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Original Research Article

Association between GSTT1 and GSTM1 genes polymorphisms and Type II diabetes miletus patients in Basra – Iraq

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A B S T R A C T

According to International Diabetes Federation (2011), Iraq is one of the world's top 10 countries for highest prevalence of diabetes. Type 2 diabetes mellitus (T2D) is caused by a combination of genetic factors and lifestyle, one of the most dangerous factors is the impact of the oxidative stress which induced by ROS. Glutathione S-transferases play a major role in the detoxification of metabolites of oxidative stress resulting in inflammation and certain diseases such as type 2 diabetes mellitus. We evaluated the association between *GSTT1* and *GSTM1* gene polymorphisms with susceptibility to T2D and investigated the effect of age, gender, smoking and emotional stress status combined with these polymorphisms on T2D. Number of studies and researches has been conducted in Iraq to determine the association between *GSTT1* and *GSTM1* gene polymorphisms with many diseases, but this is the first study to investigate the role of *GSTT1* and *GSTM1* gene polymorphisms in T2D pathogenesis in Iraqi population. A total of 232 subjects were enrolled in this study, of which 125 were T2D patients and the other 107 were non-diabetic volunteers from Basrah population. Multiplex PCR was used for genotyping the *GSTT1* and *GSTM1* gene polymorphisms. The both *GSTT1* and *GSTM1* null allele was more prevalent in diabetic patients than in the control subjects which result in 2-fold risk toward T2D. *GSTM1* polymorphism revealed a greater prevalence than *GSTT1* polymorphism in diabetics and control group. The younger age groups were more likely to develop T2D as well as males in case of GSTs polymorphisms, also there was a significant effect of smoking and emotional stress combined with these polymorphisms on diabetic patients.

Introduction

Type 2 diabetes mellitus is a multifactor disease, one of the most dangerous factors is the impact of the oxidative stress which can participate in diabetogenesis (Ho and Bray, 1999), reduction of insulin secretion (Carlsson *et al.,* 1999), and development of late diabetic complications (Baynes and Thorpe, 1999). Oxidative stress (high levels of reactive oxygen species (ROS) compared to antioxidant defenses) can arise from

various sources (Maritim *et al.,* 2003). Mitochondria are the main site of intracellular source of ROS formation (Ames, 2004). Non-mitochondrial sources of ROS include environmental pollutants (Halliwell and Gutteridge, 2007), unhealthy food (Sies *et al.,* 2005) and psychophysical stressful situations (Eskiocak *et al.,* 2005). Oxidative stress has been implicated in over 100 diseases, more as a consequence of the pathology than as the causative factor (Halliwell and Gutteridge, 2007). β-cells are particularly sensitive to ROS because they are low in antioxidant factors such as glutathione peroxidase, catalase and super oxide dismutase (Zhang *et al.,* 2010). Therefore, increased oxidative stress have an important causal role in β-cell failure and the development of insulin resistance and T2DM (Stephens *et al.,* 2009).

In healthy subjects, antioxidant compounds counter the effects of free radicals which include reactive oxygen species (ROS), antioxidants which are produced either endogenously or are derived from dietary sources, are categorized into two groups: enzymatic and non-enzymatic (Esteghamati et al, 2008). One of the most important antioxidant enzymes is glutathione (GSH) which provides the cell with multiple defenses not only against ROS but also against their toxic products (Pompella *et al.,* 2003). GSH is known as a substrate in both conjugation and reduction reactions, catalyzed by glutathione S-transferase (GST) enzymes in the cell, however, it is also capable of participating in non-enzymatic conjugation with some chemicals (Tesfamariam, 1994).

Glutathione S-transferases (GSTs) isoenzymes represent an important family of phase II drug metabolizing enzymes, these transferases inactivate endogenous end products formed as secondary metabolites during oxidative stress (Habdous *et al.,* 2004). In humans, eight gene families of soluble (or cytosolic) *GSTs* have been described: the alpha, mu, pi, theta, kappa, sigma, zeta and omega gene families (Board *et al.,* 1997).

Two of these subfamilies, *GST(mu)* and *GST(theta),* show deletion polymorphism (Habdous *et al.,* 2004). Polymorphisms in *GSTs* genes are often correlated with susceptibility to certain diseases such as various cancers (Coughlin and Hall, 2002; Al-Badran, 2003; Hayes *et al.,* 2005; Parl, 2005). In Iraq, number of studies have been implicated the association of these genes with cancers susceptibility in Iraqi population (Al Hasani, 2004; Al-Laaeiby, 2009; Al-Badran, 2011).

Association of these polymorphisms with type 2 diabetes mellitus (T2D) risk have been widely studied, however, the results were somewhat conflicting, a meta-analysis was performed before October, 2012, the accumulated evidence proved the obvious associations of *GSTM1* and *GSTT1* polymorphisms with an increased risk of T2D (Tang *et al.,* 2013). To our knowledge this study is the first in Iraq.

