RESEARCH ARTICLE

Predominant Genotypes and Alleles of Two Functional Polymorphisms in the Manganese Superoxide Dismutase Gene are Not Associated with Thai Cervical or Breast Cancer

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Abstract

Background: Defects of manganese superoxide dismutase (MnSOD) have long been implicated in generation of oxidative stress and risk susceptibility to various cancers. Two functional polymorphisms within the MnSOD gene, including the Val-9Ala of the mitochondrial targeting sequence (MTS) and the Ile58Thr of the exon-3, have been proposed to reduce its enzyme activity and antioxidant potential. Materials and Methods: A high-throughput multiplex SNaPshot® system was developed herein for simultaneous analyses of Val-9Ala and Ile58Thr in a single reaction. Genomic DNA extracted from each whole blood sample of 248 patients including 107 with cervical cancer and 141 with breast cancer and from 136 healthy women as controls was analyzed by the multiplex SNaPshot® system. Results: The Val/Val, Val/Ala genotypes and the Val allele of the MTS were predominant in patients with cervical or breast cancer as well as healthy women in Thailand. The Ile/Ile genotype and the Ile allele of the exon-3 were found in all of them whereas none of the Ile/Thr, the Thr/Thr genotypes and the Thr allele was detected. Genotypic association of both Val-9Ala and Ile58Thr polymorphisms with cervical cancer and breast cancer of these patients comparing to healthy women was not statistically significant ($p<0.05$). Conclusions: The Val/Val, Val/Ala genotypes and the Val allele of the MTS were found predominantly but the Ile/Ile genotype and the Ile allele of the exon-3 were detected in patients with cervical cancer, breast cancer and healthy women in Thailand. These two functional polymorphisms (Val-9Ala and Ile58Thr) in MnSOD gene did not associate with susceptibility risk of these cancer patients in Thailand.

Keywords: Two functional polymorphisms - MnSOD gene - cervical cancer - breast cancer - Thailand

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Introduction

Oxidative stress in human cells is led by an imbalance between production and detoxification of reactive oxygen species (ROS) (Halliwell, 2007). The ROS can initiate the onset of oxidative damage and apoptosis (Kannan and Jain, 2000). Extensive research in the field of oxidative stress caused by ROS has linked them to susceptibility risk of cancer (Kregel and Zhang, 2007; Wu et al., 2007). To protect against toxic effects and control the ROS level at the required physiological concentration, human cells have developed their intrinsically regulated antioxidant system. Single Nucleotide Polymorphisms (SNPs) in candidate genes diminishing antioxidant potential are thought to play an important role in individual variation in cancer susceptibility. Genetic association studies focusing on these SNPs are important tools for targeting the genes responsible for cancer susceptibility.

Manganese superoxide dismutase (MnSOD) is a key antioxidant enzyme which is the first line of defense against ROS or free radicals in human mitochondria (Wan et al., 1994). MnSOD is a nuclear encoded antioxidant enzyme by a nuclear gene on the short arm of chromosome 6 (6q25) as a precursor leading with mitochondrial targeting sequence (MTS) of 24 amino acids. The transport of MnSOD into mitochondria is mediated through the interaction of the MTS with specific receptors on the outer and inner mitochondrial membrane which is finally cleaved from the active enzyme by a matrix-processing peptidase (Church et al., 1992). Mature human MnSOD is a homotetrameric enzyme consisting of 198 amino acids in each subunit required Mn2+ as its cofactor. SNPs in the MnSOD gene, thus diminishing antioxidant potential, can result insufficient for protecting human cells from oxidative damage and apoptosis (Kannan et al., 2000). Two functional SNPs within the MnSOD gene, Val-9Ala in the MTS (Shimoda-Matsubayashi et al., 1996; Rosenblum et al., 1996) and Ile58Thr in exon-3 (Borgstahl et al., 1996), have been previously proposed to associate with various cancers (Han et al., 2007).

