INTRODUCTION

Human CYPs are membrane-bound proteins located to the mitochondria or the endoplasmic reticulum [1]. The cytochromes P450 (CYPs) are the major superfamily of the enzyme catalyzing the oxidative biotransformation of lipophilic xenobiotics including drugs [2]. This superfamily consists of 57 CYP genes and 58 pseudogenes [3]. Most of the CYP genes in human are grouped according to the similarity of the amino acids sequence into 18 families and 44 subfamilies [2]. In addition to their role in metabolizing the xenobiotics, they have specific endogenous functions, as they are involved in the biosynthesis of steroid hormones, prostaglandins, bile acids, Vitamin D3, and others [2]. Genetic polymorphisms of CYPs influence the metabolism of their drugs substrate. Influencing the drug metabolism may lead to an adverse drug reaction, or inter-individual variation in drug response [4,5]. Most members of the CYP families are polymorphic. The most common genetic mutation in human CYP genes is single-nucleotide polymorphisms (SNPs) that may change the amino acid in the corresponding CYP protein [6]. This change in the amino acids sequences of the protein may alter its expression, or the catalytic activity, which may induce diseases as a phenotype [6]. The crystal structure of the CYP1B1 was determined [7], it has a narrow slot-like active site that is similar to that of CYP1A1; however, the residues around the edge of the active sites are different for specific binding of substrates and inhibitors [7]. Using the reverse transcriptase-coupled polymerase chain reaction (RT-PCR), CYP1B1 mRNA was detected in hepatocytes, lymphocytes, and uterus [8]. CYP1B1 gene is expressed in different amount among tissues, the highest mRNA expression levels were found in extrahepatic tissues such as renal, uterine, heart, brain, lung and skeletal muscle [9].

CYP1B1 is highly expressed in estrogen-related organ such as uterus, ovaries, and mammary glands [10]. The CYP1B1 catalyzes the metabolism of endogenous substrates such as 17β-estradiol into reactive metabolites, such as 4-hydroxyestradiol [11]. The 4-hydroxyestradiol, a catechol metabolite reduces the activity of estrogen. However, it generates free radicals from the reductive-oxidative cycling with the corresponding quinone and semiquinone forms, which cause cellular damage. Therefore, 4-hydroxyestradiol is toxicologically active and may have a role in the induction of cancer [12]. The 4-hydroxyestradiol level is elevated in human uterine and breast cancers in comparison to normal tissue [12]. Mutations in CYP1B1 were reported to be causes of disease phenotype such as the primary congenital glaucoma [13]. Moreover, CYP1B1 gene polymorphisms L432V and A119S (rs1056827) increase the risk of developing endometrial [14] and laryngeal cancers [15], respectively.

In Kingdom of Saudi Arabia (KSA), the prevalence of DM in adult was estimated to be 23.7% in 2004 [19]. Recently, it was reported that in...
Methods

Study population

The study was approved by the Research Committee of Armed forces hospitals, Northwestern region, Tabuk. It included two groups consisted of 120 healthy controls and 113 T2D patients visiting armed forces hospitals, Northwestern region and King Fahad Specialist Hospitals Tabuk, for routine check-up during the period from January to June 2017. The study was conducted in the Department of Biochemistry, Faculty of Science in collaboration with Prince Fahd Bin Sultan Research Chair, Department of Medical Lab Technology, Faculty of Applied Medical Sciences, University of Tabuk, KSA.

DNA extraction

Peripheral blood samples were collected in EDTA vials from the T2D patients, or from the healthy controls. Genomic DNA from blood was isolated using DNA extraction kit (Qiamp DNA Blood Mini Kit S 1106, Qiagen, Germany). The quality and integrity of isolate genomic DNA were checked by NanoDrop™ (Thermo Scientific, USA). The isolated DNA was stored at −20°C until further use.

Allele-specific PCR for CYP1B1*2 codon 119 (Ala119Ser) (rs1056827, 119G>T) gene

The genomic DNA genotypes were analyzed using single tube multiplex PCR assay which was performed in a reaction volume of 25 µL containing template DNA (50 ng), 0.2 µM of each primers which were designed by the primer 3 software listed in Table 1 and 12.5 µL from GoTaq® Green Master Mix (cat no M7122, Promega, USA) that is composed of GoTaq® DNA Polymerase, 2X Green GoTaq® Reaction Buffer (pH 8.5), 400 µM dATP, 400 µM dCTP, 400 µM dGTP, 400 µM dTTP, and 3 mM MgCl2. Final volume of 25 µL was adjusted by adding nuclease-free ddH2O.

Thermocycling conditions

The reaction mixture was subjected to initial denaturation at 95°C for 3 min, followed by 40 cycles of 94°C for 30 s, 56°C for 35 s, and 72°C for 35 s with a final extension of 72°C for 10 min.

