ORIGINAL INVESTIGATION

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Evaluation of the microsomal glutathione S-transferase 3 (*MGST3*) locus on 1q23 as a Type 2 diabetes susceptibility gene in Pima Indians

Abstract Elevation of plasma glucose concentration may induce generation of oxygen-free radicals, which can play an important role in the progression of diabetes and/or development of its complications. Various glutathione transferases utilize the availability of reduced glutathione for the cellular defense against oxygen-free radicals. One such enzyme is microsomal glutathione S-transferase 3 encoded by *MGST3*, which maps to chromosome 1q23, a region linked to Type 2 diabetes mellitus (T2DM) in Pima Indians, Caucasian, and Chinese populations. We investigated the *MGST3* gene as a potential susceptibility gene for T2DM by screening this locus for single nucleotide polymorphisms (SNPs) in diabetic and non-diabetic Pima Indians. We also measured the skeletal muscle *MGST3* mRNA level by Real-Time (RT) PCR and its relationship with insulin action in non-diabetic individuals. We identified 25 diallelic variants, most of which, based on their genotypic concordance, could be divided into three distinct linkage disequilibrium (LD) groups. We genotyped unique representative SNPs in selected diabetic and non-diabetic Pima Indians and found no evidence for association with T2DM. Furthermore, inter-individual variation of skeletal muscle *MGST3* mRNA was not correlated with differences in insulin action in non-diabetic subjects. We conclude that alterations of *MGST3* are unlikely to contribute to T2DM or differences in insulin sensitivity in the Pima Indians.

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Introduction

Generation of free radicals such as superoxide anion ($O_2^·$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($OH^·$) occurs continuously in most cells as part of their normal function. However, excess of free radicals originating from endogenous or exogenous sources might play a role in the pathogenesis of some common diseases. Oxidative stress, arising as a result of an imbalance between free radicals and anti-oxidant defenses, is associated with damage to lipids, proteins and nucleic acids, which could contribute to cellular dysfunctions leading to the pathophysiology of various diseases including atherosclerosis, cancer, and diabetes mellitus (West 2000; Young and Woodside 2001). Regarding glucose homeostasis, it has been shown that micromolar concentrations of H_2O_2 impair the insulin responsiveness of 3T3-L1 adipocytes and L6 muscle cells, thus leading to insulin resistance (Rudich et al. 1997; Blair et al. 1999; Hansen et al. 1999). Furthermore, accelerated production of reactive oxygen derivatives due to hyperglycemia in diabetic patients could play an important role in the etiology of diabetic complications such as nephropathy, retinopathy, neuropathy and diabetes-accelerated atherosclerosis (Baynes and Thorpe 1999; Aronson and Rayfield 2002). This hypothesis is also supported by the fact that many biochemical pathways linked to hyperglycemia, including glucose auto-oxidation, activation of polyol pathway, prostanoid synthesis and protein glycation can increase the production of free radicals (Brownlee 2001).

One of the main defense mechanisms against oxidative stress involves reduced glutathione (GSH), which is a ubiquitous γ-glu/cys/gly tri-peptide moiety (Lu 1999). GSH is conjugated with various xenobiotic and endobiotic reactive compounds by different isoforms of glutathione S-transferases (GST). One such enzyme in humans is the microsomal glutathione S-transferase 3 (EC.2.5.1.18) encoded by *MGST3*, located on chromosome 1q23 (Jakobsson et al. 1997).

The deduced 152 amino acid of the *MGST3* protein has a molecular mass of 16.5 kDa and shares 36% and 22% amino acid sequence identity with *MGST2* and *MGST1*,

respectively. *MGST3* was found to be predominantly expressed in skeletal muscle, heart and adrenal cortex. In the presence of reduced glutathione, *MGST3* was found to possess glutathione-dependent peroxidase activity (Jakobsson et al. 1997) and several isoforms of this enzyme are known to prevent the destruction of cell membranes by removing lipid peroxides (Young and Woodside 2001). *MGST3* also has a leukotriene C4 synthase activity, producing mediators of various inflammatory and immediate hypersensitivity processes (Ford-Hutchinson 1990), where oxidative stress is a component.