Val-9Ala polymorphism in the MTS was previously predicted to be essential for correct transport from cytoplasm to mitochondria. The Val-MnSOD had a beta-sheet structure, while the Ala-MnSOD might change its

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conformation from beta-sheet to alpha-helical structure, a common conformation of mitochondrial leader signals (Shimoda-Matsubayashi et al., 1996; Rosenblum et al., 1996). Sutton et al. (2003) suggested that substitution of Ala to Val may decrease the mitochondrial uptake and MnSOD activity. Study conducted by Sutton et al. (2005) found that the Ala-MnSOD activity was approximately 40% higher than the Val-MnSOD activity in the mitochondrial matrix after import to mitochondria. Lower activity of the Val-MnSOD associated with higher levels of ROS and thus predisposed to a greater risk of cancer has been previously predicted (Bag and Bag, 2008).

The Ile58Thr polymorphism in exon-3 has been suggested to destabilize the human MnSOD tetramer that were responsible for its tetrameric disassembly, decreased thermostability, and increased thermal inactivation (Borgstahl et al., 1996; Grasbon-Frodl et al., 1999). These structural defects could result in ineffective levels of MnSOD activity in vitro and in vivo (Martin et al., 2005). Borgstahl et al. (1996) reported that Thr58-MnSOD exhibited only half the enzymatic activity of the Ile58-MnSOD in mutagenetic analysis. The Ile58 MnSOD not only had three times the specific activity of the Thr58 MnSOD, but also had a higher tumor suppressive effect because they had higher MnSOD activity (Zhang et al., 1999).

A number of techniques have been previously described for separately genotyping Val-9Ala and Ile58Thr polymorphisms within MnSOD gene such as PCR amplification of the polymorphic region, followed by restriction endonuclease analysis or PCR-RFLP (Akyol et al., 2004; Gałecki et al., 2010), heteroduplex-single stranded conformational polymorphism (HEX-SSCP) analysis (Hitzeroth et al., 2007), single-strand conformational polymorphism analysis of the product amplified by PCR (PCR-SSCP) (Knight et al., 2004; Ventriglia et al., 2006), allele-specific PCR (ASP) assay (Rosenblum et al., 1996), the PCR product hybridized with allele-specific oligonucleotide (ASO) probes (Hiroi et al., 1999), DNA sequencing (Wang et al., 2010) and a real-time PCR using TaqMan allelic discrimination assay (Martin et al., 2005; Bastaki et al., 2006). Automated DNA sequencing (Sanger,1997) represents the "gold standard" system of SNPs analysis; however this procedure is time-consuming and expensive.

Cancer in Thailand is becoming a significant health problem. Since 2000, cancer has ranked as the primary cause of deaths among the Thai citizens. With regard to leading cancers in Thailand for female population, the highest incidence falls into cervical cancer, with an estimated 10,000 new cases and 5,000 deaths each year. The incidence of cervical cancer in Thailand is relatively high in comparison with other developing countries in Southern and Southeastern Asia. Breast cancer is the second most common form of cancer among Thai women after cervical cancer. The incidence rate of breast cancer has increased gradually over the past 5-10 years, which may be related to the change of lifestyle and diet (Khuhaprema, 2008).

In our study, two targeted SNPs of both MTS and exon-3 within MnSOD gene of patients with cervical cancer, breast cancer comparing to healthy women in Thailand were simultaneously genotyped by the high-throughput multiplex SNaPshot® system recently developed herein. The chemistry is based on the dideoxy single-base extension of an unlabeled oligonucleotide primer (or primers). Each primer binds to a complementary template in the presence of fluorescently labelled ddNTPs and AmpliTaq DNA polymerase and the polymerase extends the primer just by one nucleotide, adding a single ddNTP to its 3'end. Results were visualized by electrophoreses of samples on ABI PRISM 310 Genetic Analyzer (Applied Biosystems) and data were analyzed by Genotyper version 3.7 software (Applied Biosystems). The SNaPshot® system involved differential fluorescent labeling of the four ddNTPs in a single base extension reaction allowing fluorescent detection of the incorporated nucleotide. The capillary electrophoretic mobility of the extension product was usually present in the homozygous state yielding one peak and the heterozygous state yielding two peaks. Predominant genotypes and alleles of two functional SNPs at MTS and exon-3 within MnSOD gene of patients with cervical cancer, breast cancer comparing to healthy women in Thailand were analyzed and their association with each cancer risk were statistically compared.