Materials and Methods

One hundred and twenty five blood samples of type 2 diabetes patients (47 males and 78 females) were collected from Almoani Hospital in Basra city, their ages range between 25 and 84 years old. Other 107 blood samples of non-diabetic as control group were collected from volunteers, their age and gender matched the diabetic subjects. Subject's histories were recorded using standard questionnaire categories: age, sex, smoking, family history of T2D and psychological status as for diabetic patients.
The genomic DNA for genotyping was

isolated from 2ml of peripheral blood lymphocytes which were collected, using genomic DNA extraction kit (Geneaid, New Taipei city, Taiwan), in an EDTA containing a vacutainer.

The *GSTM1* and *GSTT1* null genotypes were detected using a multiplex PCR method of a total 25 ml multiplex PCR mixture containing 5ml genomic DNA,10pmol of each following *GSTM1* primers (F-5′-GAACTCCCTGAAAAGCTAAAGC-3′) / (R-5′-CTTGGG CTCAAATATACGGT GG-3′) and *GSTT1* primers (F-5′-TTCCTTACT GGTCCTCACATCTC-3′) / (R-5′-TCACCGGATCATGGCCAGC A-3′). As an internal control, the *ALBUMIN* gene (F-5'-GCCCTCTGCTAACAA GTCCTAC-3') / (R-5'-GCCCTAAAAAG AAA ATCGCC AATC-3') in 5ml of AccuPower PCR PreMix (Bioneer, Korea) was used.

The PCR protocol included an initial melting temperature of 95°C (3 minutes) followed by 30 cycles of amplification (1 minute at 95°C, 1 minute at 58°C and 1 minute at 72°C). A final 7 minutes extension step (72°C) terminated the process.

The PCR products were analyzed on 2% agarose gel. A fragment of 219pb indicated the presence of *GSTM1*; a fragment of 459pb indicated the presence of *GSTT1*; and a fragment of 350pb indicated the positive internal control *ALBUMIN*. The subjects were classified as (+) when the gene was detected, or (−) when they showed a null genotype.

Descriptive statistic has been used to describe characteristics of the study subjects by using mean, standard deviation and percentage. ORs and the 95%CIs were calculated online [http://www.medical .org/ calc/ odds _ratio.php].

Results and Discussion

Our case-control study has been conducted to evaluate the association between *GSTs* polymorphisms with susceptibility to type II diabetes mellitus and the effect of age, gender, smoking and emotional stress on these polymorphisms in 125 unrelated T2D patients, using control group of 107 non-diabetics, residing in Basrah city southern of Iraq. The subjects' characteristics are presented in Table 1. It has shown that the average age of patients with diabetes was higher than in the control group OR (95%CI) = 5.7 (3–11.1). Smoking has increased the risk of T2D to fivefold, and having family history was more prevalent in diabetics than in the control group in around thirteen-fold.

Diabetic patients were distributed depending on the age at diagnosis and the onset of the disease. The incidence of type II diabetes increased over the latest two decades for all age groups (Figure 1).

Amplified *GSTT1* and *GSM1* genes shown in Figure (2), the PCR products have been visualized on 2% agarose gel electrophoresis in order to detect present and absent of both genes bands.

Table 2 summarizes the *GSTT1/GSTM1* gene polymorphisms distribution in cases and control. Type II diabetic patients had higher frequency of *GSTM1/GSTT1* null genotype in comparison to the control group as (41.6%) of them showed *GSTM1* null genotype in comparison to (28%) in the control group with an OR (95% CI) =1.8 (1-3.1). Regarding *GSTT1* null genotype , 24.8% of cases showed *GSTT1* null genotype in comparison to 16.8 % in control with an OR (95%CI) = 1.6 (0.8 – 3) while there was 2-fold increased type 2 diabetes mellitus risk with both null

genotype OR (95%CI) = 2 (0.7-5) (figure2). Tables 3, 4 and 5 reveal the effect of age of clinical onset on *GSTT1, GSTM1* and both *GSTT1/GSTM1* null genotype, respectively. There was a 2.4 fold increased risk of T2D in the younger age group (24–36) year and 1.8 fold in the older age group (37–47) year with *GSTT1* null genotype as in the Table 3.

The Table 4 shows that *GSTM1* null genotype was significantly higher in the age group (24–36) years than the other age groups in diabetic patients.

Both *GSTT1* and *GSTM1* null genotype was significantly higher in the age group (24–36) years, also there was a-3.3 fold increase risk of T2D in the age group (37–47), (Table 5).

Males were significantly more likely to have type II diabetes than females in case of *GSTM1* null genotype, that males with both null genotype registered a-2.6 fold higher risk of T2D than females , as indicated in Table 6.