Due to the localization of *MGST3* on 1q21–23, a region linked to T2DM in Pima Indians, several Caucasian populations and Chinese people (Hanson et al. 1998; Elbein et al. 1999; St. Jean et al. 2000; Vionnet et al. 2000; Wiltshire et al. 2001; Xiang et al. 2002), and the apparent role of this enzyme in scavenging lipid peroxides (Jakobsson et al. 1997), we investigated the *MGST3* as a potential susceptibility gene for T2DM. In this paper, we report the screening of this locus for variants in diabetic and non-diabetic Pima Indians, and quantitative measurements of skeletal muscle *MGST3* mRNA level with respect to insulin action in non-diabetic subjects.

Materials and methods

Subjects

Diabetic and non-diabetic Pima Indians selected for the genomic screening of *MGST3* were participants of the ongoing longitudinal studies of T2DM conducted among members of the Gila River Indian Community since 1965 (Hanson et al. 1998). Diabetes was diagnosed according to World Health Organization (WHO) criteria as described previously (Hanson et al. 1998). This study was approved by the Institutional Review Board of the National Institutes of Diabetes and Digestive and Kidney Diseases (NIDDK) and the Tribal Council of the Gila River Indian Community, and all subjects gave written informed consent before participation.

Clinical measurements

Non-diabetic volunteers were admitted to the clinical research unit and after 2 to 3 days on a weight maintaining diet (containing 50% carbohydrates, 30% fat, and 20% protein), followed by a 12-h overnight fast, oral glucose tolerance tests were carried out. Plasma glucose and insulin concentrations were measured in blood samples drawn prior to 75 g glucose ingestion, and at 30, 60, 120, and 180 min thereafter.

Insulin action was measured at physiologic and supraphysiologic insulin concentrations during a two-step hyperinsulinemic-euglycemic glucose clamp as previously described (Lillioja et al. 1993). After an overnight fast, a primed continuous intravenous insulin infusion was administered for 100 min at a constant rate of 40 mU/m^2 body surface area/min (low dose; M-low), followed by a second insulin infusion for 100 min at 400 mU/m^2/min (high dose; M-high). These infusions achieved steady-state plasma insulin concentrations of $151 \pm 52\,\mu$U/ml and $2{,}699 \pm 1{,}158\,\mu$U/ml (Mean $\pm$ SD) at low and high doses, respectively. Plasma glucose concentrations were maintained at about 100 mg/dl with a variable infusion of a 20% glucose solution. The rates of insulin-stimulated glucose disposal for low and high insulin infusion dosages were calculated for the last 40 min of each phase as previously described (Lillioja et al. 1993). Insulin secretion was measured during intravenous glucose tolerance test as described (Lillioja et al. 1993).

RNA extraction from skeletal muscle biopsies and cDNA synthesis

In the morning after an overnight fast, percutaneous skeletal muscle biopsies of the vastus lateralis muscle were carried out using Bergstrom needles (Depuy) under local anesthetic with 1% lidocaine. The biopsy was cleaned of any visible fat, rinsed in sterile 0.9% NaCl solution, snap-frozen in liquid nitrogen and stored at –70°C. Total RNA was isolated from the frozen tissues using Trizol Reagent (Life Technologies, Gaithersburg, MD, USA). The quality of the RNA was assessed by agarose gel electrophoresis. For a few samples, polyA$^+$ mRNA was extracted from the total RNA using Oligotex direct mRNA Midi/Maxi kit (Qiagen, Santa Clarita, CA, USA). Oligo dT-primed cDNA was subsequently synthesized using the Advantage RT-for-PCR kit (Clontech, Palo Alto, CA, USA) from $1\,\mu$g of RNA or $0.2\,\mu$g of polyA$^+$ mRNA samples. PCR amplification of *GAPDH* transcript using *GAPDH* primers provided in the Advantage RT-for-PCR kit was carried out to confirm successful cDNA synthesis.