Materials and Methods

Study subjects

The study was approved by Research Review Board and Ethical Committee of Rajavithi Hospital, Bangkok, Thailand. All the presented subjects were accepted to participate and written informed consent was obtained from each subject. Thai patients with histopathologically confirmed cancer (N=248) including cervical cancer (N=107) and breast cancer (N=141) were recruited from Rajavithi Hospital. Thai healthy women (N=135) aged 20-75 years had applied in check-up program of Rajavithi Hospital. These healthy women were defined by physical examination, laboratory examination and historical questionnaires without consideration of occupation and hometown origin. They were biologically unrelated to the study patients and were cancer free participants. The following serum quantifications were conducted: glucose, urea, creatinine, uric acid, albumin, cholesterol, triglycerides, total protein, total bilirubin, directed bilirubin, high-density lipoproteins (HDL) cholesterol, low-density lipoproteins (LDL), aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP). All these clinical chemistry tests were analysed by an automatic analyzer (Hitachi 917) using commercial diagnostic reagents obtained from Hoffmann-La Roche Ltd, Switzerland. These tests were used as screening profile measurements for the diagnosis of the clinically healthy subjects.

DNA extraction and amplification of two polymorphic regions in MnSOD gene

Two ml of each whole blood sample was collected from each subject in sterile EDTA-coated vacutainer. DNA was extracted by standard procedure of guanidinium thiocyanate-silica based method (Boom et al., 1990) and

stored at -20°C until used for genotyping. Genotypings of two SNPs (Val9Ala and Ile58Thr) within MnSOD gene were carried out employing our high-throughput multiplex SNaPshot® system newly developed in this study. According to the map of Val-9Ala in MTS and Ile58Thr in exon-3 of MnSOD gene (Figure 1), all the primers for PCR amplification (Table 1) and multiplex SNaPshot® reactions (Table 2) were designed following the published sequence of human MnSOD gene (Genbank accession number S77127) (Wan et al., 1994). The primer sequences for PCR amplification (Table 1) as well as SNaPshot® extension reactions (Table 2) were designed to have an annealing temperature around 60°C using Primer 3 and BLAST web-based program (http://www.ncbi.nlm.nih.gov/tools/primer-blast). The primers for multiplex SNaPshot® reactions were designed to anneal specifically adjacent to the nucleotide at each targeted SNP. Two polymorphic regions (Val9Ala and Ile58Thr) of MnSOD gene were amplified using 9700 Thermalcycler (Applied Biosystem). The 246 bp-polymorphic region of Val-9Ala in MTS was amplified using the F-MTS and the R-MTS primers (Table 1). The 248 bp-polymorphic region of Ile58Thr in exon-3 was amplified using the F-Exon3 and R-Exon3 primers (Table1). The PCR reactions were carried out in a 25 μl volume of 1X PCR buffer pH 8.4, 1.5mM $MgCl_2$, 50mM KCl, 0.2mM of dNTPs, 0.4 pM of each primer and 2.5 U HotStart Taq DNA polymerase (Qiagen) with a denaturation of 95°C for 15 min, followed by 30 cycles of amplification at 95°C for 30 sec, 56°C for 30 sec, 72°C for 1 min and finally 10 min at 72°C.

Genotyping of Val-9Ala and Ile58Thr by high-throughput multiplex SNaPshot® system

The high-throughput multiplex SNaPshot® system was developed herein using the SNaPshot™ kit following the manufacturer's instruction (Applied Biosystem) and a 9700 Thermalcycler (Applied Biosystem). Primer extension reaction (10 μl) was performed with 4 μl of treated PCR product, 5 μl of SNaPshot® reaction mix and 0.5 μM of extension primer for each SNP (Table 2). The following cycling program was conducted: 25 cycles of denaturation at 95°C for 5 sec, annealing at 50°C for 5 sec and extension at 60°C for 30 sec. After the primer extension, a post treatment to prevent unincorporated terminators from co-migrating with the extended primers and producing high background signal was done by removing the 5'-phosphoryl group of the ddNTPs. For the treatment, a 10 μl of the reaction products was incubated with 1U shrimp alkaline phosphatase (Amersham Biosciences) and 2 units of exonuclease I (Amersham Pharmacia Biotech) for 45 min at 37°C followed by 15 min at 75°C for enzyme inactivation. The SNaPshot®

