese-containing SOD leading to a decrease in the defense capacity of mitochondria. Ala/Val variation in the MnSOD leader signal affects the activity of the enzyme [6, 8]. The conformations of the Ala- and Val-type leader signals have been predicted: the Ala form has an alpha helical structure, a common conformation for mitochondrial leader signals, whereas the Val form might change the conformation from alpha helix to a beta sheet starting from position 16 owing to amino-acid substitution [10, 11]. The Val form is less efficiently transported from mitochondria than the Ala form of the enzyme. Poor signal sequence recognition by a

receptor in the inner mitochondrial membrane results in mistargetting. In addition, inefficient cleavage of a particular signal may reduce the level of enzyme activity of an imported protein, such as MnSOD. Shimoda-Matsubayashi et al. [23] suggested that the Ala allele was associated with higher activity, and that higher activity can induce antioxidant enzyme protection, inducing production of $H_2O_2$ [24–26]. In our study, we found an increased frequency of the Ala allele in the control group (Table 2). Based on the results of the previous studies, it is believed that the reason why Ala allele was more likely to be associated with a low risk for disease development than the Val allele was that the former may induce higher MnSOD activity, thereby leading to an increased capacity for defense against antioxidants [23–27]. If Ala allele frequency was high, this might indicate that the patient would be resistant to oxidative stress and due to this fact, it might provide a clue that the stone-forming risk in the patient would be less.

Wang et al. [26] found statistically significantly increased risks of lung cancer for individuals heterozygous or homozygous for the MnSOD Val allele as well as a statistically significant gene dose–response effect with increasing risk for each additional Val allele. In our study, we found an increased frequency of Val allele in the patient group with high levels. There are many studies that have investigated MnSOD polymorphism related to various diseases [12, 27].

Ambrosone et al. [27] reported that the Val/Val genotype was significantly associated with an increased risk of breast cancer among premenopausal Caucasian women, particularly those who had a low intake of fruits and vegetables and of dietary ascorbic acid and α-tocopherol. We found that in the patient group the frequency of the Val/Val allele was 2.5-fold increased compared to the control groups.

## Conclusion

The genetic predisposition of the MnSOD gene may provide a tool to identify individuals who are at risk for urolithiasis. This study will be a starting point for studies on urolithiasis and antioxidant system failure. Therefore, although we have a limited population number to explain detailed genetic risk factors in disease pathophysiology, we evaluated that an aminoacid exchange in MnSOD protein may be a reason for diminished SOD activity.

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