Quantitative Real-Time RT-PCR

Transcript level quantification for *MGST3* was done using the ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). The primer pairs and probe for *MGST3* were designed using Primer Express software (Applied Biosystems); the forward primer was 5′-ACCCTGAAAATGGGCA-CATCT -3′, reverse primer 5′-TGGTAAACACCTCCAACAGC-TAGA-3′, and probe 5′(Fam)-CATTCAGCGAGCCCACCAG-AACACGT-(Tamra)p-3′. The relative abundance of *MGST3* was normalized to the expression level of human β-actin, since the latter did not significantly correlate with any clinical parameters of the studied subjects and the primers did not amplify any detectable product from RNA alone (data not shown). RT-PCR of the β-actin transcript utilized forward primer 5′-TCACCCACACTGTGCC-CATCTACGA-3′, reverse primer 5′-TGCCATGACGGGAGAC-CTCTT-3′, and probe 5′(Vic)-ATGCCCCCCCCATGCCATCCT-GCGT(Tamra)p-3′. A standard curve for each primer-probe set was generated by serial dilution of a cDNA from one healthy subject and the measurements were done in triplicate. Subsequently, each individual sample was run in duplicate and the mean values were used to calculate a relative transcript level as a ratio between *MGST3* and β-actin. Observed differences were assessed for statistical significance using the Statistical Analysis System of the SAS Institute (Cary, NC, USA).

PCR, variant screening and sequencing

The *MGST3* locus was screened for variants by denaturing HPLC (d-HPLC) and direct sequencing of PCR products amplified from genomic DNA. To amplify products for d-HPLC, we used two separate pooled DNA samples (case/control and sib-pairs), each comprised of a mix of DNA from five different diabetic and five non-diabetic Pima Indians. Human chromosome 1-specific somatic cell hybrid DNA (#NA13139; Coriell Cell Repositories, Camden, NJ) was used as a „hemizygous" non-polymorphic control representing a single allelic version of all variants on this chromosome. PCR products were scanned for sequence variants by d-HPLC using the WAVE DNA fragment analysis system (Transgenomic, Omaha, NE, USA) as previously described (Wolford et al. 2000). SNPs were validated by sequencing of PCR products from individual DNA samples with an ABI Automated Sequencer model 3700 using the ABI Prism Big Dye Terminator Cycle Sequencing kit (PE Applied Biosystems, Foster City, CA, USA).

Variants genotyping and association analysis

Variants that created restriction sites were genotyped by PCR-RFLP using the appropriate combination of primers and restriction enzyme. For the present study, association analyses were conducted

using groups of individuals (50 cases and 50 controls) who contributed most significantly to the linkage in Pima Indians. The polymorphism showing a significant association with T2DM ($p<0.05$) in the cases and controls was further genotyped in the entire linkage set comprising 1,338 subjects. Differences in allelic frequencies between the affected and unaffected groups were analyzed by Chi-square test (SAS Institute, Cary, NC, USA).

Results and Discussion

In diabetes, oxidative stress seems mainly due to an increased generation of plasma free radicals and a decreased antioxidant scavenging mechanism. Lipid peroxidation is one of the free radical damage products and glutathione dependent peroxidase isoforms are the main scavengers of hydrogen peroxide and/or lipid peroxides in the cell. In this study, we investigated *MGST3* as a potential susceptibility locus for T2DM because of its glutathione dependent peroxidase function and its position within a region on 1q linked with T2DM in several populations.