Statistical analysis

T2D patients and controls were compared by statistical analysis performed using the SPSS 16.0 software package. Chi-square analysis and Fisher exact test were carried out to compare the observed genotype frequencies of CYP1B1*2 (GG, GT, and TT) variant genotypes and risk of T2D in the case–control groups. The associations between CYP1B1*2 (rs1056827G>C) variant genotypes and risk of T2D were estimated by computing the odds ratios (ORs) and their 95% confidence intervals (CIs) from both univariate and multivariate logistic regression analysis. p ≤ 0.05 was considered statistically significant.

Results

CYP1B1 (rs1056827G>T) genotyping

CYP1B1 (rs1056827G>T) genotyping was analyzed by allele-specific PCR. The PCR products were separated on a 2% agarose gel stained with ethidium bromide. Both of the alleles G homozygote and T homozygote generated a single fragment of 203bp as shown in Fig. 2.

Allelic frequencies and genotype distribution of CYP1B1*2 rs1056827 G>T

The association of CYP1B1*2 rs1056827 gene polymorphism between cases and controls is summarized in Table 2. The result of this study indicated that there was a high percentage of GG genotype (43.36%) in patients compared to 15.83% in the controls (Table 2). The frequency of the GT genotypes was 55.75% in the patients group, whereas it was 83.33% in the control group (Table 2). This study revealed the same percentage of TT genotype (<1%) in both patients as well as controls. The observed genotype frequencies of CYP1B1*2 (GG, GT, and TT) were statistically significantly different among patients and healthy controls (χ²=20, p=0.0001) (Table 2).

Table 1: Primers used for CYP1B1*2 (rs1056827 G>C) genotype analysis

<table>
<thead>
<tr>
<th>Allele-specific PCR primer for CC genotype</th>
<th>Wild type reverse primer</th>
<th>Common forward primer</th>
<th>Allele-specific PCR primer for AA genotype</th>
<th>Mutant reverse primer</th>
<th>Common forward primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>5'-ACACGGAAGGAGGCGAAGAC-3'</td>
<td>5'-CCACTGATCGGAAACGC-3'</td>
<td>5'-ACACGGAAGGAGGCGAAGAC-3'</td>
<td>5'-CCACTGATCGGAAACGC-3'</td>
<td>5'-CCACTGATCGGAAACGC-3'</td>
<td></td>
</tr>
</tbody>
</table>


The higher frequency of G allele was observed in cases than the healthy controls (G= 0.71 vs. 0.57). In controls the higher frequency of T allele was reported that the cases (TT= 0.43 vs. 0.29) as summarized in Table 3.

### Risk of T2D is associated with the CYP1B1 rs1056827 G>T variant

Odds ratio (OD) and risk ratio (RR) with 95% CI were calculated for each group to estimate the degree of association between the CYP1B1*2 rs1056827G>T variant and risk of T2D in Saudi patients as depicted in (Table 4). When it was compared the GG homozygous genotype with the TT homozygous genotype, we did not find a significant association with the OR 0.39 (0.25-0.53) RR 0.46 (0.30-0.71) p<0.04. In a comparison of GG homozygous genotype with the GT heterozygous genotype, it was found a highly significant association with the OR 0.24 (0.13-0.45) RR 0.45 (0.30-0.67) p<0.0002. During the allelic comparison, the G allele was compared with the T allele, and it was found a highly significant association with OR 0.54 (0.37-0.80) and RR 0.75 (0.63-0.897) p<0.003 indicating a possible dominant effect of this polymorphism on T2D risk (Table 4).

When CYP1B1*2 rs1056827 polymorphism was investigated the heterozygous carriers of the rs1056827 SNP were more likely to develop T2D compared to their wild-type counterparts OR 0.24 (0.131-0.45) RR 0.45 (0.30-0.67) p<0.0002 (Table 4). The combination of being homozygote for rs1056827 SNPs did not appear to be at an increase risk of disease. The present study demonstrates that the CYP1B1*2 rs1056827 gene polymorphism (heterozygosity) may contribute to the risk of developing T2D in Saudi population of Tabuk region. The individuals who were carrying GT genotype (homozygous) of rs1056827G>T were not associated with an increased risk of T2D disease.