Table 1. Oligonucleotide Sequences of Primers for PCR Amplification of the Val-9Ala in MTS and the Ile58Thr in exon-3 of Human MnSOD Gene Designed Following the Published Sequence of Human MnSOD Gene (Genbank accession number S77127)

Human MnSOD Gene	PCR primers	PCR products (bps)
F-MTS	5'-AGCCCAGCCTGCGTAGAC-3'	246
R-MTS	5-TACTTCTCCTCGGTGACG-3'	
F- Exon3	5'-AGCTGGTCCCATTATCTAATAGC-3'	248
R-Exon3	5'-TGTAGATAAGGGTGCACC-3'	

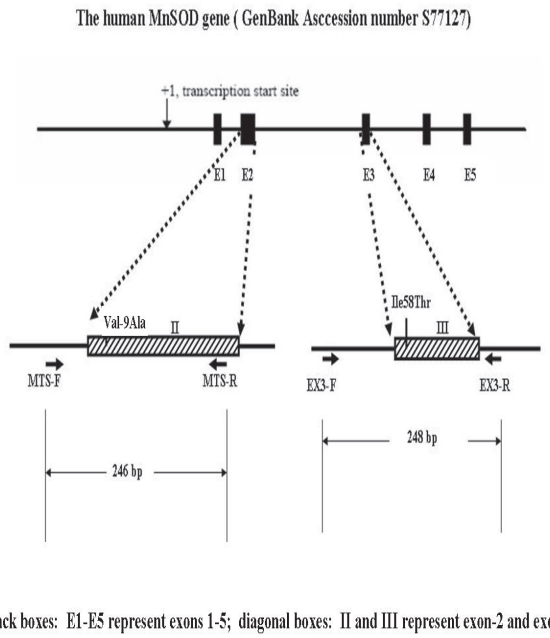

Figure 1. Mapping of Human MnSOD Gene. Two regions of interest were amplified by PCR using the primers (F-MTS and R-MTS) and the primers (F-Exon3 and R-Exon3) which generated the 246 and 248 bp products containing the Val-9Ala in MTS and Ile58Thr in Exon-3 respectively. The oligonucleotide sequences of all these four primers (Table 1) were designed following the published sequence of human MnSOD gene from Genbank accession number S77127 (Wan et al., 1994)

Table 2. Oligonucleotide Sequences of the Extension Primers for Multiplex SNaPshot® System Designed Following the Published Sequence of Human MnSOD Gene (Genbank accession number S77127)*

Human MnSOD Gene	Extension primers		Size (bps)	Polymorphisms SNPs	Amino acids
MTS (Val-9Ala):	S-MTS	F: 5'-ACCAGCAGGCAGCTGGCTCCGG-3'	22	T	Val
				C	Ala
	RS-MTS	R: 5'-CTTCTGCCTGGAGCCCAGATACCCCAAA-3'	28	A	Val
				G	Ala
Exon-3 (Ile58Thr):	S-Exon3	F: 5'-GAGATGTTACAGCCCAGA-3'	18	T	Ile
				C	Thr
	RS-Exon3	R: 5'-AACTTCAGTGCAGGCTGAAGAGCT-3'	24	A	Ile
				G	Thr

*Both the sense and the antisense primers for multiplex SNaPshot® reactions were designed to anneal immediately adjacent to the nucleotide at each targeted SNP of the Val-9Ala in MTS and the Ile58Thr in exon- 3 of human MnSOD gene

product (2 µl) of each sample was mixed with 9 µl of Hi-Di™ formamide and 0.5 µl of GenScan-120LIZ size standard (Applied Biosystems) and denatured at 95°C for 5 min. The SNaPshot® products were detected based on four different fluorochromes to identify each base. The fluorescently labeled fragments were resolved by capillary electrophoresis on ABI PRISM 310 Genetic Analyzer (Applied Biosystems). The SNaPshot® products were injected electrokinetically in a capillary using POP 4™ polymer and electrophoresed at 15 kV and 9mA for 20 min at 60°C. The resulting data was analyzed with Genotyper version 3.7 software (Applied Biosystems).