The genomic structure of the *MGST3* locus was determined by comparison of the human *MGST3* cDNA (GenBank accession #NM_004528) with available genomic sequences. The reported cDNA encoding the *MGST3* has a 459 bp open reading frame encoding a protein of 152 amino acids (Jakobsson et al. 1997). Using public database searches, we identified a BAC clone 306I1 (#AL356441) containing the entire *MGST3* locus and determined that the gene is composed of six exons and spans approximately 28 kb (Fig. 1). The sequence analysis showed that all exon-intron boundaries comply with the gt/ag splice donor/acceptor site consensus sequences (Shapiro and Senapathy 1987). The exon sizes varied from 58 bp (exon 4) to 422 bp (exon 1). The putative transcription start site

of *MGST3* (exon 1) was arbitrarily designated as the first base of the dbEST entry # BG491134, which includes the largest 5′ part of exon 1. The translation start codon (ATG) is located in exon 2, and the translation stop codon and the 3′UTR sequences of *MGST3* are located in exon 6 (Fig. 1). Analysis of the adjacent genomic sequences revealed that *MGST3* is flanked approximately 6 kb upstream by *ALDH9A1* and 45 kb downstream by the putative gene *LOC200138* (data not shown).

To characterize the promoter region, the sequence extending 1.5 kb upstream of the putative transcript start site of *MGST3* was analyzed by the computer-assisted TransFac program (Wingender et al. 2000). Analysis of the 5′-upstream region failed to detect the presence of either a TATA or CAAT box (Fig. 2), but the area surrounding the transcription start site has an elevated GC content (54%) and contains two SP1 binding sites, which are thought to be critical for initiation of transcription in genes without TATA (Pugh and Tjian 1990). In addition, this program predicted several potential cis-acting binding elements for multiple transcription regulators including AP1, GATA-1, and C/EBPbeta (Fig. 2). However, the functional significance (if any) of these sites remains to be established in further studies.

All exons including their flanking intronic sequences, 2.0 kb upstream from exon 1 and 2.0 kb downstream from the translation stop codon were amplified by PCR and scanned for mutation by d-HPLC followed by sequencing. This analysis identified 25 diallelic variants, including 12 in the putative promoter region, five in intron 1, and four each in intron 4 and 5 (Fig. 1). However, our mutation analysis did not identify any variants in the exonic regions. Out of the 25 diallelic variants, two are length variants (LVTs), including a 5-bp deletion/insertion in the pu-