### DISCUSSION

This study examines the potential influence of CYP1B1*2 (rs1056827G>T) genetic variants on T2D risk. The CYP1B1*2 rs1056827 heterozygosity appeared to be associated with increasing risk of developing T2D in Saudi population. CYP1B1 is a major enzyme involved in the hydroxylation of estrogens and activation of potential carcinogens [25] and is regulated by several key transcriptional factors, including aryl hydrocarbon receptor and estrogen receptor [26]. It has been reported that CYP1B1 expression increases in the early phase of in vitro adipogenic differentiation in parallel with peroxisome proliferative activated receptor gamma (PPAR gamma) expression in tissue [27,28]. PPAR gamma is an important transcriptional factor for adipocyte differentiation and glucose homeostasis [29]. In CYP1B1 KO mice, the expression of PPAR gamma was reduced at both mRNA and protein levels in adipose tissues as well as liver [28]. The same study demonstrated that the CYP1B1 KO mice exhibited improved glucose intolerance than the wild-type mice [28]. This may be the reason why the highly active CYP1B1*2 rs1056827 [30] may be the cause of induction of T2D in the GT genotype carriers.

In addition, the role of the CYP1B1 in the occurrence of obesity and glucose intolerance and hence insulin resistance can be attributed to other effects. It has been reported that in the liver and fat tissues of CYP1B1 deficient mice there were suppression of genes that promote lipogenesis [28]. For instance, the expression of the gene of Acetyl CoA carboxylase that catalyzes the synthesis of malonyl-CoA, a key step fatty acids synthesis is reduced [31]. There will also be decreased expression of fatty acid synthase, an important a multi-enzyme protein that catalyzes synthesis of fatty acids [28]. The expression of gene of the Stearyl-CoA desaturase-1 that catalyzes monounsaturated fatty acids synthesis is decreased [28]. Furthermore, in the CYP1B1 KO mice, there was enhancement of expression of the genes that increase lipolysis [28], the carnitine palmitoyltransferase 1a and the uncoupling protein 2 which both promotes mitochondrial fatty acid beta-oxidation [29]. Therefore, the highly active version [30] of CYP1B1*2 rs6510827 can be a cause of T2D as indicated in this study results.

The major phenotypic features associated with CYP1B1 deletion include protection from the deleterious effects of excess dietary fat, including oxidative stress, hepatic steatosis, and T2D [32]. Recently microarray data from the livers of the CYP1B1 KO mice that are resistant to diet-induced obesity indicated that CYP1B1 deletion substantially alters the expression of genes associated with fatty acid homeostasis [23]. Several studies have examined the relationship between individual CYP1B1 polymorphisms and cancer risk [14,15,34-37]. However, very limited data are available for CYP1B1*2 (rs1056827G>T) genetic variants in T2D. The aim of the current study was the investigation of allelic distribution of CYP1B1*2 (rs1056827G>T) genetic variants in an ethnically-homogeneous population of controls and patients with one of the most frequently observed diseases in Saudi Arabia [20,22,38]. The effects of the CYP1B1*2 (rs1056827G>T) genetic variants appear to be modest (Table 4). The variants in codons 119 of CYP1B1*2 (rs1056827) were associated with different substrate specificities and consequently the catalytic activity of the enzyme [39]. Several studies have been given an evidence that the CYP1B1 variants codons 119 of CYP1B1*2 rs1056827 exhibit greater catalytic 4-hydroxylation activity than the wild-type enzyme [30,40]. This may give a functional evidence as to why there appears to be an, albeit small, association with the risk of developing T2D in GT carriers.

### Allelic frequencies of CYP1B1*2 rs1056827G>T genetic variants in different cases in different populations

Several population-based studies have reported the frequency of CYP1B1*2 rs1056827G>T gene polymorphism in different populations. The distribution of CYP1B1*2 rs1056827G>T genotypes varies among different ethnic groups with different diseases (Table 5). The range of GT genotypes frequency reported in different cases of different populations was in the range from 26% to 70%. The WT genotypes of CYP1B1*2 rs1056827 polymorphism were ranged from 1% to 13% except in Iranian population (Table 5). The prevalence of GG genotypes of CYP1B1*2 rs1056827 polymorphism is ranged from 20 to 54%. In this study, the prevalence of GG, TT, and GT genotypes in cases was 43%, 1%, and 56%. Therefore, results were in accordance with the result of the other populations (Table 5).

### Allelic frequencies of CYP1B1*2 (rs1056827G>T) genetic variants in control populations

Several population-based studies have reported a prevalence of CYP1B1*2 rs1056827G>T gene polymorphism in healthy controls of different populations (Table 5). The prevalence of GG, TT, and GT genotypes of CYP1B1*2 rs1056827 G>T in different populations is ranging from 43% to 79%, 0% to 23%, and 16% to 55%, respectively. In this study, the prevalence of GG, TT, and GT genotypes of CYP1B1*2