Statistical analysis

Allele frequencies were calculated by the gene-counting method. The genotypes and allelic frequencies were compared among two groups using the chi-square statistical test. Statistical analyses were performed using SPSS16.0 (SPSS Inc. Michigan, IL, USA). Confidence intervals of 95% were determined for genotype comparisons. A value of $p<0.05$ was considered as statistically significant.

Results

Two functional SNPs in MnSOD gene of 248 cancer patients including 107 cervical cancer and 141 breast cancer as well as 135 healthy women in Thailand were analysed by the multiplex SNaPshot® system recently developed herein. At first, the attempts were done on development of the SNaPshot® system for detecting a single SNP, the Val-9Ala of MTS or the Ile58Thr of Exon3 separately. The multiplex SNaPshot® system for detecting two directions of the Val-9Ala in MTS was performed by adding both the sense (S-MTS) and the anti-sense (RS-MTS) extension primers (Table 2) into the same SNaPshot® reaction. The multiplex electropherogram of the heterozygous Val/Ala genotype obviously showed four peaks of 22C (black), 22T (red), 28G (blue) and 28A (green) (Figure 2). For detecting two directions of the Ile58Thr in the exon-3, the multiplex electropherogram of the homozygous Ile/Ile genotype adding both the sense (S-Exon3) and anti-sense (RS-Exon3) extension primers (Table 2) into the same SNaPshot® reaction showed two peaks of 18T (red) and 24A (green) (Figure 3). In the present study, the heterozygous Ile/Thr and the homozygous Thr/Thr genotypes were not found in all of these patients and healthy women. Finally, genotyping two SNPs simultaneously was developed by adding the anti-sense extension primer of the Val-9Ala of MTS (RS-MTS) and the anti-sense extension primer of the Ile58Thr of the exon-3 (RS-Exon3) (Table 2) into the same SNaPshot® reaction. The multiplex electropherograms of the heterozygous Val/Ala with the homozygous Ile/Ile, the homozygous Val/Val with the homozygous Ile/Ile and the homozygous Ala/Ala with the homozygous Ile/Ile showed

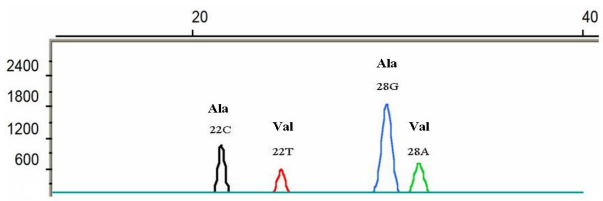

Figure 2. Electropherogram Showing Multiplex SNaPshot® Reaction Adding Template of MTS (FR-MTS) with 2 Primers of the Sense (S-MTS) and the Antisense (RS-MTS) into a Single Reaction Tube of a Known Sample with Heterozygous Val/Ala Genotype. The SNaPshot® products were analyzed by ABI PRISM 310 Genetic Analyzer and GeneScan v3.7. Two peaks of the sense primer (black and red) and two peaks of the antisense primer (blue and green) of MTS were shown

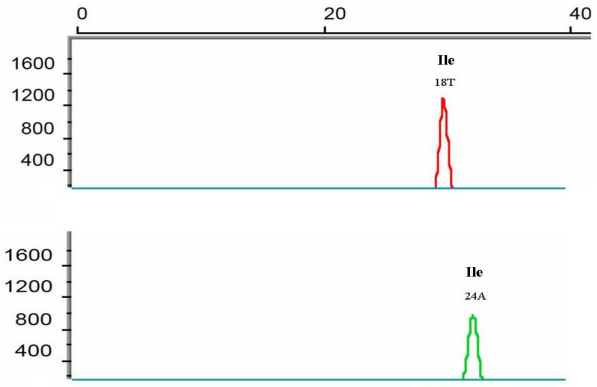

Figure 3. Electropherograms Showing the Multiplex SNaPshot® Reaction Adding Template of exon-3 (FR-Exon3) with 2 Primers of the Sense (S-Exon3) and the Antisense (RS-Exon3) into a Single Reaction Tube of a Known Sample with Homozygous Ile/Ile Genotype. The SNaPshot® the sense primer (S-Exon3) for 18T (red) and only one peak of the antisense primer (RS-Exon3) for 24A (green) were shown