Fig. 1 Schematic diagram of human *MGST3* gene structure, and DNA variants. The exons (nos. *1–6*) are represented by *solid boxes* (sizes in bp indicated below) and the introns by a *thin line* (approximate sizes in kb indicated below). Variants are marked by *vertical dotted arrows* and the exact base positions and the base changes are given in parentheses (*positive numbers* show distance from the first base of the corresponding intron; *negative numbers* indicate to the distance proceeding from the following exon). Clusters of markers in linkage disequilibrium (*I*, *II*, and *III*) are indicated below the gene structure
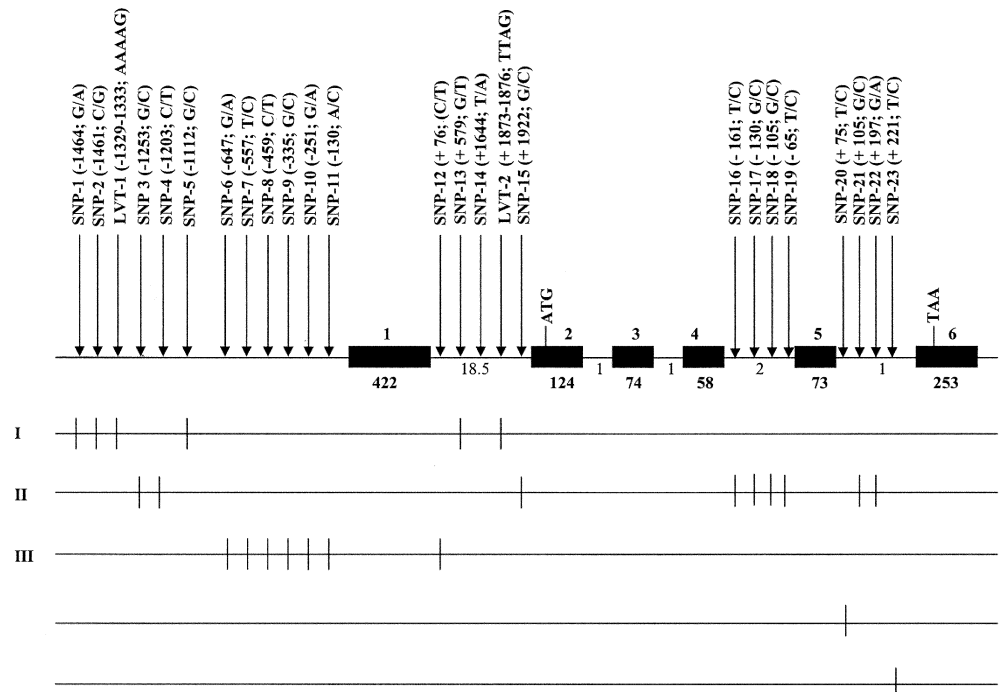
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CATGAACCGTTCAAGAAATGAAAAGCGTCCTACATGAAAAAAATACACTGAGTGCATGACAGGAGTTTTCTAGTCCACAA
CATACTTAGGTTCCCAGTTGCAAATCAATAAAGTGACACTCTGGTTTATGTATCAAAAGAAAAGAAAAAGAAATGAAAAG
CAAAAAAAGGGTTTACATTTTATGAAGTGAACAGAGCAGACCACAAAACAGAATATACAGTATGATTCTATTGTGTGTGT
ATGTAATTTTTTTGTGGGCTTTTCTGTATCTCCGAAAATTCTAATATAAAGGAATACTGCTTTTGTAATAAGATTTTTAA
                                      ◄─── GATA-1
AAGAAGGTCCTCAGTGGTTAAGTACTTGTCTGTGGTCAGAGATAACTGGCAAAGAAGAGGCCAAATTTTTTTTGAGACTG
CAGTCTCCGGTGTAAAATGAGGGTGCAAGGTGCATCCCCGCTCCTGATTTTTGAATCATCCCTTCCACCACCTTGGAATG
ACTTCTTACGTAGAACAGCCAGCAAGTGGAGAAACTTGCCTCAATAATGCAAAACTGTCTCCCTAACTTACTTCCTTTCA
ATGTTCAAGCAAAACAAAGCCCTCTCTACATCCAACATGACAGAAACCCAACATGACAGAAAGTATGAGAATGGTTGGAA
ATTGCTGAAACAGCAACAGTAAAGATTTAATGAGCTCTCAAAGTACCTCTAGATGTGCAAATATGACAACCCAATAAAAA
GAAACACACACATAACCTAGAGCCTTCAAAAATCTAGATTTCTGCCTTTCCACACCTTAACTTGGAATCATTCAGGTGGG
                                        ◄──── IgPE-1    ──── C/EBPbeta ─►
TTCCTAAGCATTCTCTCGGAGGTGAGGAATCCTCCAAAGAGGAGACTCTAGAGGGGCCCAGGTGTGGCAATCTTCAAAAG
GATGAGGGATGTCCAGAGGAGAGGTGGCGGGAGGGTGCACTTAAAACACGCGAAAATTGGAAACGTGCCTTCTGCAGTCA
GTTCCTTCGGATTTTCCGGCATTTAGAAGCCAGTTCTACTCCTTCAGCCCGACCGTATCTGCAGGTCTCACCCAAGACCA
      AP-1,Sp-1 ─►                                         Sp-1 ─►
AGACCCCCGCCCCCACCCCTCCTCCACCCGGCCTCGAGCCCCGCCCCGGACGCTGGACCCGAGCGTAACGGGAGCAGGTG
      Sp-1                                    MAZ, TBP
TTCACATCAAAGGTTGGGTCGCCAAGGCAGAGACTCCATGTGCCGCAAAAAAAACTCCACGGACAATTGGGAGACTCCTT
TCAAAAAAACAAAACTCCGGAGCCACGCGACTCCGCGCTCAGGGGGCGTGGGTACGGCCCACCACGAGGGCAGGGCTCGG
      Sox-5 ─►                    SRY ─►
CGCCAGCTCTCGGCAAAACTAACAATATTTGTTAGGCCCTAAACAAAAAGGCCCTAACAGCGATAAATCCATTCACTTCG
GGAGTGTAGCAACCTGGGACACCAAGCGCCGCCCACGCCCCAGCTACGCGCCTCAGGGACACATCGATTGAGGCGCGGCT
ACTTTCGCGCACGCGCCTTGCATGCCCGTGCTTCGCCCTTACAGCAGAGCGCCTAAAACCGCTACGGTGGCTCTGCTGGG
                            └──────► exon-1
GACAAATTATTCACGTCCCACTGTTCTGCAAACCCAATCCGGGAGGTATGACCTGGCTGGGACAGTTTAGGAAGATGGCC
ACGAAGGCACCTGGGGGAGTAGATATATGTTTTTGAGAATGAGAGGAGTAAATCGCTGGACGACCATGAGCAGGAAGAAA
ATTAGGCCTCCACCTCCCAACCAGTGGCCCTGAGTTGGCAACTTAGACAGCGCGCGCCAGCGCCAGGAGCCCCGCCTTGG