When psychological stress was taken in consideration (Table 7), the risk of type II diabetes increased to 4-fold when diabetic patients have *GSTT1* null genotype OR (95%CI) = 3.5(1.1–10.8), this risk increased to around 10-fold in case of both *GSTT1* / *GSTM1* null genotype OR (95% CI) = 10.3 (1.1– 89.8).

Smokers possessing twofold risk *GSTT1* null genotypes exhibited a statistically significant increased risk of T2D compared with non-smokers OR (95% CI) = 2.3 (1.04–2.48), and around threefold with both null genotype OR (95%CI) = 2.9 (0.7–11.8), (Table 8).

Type 2 diabetes is an elderly disease, normally, the regenerative capacity of most organs decreases with age (Zhang *et al.,* 2010), long-term hyper secretion of insulin results in increased co-secretion of amylin which helps to regulate glucose homeostasis, this amylin which aggregates into amyloid plaques and oxidative stress which increases with age can subsequently lead to increase beta cell apoptosis and cause progression of diabetes (Law *et al.,* 2010). Our results showed that the risk of T2D increased to 6-fold by age progress. Exposure to tobacco smoke can accelerate beta-cells apoptosis by the toxic effect of nicotine (Lynch *et al.,* 2009), we found that smokers prone to develop T2D to 5-fold than non-smokers. However, most of our diabetic patients were have a family history around 13-fold than other patients who weren't have. Having family history is considered as an independent risk of T2D. Harrison *et al.,* (2003) have documented that having one or more first degree relatives with T2D increase the odds 2–6 times compared with someone without such relatives (Harrison *et al.,* 2003). It has been shown in the present study that the incidence of T2D has increased over the last decade, this increase included all age groups. After 2003, Iraq faced a change in lifestyle, for example, import of genetically modified foods, increased number of cars, use of diesel generators in addition to the entry of modern technologies such as cellular phones, laptop computers, etc. Experts from the United Nations Environment Program UNEP estimated that contaminated sites with remnants of war are thousands (Al-Saad *et al.,* 2010). Basrah city, specially, have too high concentrations of ROS; CO, NO_2, SO_2, and hydrocarbons (HCs) within the industrial area (Al-Saad *et al.,* 2010; Al-Hassen, 2011) all of these are sources of oxidative stress (Schroder and Krutmann, 2005). Increased oxidative stress appears to be a deleterious factor leading to insulin resistance, β-cell dysfunction, impaired glucose tolerance, and ultimately, type 2

diabetes (Evans *et al.,* 2003; Ceriello and Motz, 2004; Halliwell and Gutteridge, 2007; Shah *et al.,* 2007; Bashan *et al.,* 2009; Stephens *et al.,* 2009).

The development of type 2 diabetes is caused by a combination of genetic and environmental factors (Murea et la., 2012). Since people vary genetically in their susceptibility to the effects of environmental risk factors for many diseases therefore genes modulating oxidative stress are good candidates for investigating gene × oxidative stress interactions (Risérus *et al.,* 2009; Minelli *et al.,* 2011).

GSTM1 and *GSTT1* genes defend against oxidative stress by conjugating ROS with glutathione, which detoxifies and eliminates them, they show deletion polymorphisms (Habdous *et al.,* 2004). According to our study, it has been observed that deletion in *GSTM1*, *GSTT1* genes and both null genotype were higher in type 2 diabetics than the control group, OR (95%CI) = 1.8 (1–3.1), 1.6 (0.8–3) and 2 (0.7–5.6), respectively, going with (Hori *et al.,* 2007; Nowier *et al.,* 2009, Bid *et al.,* 2010; Amer *et al.,* 2011; Moasser *et al.,* 2012; Zhang *et al.,* 2012).

Depending on our results, the polymorphic frequency of *GSTM1* and *GSTT1* null genotype in the control group was (28% and 16.8%, respectively), while the respective frequencies reported for Egyptian (47 and 21%) (Amer *et al.,* 2011), Spanish (49.7 and 20.5%) (Gsur *et al.,* 2001), Turkish (51.9 and 17.3%) (Ada *et al.,* 2004), Italian (46.9 and 19%) (D'Alò *et al.,* 2004) and Caucasian (48.8 and 19.9%) (Gsur *et al.,* 2001) control populations, in agreement with (Al-Awadi *et al.,* 2008). It has been reported that prevalence of these candidate genes can vary considerably by ethnicity (Garte *et al.,* 2001). Kim and Hong (2012) suggested that *GSTM1* and *GSTT1* null genotypes may increase susceptibility to potential effects of ambient air pollutants on insulin resistance in elderly Koreans, they didn't take in consideration all age groups (Kim and Hong, 2012a).