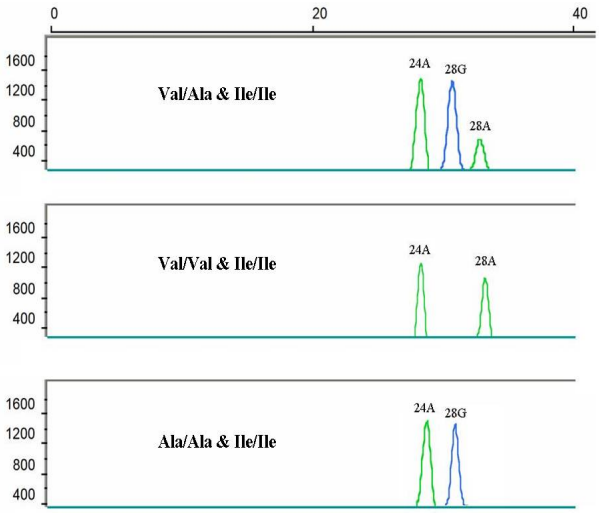

Figure 4. Electropherograms Showing the Multiplex SNaPshot® Reaction Adding Mixed Templates of MTS (FR-MTS) and exon3 (FR-Exon3) Fragments and 2 Primers of the Antisense (RS-MTS) for the Val-9Ala and the Antisense (RS-Exon3) for the Ile58Thr into a Single Reaction of a Known Sample with Heterozygous Val/Ala Genotype and Homozygous Ile/Ile Genotype. The SNaPshot® products were analyzed by ABI PRISM 310 Genetic Analyzer and GeneScan v3.7 of the antisense (RS-MTS) and the antisense (RS-Exon3). Two peaks of 28G (blue) and 28A (green) represented for the Val and Ala allele respectively and only one peak of 24A (green) represented for the Ile allele

all corresponding peaks of Val, Ala and Ile alleles (Figure 4).

Genotypic and allelic distributions of both MTS (Val-9Ala) and exon-3 (Ile58Thr) of patients with cervical cancer and breast cancer comparing to healthy women in Thailand have been firstly reported herein (Table 3). The genotypes of 135 healthy women were 62.2% for the Val/Val, 35.6% for the Val/Ala and 2.2% for the Ala/Ala. Their allelic frequencies of the Val and Ala alleles were 0.80 and 0.20 respectively (Table 4). The genotypes of 248 patients including 107 cervical cancer and 141 breast cancer in Thailand were 58.2-59.8% for the Val/Val, 36.5-38.3% for the Val/Ala, and 3.5-3.7% for the Ala/Ala. Their allelic frequencies of the Val and Ala alleles were 0.77-0.78 and 0.22-0.23 respectively (Table 4). In both cancer patients and healthy women in Thailand, the Val/Val, Val/Ala genotypes and the Val allele frequency were found predominantly rather than the Ala/Ala genotype and the Ala allele. Regarding the second polymorphism of MnSOD gene in all 248 cancer patients and 135 healthy women in Thailand, only the Ile allele and the Ile/Ile genotype were detected whereas the Thr allele or the Ile/Thr or the Thr/Thr was not found (Table 4). Genotypic and allelic frequencies of both MTS (Val-9Ala) and exon-3 (Ile58Thr) of patients with cervical cancer and breast cancer comparing to healthy women in Thailand were not significantly different at p-values <0.05 (Table 4).