GGGCGTGGCCGCGCGCTGGTCGGCTGGGGCGGGGCCTGCTTCTGGCCTCATCTAGCCCCGCCCCAGGCGAGGGCGCCGCA

CCCACACCGCGCTGCGCAGTTTTGTTCTGCTCCAGCTGTTCGAAGGTGATCCAG/ gtgagtgctagc... intron-1

(18.5 kb)... tttcttccacag /ACGCAAGATGGCTGTC
                        └──────► exon-2
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Fig. 2 Genomic sequences of the 5′ upstream region of *MGST3*. *Bold and capital letters* mark the nucleotides in exons. The start codon (ATG) is in *boldface and underlined*. The first nucleotide of exon-1 (transcription start site) corresponds to the first base of EST entry #BG491134. Sequences of intron-1 are shown as *lowercase* and the splice sites are in *italics*. *Arrows* indicate the predicted binding sites for transcription factors and the forward and reverse orientation corresponds to the sense and the complementary sequences, respectively

Table 1 Association analysis of *MGST3* SNPs with diabetes in 50 cases and 50 controls

Representative SNPs[a] (Clusters)	Typing method[*]	P value Case-control
SNP13 (Cluster I)	Bse I	0.44
SNP3 (Cluster II)	*Hpy* CH4 III	0.03
SNP11 (Cluster III)	*Afe* I	0.08
SNP20	Seq[b]	0.53
SNP23	Seq	0.59

[*]Listed are endonucleases used for genotyping of SNPs causing a gain or loss of the corresponding restriction site
[a]See Fig.1 for the location of each variant
[b]Genotyped by direct sequencing

tative promoter region (LVT-1) and a 4-bp deletion/insertion in intron 1 (LVT-2), whereas the remaining variants are single nucleotide polymorphisms (SNPs).