<table>
<thead>
<tr>
<th>Subjects</th>
<th>N</th>
<th>GG Genotype</th>
<th>TT Genotype</th>
<th>G/T Genotype</th>
<th>χ²</th>
<th>df</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cases</td>
<td>113</td>
<td>49 (43.36)</td>
<td>1 (0.88)</td>
<td>63 (55.75)</td>
<td>20</td>
<td>2</td>
<td>0.0001</td>
</tr>
<tr>
<td>Controls</td>
<td>120</td>
<td>19 (15.83)</td>
<td>1 (0.83)</td>
<td>100 (83.33)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Number</th>
<th>GG</th>
<th>TT</th>
<th>G/T</th>
<th>allele</th>
<th>T allele</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cases</td>
<td>113</td>
<td>49</td>
<td>01</td>
<td>63</td>
<td>0.71</td>
<td>0.29</td>
<td>0.0001</td>
</tr>
<tr>
<td>Controls</td>
<td>120</td>
<td>19</td>
<td>01</td>
<td>100</td>
<td>0.57</td>
<td>0.43</td>
<td>0.03</td>
</tr>
<tr>
<td>Total</td>
<td>233</td>
<td>68</td>
<td>02</td>
<td>163</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 4: Risk of T2D is associated with the CYPs1b1 (rs1056827) G>T variant

<table>
<thead>
<tr>
<th>Genotype</th>
<th>T2D patients</th>
<th>Healthy controls</th>
<th>OR (95% CI)</th>
<th>RR</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYPs1B1-GG</td>
<td>n=113 (%)</td>
<td>n=120 (%)</td>
<td>1 (ref.)</td>
<td>1 (ref.)</td>
<td>&gt;0.0002</td>
</tr>
<tr>
<td>CYPs1B1-CT</td>
<td>120 (53.6)</td>
<td>35 (10.2)</td>
<td>0.24 (0.13–0.45)</td>
<td>0.45 (0.30–0.67)</td>
<td>&lt;0.0002</td>
</tr>
<tr>
<td>CYPs1B1-TC</td>
<td>61 (49.36)</td>
<td>65 (10.2)</td>
<td>0.38 (0.02–6.51)</td>
<td>0.55 (0.13–2.35)</td>
<td>&lt;0.49</td>
</tr>
<tr>
<td>CYPs1B1-CTT</td>
<td>236 (78.7)</td>
<td>22 (9.6)</td>
<td>0.54 (0.07–0.80)</td>
<td>0.75 (0.63–0.897)</td>
<td>p&gt;0.003</td>
</tr>
</tbody>
</table>

Table 5: Frequency of CYP1B1*2 rs1056827 gene polymorphism in different populations

<table>
<thead>
<tr>
<th>Country</th>
<th>Year</th>
<th>N=</th>
<th>GG %</th>
<th>TT %</th>
<th>G/T %</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iran</td>
<td>2017</td>
<td>79</td>
<td>48 (60.8)</td>
<td>18 (22.8)</td>
<td>13 (16.4)</td>
<td>Afzali et al. [41]</td>
</tr>
<tr>
<td>China</td>
<td>2015</td>
<td>300</td>
<td>129 (43)</td>
<td>6 (2)</td>
<td>165 (55)</td>
<td>Yu et al. [15]</td>
</tr>
<tr>
<td>Australia</td>
<td>2010</td>
<td>597</td>
<td>277 (46.4)</td>
<td>61 (10.2)</td>
<td>259 (43.4)</td>
<td>Srer et al. [34]</td>
</tr>
<tr>
<td>Bulgaria</td>
<td>2015</td>
<td>257</td>
<td>129 (50.2)</td>
<td>20 (7.8)</td>
<td>108 (42)</td>
<td>Kachkovka et al. [35]</td>
</tr>
<tr>
<td>South Korea</td>
<td>2014</td>
<td>480</td>
<td>266 (55.4)</td>
<td>20 (5.8)</td>
<td>194 (40.8)</td>
<td>Wang et al. [21]</td>
</tr>
<tr>
<td>Chinese</td>
<td>2014</td>
<td>300</td>
<td>236 (78.7)</td>
<td>0 (0)</td>
<td>64 (21.3)</td>
<td>Shen et al. [36]</td>
</tr>
<tr>
<td>This study</td>
<td>2017</td>
<td>120</td>
<td>19 (15.83)</td>
<td>1 (0.83)</td>
<td>100 (88)</td>
<td></td>
</tr>
</tbody>
</table>

(rs1056827) gene polymorphism is about 16%, <1%, and 83%, respectively (Table 5).

Limited data exist about status of CYP1B1*2 rs1056827 polymorphism among patients with T2D especially among the population of Northwestern region of Saudi Arabia.

CONCLUSION

The CYP1B1 variants examined in this study suggest that CYP1B1*2 (rs1056827) contribute to inter-individual differences in T2D risk and may be potentially valuable in genetic risk assessment and prevention studies. However, it is important to be confirmed in other T2D populations before introducing it in the clinical setting.

ACKNOWLEDGMENT

We are very grateful to all participants who have provided blood samples for this project. This study was funded by the University of Tabuk, Deanship of Scientific Research for IE and FMA (Grant for project No S-1437-0178).

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