Table 3. Genotypes of the Val-9Ala Polymorphisms in MTS and the Ile58Thr Polymorphisms in exon-3 of MnSOD Gene Comparing between 248 Cancer Patients with Cervical Cancer (N=107), Breast Cancer (N=141) and 135 Healthy Women in Thailand Analyzed by the High-Throughput Multiplex SNaPshot® System Recently Developed in This Study

Polymorphisms of MnSOD gene		Thai healthy women (N=135)	Thai cancer patients (N=248) Cervical cancer (N=107)	Breast cancer (N=141)
Val-9Ala Genotypes:	Val/Val	84	64	82
	Val/Ala	48	39	54
	Ala/Ala	3	4	5
Ile58Thr Genotypes:	Ile/Ile	221	107	141
	Ile/Thr	0	0	0
	Thr/Thr	0	0	0

Table 4. Statistically Analysis Comparing Genotypic as Well as Allelic Frequencies of the Val-9Ala Polymorphisms in MTS and the Ile58Thr in exon-3 of MnSOD Gene between 248 Cancer Patients with Cervical Cancer (N=107), Breast Cancer (N=141) and 135 Healthy Women in Thailand*

Polymorphisms of MnSOD gene	Thai healthy women (N=135)	Thai cancer patients (N=248) Cervical cancer (N=107)		Breast cancer (N=141)	
		Frequencies	p-values	Frequencies	p-values
Val-9Ala Genotypes					
Val/Val	62.20%	59.80%	0.98	58.20%	0.96
Val/Ala	35.60%	36.50%	0.99	38.30%	0.96
Ala/Ala	2.20%	3.70%	0.75	3.50%	0.77
Val-9Ala alleles					
Val allele	0.8	0.78	0.99	0.77	0.98
Ala allele	0.2	0.22	0.95	0.23	0.93
Ile58Thr Genotypes					
Ile/Ile	100%	100%	NA	100%	NA
Ile/Thr	0%	0%	NA	0%	NA
Thr/Thr	0%	0%	NA	0%	NA
Ile58Thr alleles					
Ile allele	1	1	NA	1	NA
Thr allele	0	0	NA	0	NA

*Confidence intervals of 95% were determined for genotype comparisons. A value of p<0.05 was considered as statistically significant

Discussion

Defect of manganese superoxide dismutase (MnSOD), the important primary antioxidant enzyme in the detoxification pathway of mitochondrial ROS, have long been discussed very often for its association with cancer susceptibility in caucasian ethnics. Two functional SNPs within the MnSOD gene, Val-9Ala in MTS and Ile58Thr in exon-3, have been proposed to reduce its antioxidant potential. In this study, the Val-9Ala genotypic frequencies of cancer patients were 58.2-59.8%, 36.5-38.3% and 3.5-3.7% for the Val/Val, the Val/Ala and the Ala/Ala respectively and the values were not significantly different between cervical and breast cancer. The frequencies of healthy women were 62.2%, 35.6% and 2.2% for the Val/Val, the Val/Ala and the Ala/Ala respectively (Table 3) and the values were as same as our previous report analyzed by real-time PCR using TaqMan allelic discrimination assay (Attatippaholkun, 2012). The allelic frequencies of these cancer patients were 0.77-0.78 for the Val and 0.22-0.23 for the Ala and those of healthy women were 0.80 for the Val and 0.20 for the Ala. The allelic frequencies in MTS of 248 cancer patients (107 cervical cancer and 141 breast cancer) and 135 healthy women in Thailand reported herein were closely similar to those previous reports in a few Asian ethnics such as China (Ho et al., 2006), Japan (Fujimoto et al., 2008) and Korea (Pae et al., 2007). Genotypic frequencies (Val/Val, Val/Ala and Ala/Ala) as well as allelic frequencies (Val and Ala) were not significantly different ($p<0.05$) among patients with cervical cancer, breast cancer and healthy women in Thailand. On the contrary, previous studies in Caucasian ethnics such as USA (Mikhak et al., 2008), United Kingdom (Elsakka et al., 2007), Germany (Osterreicher et al., 2007), Ireland (Murphy et al., 2007), Italy (Ventriglia et al., 2006), Finland (Kakko et al., 2003), Russia (Chistyakov et al., 2001), Turkey (Zejnilovic et al., 2007), South Africa-Xhosa (Hitzeroth et al., 2009), Moroccan (Ezzikouri et al., 2008) and Australia (Johnatty et al., 2007) reported that allelic frequencies of the Val (0.44-0.59) and the Ala (0.41-0.56) were approximately equal. The Val frequency of Asian ethnics as 0.75-0.79 was higher than 0.44-0.58 of Caucasian ethnics whereas the Ala frequency of Asian ethnics as 0.21-0.25 was lower than 0.41-0.59 of Caucasian ethnics (Attatippaholkun, 2012). Sutton et al. (2003) suggested that the Val-MnSOD was less efficiently transported and targeted into mitochondria than the Ala-MnSOD. So, the Val-MnSOD was previously proposed to be related to reduced its