Based on an initial genotyping in 10 diabetic and 10 non-diabetic Pima Indians, most variants could be divided into three groups, indicative of distinct linkage disequilibria (LD). These include SNPs 1, 2, 5, 13, and LVTs 1 and 2 (SNP cluster I), SNPs 3, 4, 15, 16, 17, 18, 19, 21, and 22 (SNP cluster II), and SNPs 6, 7, 8, 9, 10, 11, 12, (SNP cluster III). Therefore, SNPs 13 (cluster I), 3 (cluster II), and 11 (cluster III) were selected as representative markers for each unique cluster of variants and genotyped by

PCR-RFLP in 50 cases (diabetes onset before 25 years of age, which has a stronger heritability than the late-onset form in this population; Knowler et al. 1990) and 50 control subjects (over 45 years old with normal glucose tolerance). Also, we identified two unique markers (SNPs 20 and 23) in intron 5, which were not in LD with any of the three clusters and thus were also genotyped in these cases

Table 2 Correlation of skeletal muscle *MGST3* mRNA level and clinical characteristics in 56 non-diabetic Pima Indians

Parameters	Means± SD	R value	P value
Age (years)	30±7	–0.02	0.87
%Fat	32±7	–0.09	0.51
Fasting plasma glucose (mg/dl)	87±10	0.06	0.64
2 h plasma glucose (mg/dl)	121±34	–0.04	0.77
Log$_{10}$ of fasting plasma insulin (µU/ml)	1.6±0.14	–0.02	0.12
Log$_{10}$ of 2 h plasma insulin (µU/ml)	2.1±0.30	–0.17	0.20
M-low (mg/kg EMBS per minute)	0.4±0.13	0.20	0.12
M-high (mg/kg EMBS per minute)[a]	0.95±0.36	0.13	0.29

[a]M-low: log of glucose disposal rate at physiological insulin concentration; M-high: glucose disposal rate at maximal stimulated insulin concentration. EMBS: Estimated Metabolic Body Size

and controls. In addition, SNP14 was identified only in one subject and was not genotyped further because of its low frequency (0.025). Information about all representative SNPs, allelic frequencies, and the results of the association analyses with T2DM are summarized in Table 1.

Of all analyzed markers, SNP-3 (cluster II) was the only one associated ($p<0.03$) with T2DM in the case-control group. Hence, we extended the genotyping of SNP-3 to the entire linkage set comprising 1,338 subjects (described in Hanson et al. 1998) to assess the relationship of this marker with the original linkage to T2DM. Analysis of this SNP in the linkage set revealed no significant association between these genotypes and T2DM or any other related phenotypes including BMI, and insulin action and secretion measured in a subset of non-diabetic subjects (data not shown). In addition, neither SNP-13 (cluster I) nor SNP-11 (cluster III) was associated with T2DM in the case-control study group. Therefore, we conclude that the variants we detected in *MGST3* are not associated with T2DM in the Pima population.

The characteristics of 56 non-diabetic Pima subjects used to obtain skeletal muscle mRNA, and the results of analysis of *MGST3* expression are given in Table 2. As can be seen from this table, no statistically significant correlation was found between *MGST3* expression levels and parameters of insulin sensitivity (M-low, M-high, plasma glucose), even after adjusting for age, sex and percentage of body fat (data not shown). Interestingly, the SNP-5 that we identified in the promoter region, is within a predicted GATA-1 site, which is known to regulate the expression of *IGF-1* (Wang et al. 2000) and *Pax4* (Xu and Murphy 2000). In order to identify whether the reported variants in the promoter region could alter the expression of *MGST3* mRNA, a simple ANOVA (Analysis of Variance) was also carried out and our analysis did not identify any significant association between the *MGST3* expression and the genotypes (data not shown).

In conclusion, we have described the genomic structure of *MGST3* locus on 1q and our analyses do not indicate that variations in this locus could be a major cause of T2DM in Pima Indians. In addition, skeletal muscle *MGST3* mRNA is not significantly correlated with the insulin ac-

tion in Pima Indians. However, information on the structural organization of the gene and identification of variants in this study will make it possible to explore this locus for its possible contribution to T2DM and diseases associated with oxidative stress in other populations.

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