content in mitochondria and its activity from high to low was predicted as the Ala/Ala, the Val/Ala, and the Val/Val genotypes. Since the Val allele was the predominant allele rather than the Ala allele in Asian ethnics including Thai. Therefore, the previous prediction by Sutton et al. (2005) involving the Val-9Ala polymorphisms to be important for targeting the enzyme into the mitochondria and the Ala-MnSOD activity was approximately 40% higher than the Val-MnSOD activity in the mitochondrial matrix after import to mitochondria were still unclear. The Val-MnSOD was not associated with higher levels of oxidative stress and thus predisposed to a greater risk of cancer as previously proposed (Bag et al., 2008). In addition, our previous study reported that superoxide dismutase activity and total antioxidant status of both healthy subjects with the Val/Val and the Val/Ala genotypes in Thailand were not significantly lower than those with the Ala/Ala genotypes ($p<0.05$) (Attatippaholkun, 2012).

Regarding the polymorphisms in exon-3 within MnSOD gene, the Ile58Thr variant was proposed to affect stability of MnSOD tetramer and reduce MnSOD activity. However, the Thr allele previously studied has not been reported until now. In cancer patients and healthy women in Thailand studied herein, the Ile58Thr sequence change was not found and no other sequence variant was detected in exon-3 of MnSOD gene. Our data were in concordance with results of Grasbon-Frodl et al. (1999) who reported the Ile/Ile variant only in 63 random German subjects as well as Parboosingh et al. (1995) who failed to detect any sequence variation in exon-3 of the MnSOD gene by SSCP in 107 Parkinson disease patients including 40 sporadic and 67 familial cases.

However, much more exhaustive studies involving a large sample size and considering the variables such as polymorphisms in linkage disequilibrium, gene-gene interactions and environmental exposures are required to acquire a total knowledge on the link between MnSOD polymorphisms and cancer susceptibility risk. It would be a premature conclusion to remark that these two functional SNPs of MnSOD gene were associated with higher cancer susceptibility risk and might have role in cancer development due to the fact that MnSOD constituted a first-line defense against ROS. Some polymorphisms might be associated with cancer susceptibility risk for some ethnic communities only not for other ones (Parboosingh et al., 1995).

These two functional SNPs of the MnSOD gene were simultaneously genotyped by our recently developed multiplex SNaPshot® system designed to interrogate Val-9Ala of MTS and Ile58Thr of exon-3 on two templates in a single reaction. The primers were designed in both the forward and reversed directions to terminate directly 5'of each SNP site. The critical point was primer design and the primers used in a single reaction for multi-loci interrogation were different significantly in length to avoid overlap between the final SNaPshot® products. Automatic genotyping of each sample was analyzed by Genotyper Software v.3.7 using the 5th dye-labelled GeneScan-120LIZ (Applied Biosystems) as internal size standards. The multiplex SNaPshot® system developed herein had numerous advantages over other techniques previously reported. Firstly, a single tube reaction was used for each sample that implied the reduction number of steps and handling. Secondly, high-throughput genotyping was possible as the automated process in 96-well plates. Lastly, the interpretation of the peak patterns was very simple and the method was sensitive and low cost. So, this approach was a high-throughput SNP genotyping technology which became a useful tool economically suitable for large-scale population study.

In conclusion, the Val/Val, Val/Ala genotypes and the Val allele were predominant rather than the Ala/Ala genotype and the Ala allele whereas only the Ile/Ile genotype and the Ile allele were found in all these cancer patients with cervical cancer, breast cancer and healthy women in Thailand. Association among these genotypes comparing patients with cervical cancer and breast cancer to healthy women in Thailand was not statistically significant. Multiplex SNaPshot® system has been successfully developed for high-throughput genotyping these two targeted SNPs of MnSOD gene simultaneously in a single SNaPshot® reaction .

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