Present study found that the association between *GSTs* polymorphisms and T2D were stronger among younger adult participants than the elderly, also males were more affected by *GSTM1* and both *GSTM1*/ *GSTT1* polymorphisms than females thus increase their risk for developing T2D. AL-Youzbaki, (2008) has demonstrated in his study the significant effects of the sociological and environmental factors in developing type II diabetes in Iraqi males (AL-Youzbaki, 2008). At physiological levels in any period studied, females live longer than males, mitochondrial oxidative stress is higher in males than females and the higher levels of estrogens in females protect them against ageing, by up-regulating the expression of antioxidant, longevity-related genes, the chemical structure of estradiol confers antioxidant properties to the molecule, estrogens and phytoestrogens up-regulate expression of antioxidant enzymes via the estrogen receptor (Vina *et al.,* 2011).

As a result of the unstable circumstances experienced by Iraq, the Iraqis are suffering from a severe and chronic psychological stress. The results of our study showed that *GSTT1* and both *GSTT1* / *GSTM1* null genotypes may increase susceptibility to potential effects of emotional stress on T2D. Emotional stress can increase the risk for developing T2D through different pathways (Bonnet *et al.,* 2005; Rod *et al.,* 2009). Eskiocak *et al.* (2005) have showed in their results that emotional stress in humans associates with higher biomarkers for oxidative stress, increases catecholamine metabolism causes an acute-phase response

by inducing IL-6, TNF-α and other cytokines secretion from macrophages which induce oxidative stress by increasing the production of free-radicals (Eskiocak *et al.,* 2005). Cortisol increases in response to an acutely stressful event have the potential to either enhance or undermine psychobiological resilience to oxidative damage, depending on the body's prior exposure to chronic psychological stress, women under chronic stress had higher oxidative damage biomarkers and oxidative damage to RNA (Aschbacher *et al.,* 2013).

Present study has found that smokers have showed higher risk for developing type II diabetes than non-smokers, when they have *GSTT1* or both *GSTT1* /*GSTM1* null genotype to OR= 3-fold. Smoking is a common mechanism for generating oxidative stress and inflammation, Sliwinska *et al.* (2012) have showed in their study that the role of the oxidative stress in the induction of pancreatitis is associated with chronic cigarette smoke inhalation (Sliwinska-Mosson *et al.,* 2012). The *GSTT1* polymorphism was of interest as well, because monohalomethans and others, which are metabolized by *GSTT1*, are present in tobacco smoke (Katoh *et al.,* 1998).

Table.1 Distribution of demographic variables of patients and Control

Variables	Control (n= 107)	Patients (n= 125)	OR	CI 95%
Gender				
Male	35(32.7%)	47 (37.6%)	0.8	0.4–1.3
Female	72(67.3%)	78(62.4%)		
Age (Years)				
Mean age (± SD)	43± 11	56 ± 11	5.7	3–11.1
Range	24-72	25 -83		
Smoking Status				
Non – Smoker	103(96.3%)	104(83.2%)	5.1	1.7–15.7
Smoker	4(3.7%)	21 (16.8%)		
Social status				
Married	86 (80.4%)	99(79.2%)	1	0.5–2
Single	21 (19.6%)	26(20.8%)		
Family history				
Don't have	80(74.7%)	23 (18.4%)	13.1	7–24.6
Have	27 (25.2%)	102 (81.6%)		

Figure.1 Distribution of age criteria of diabetic patients according to the age at diagnosis and the onset of the disease

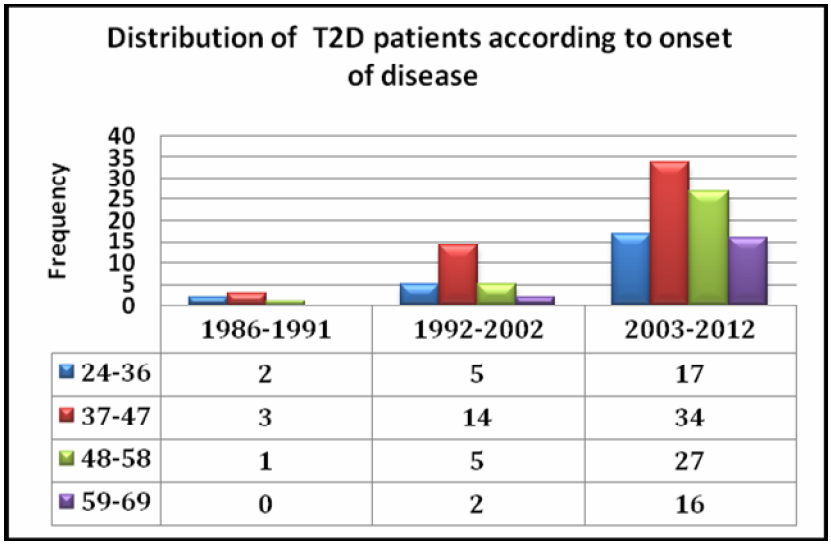
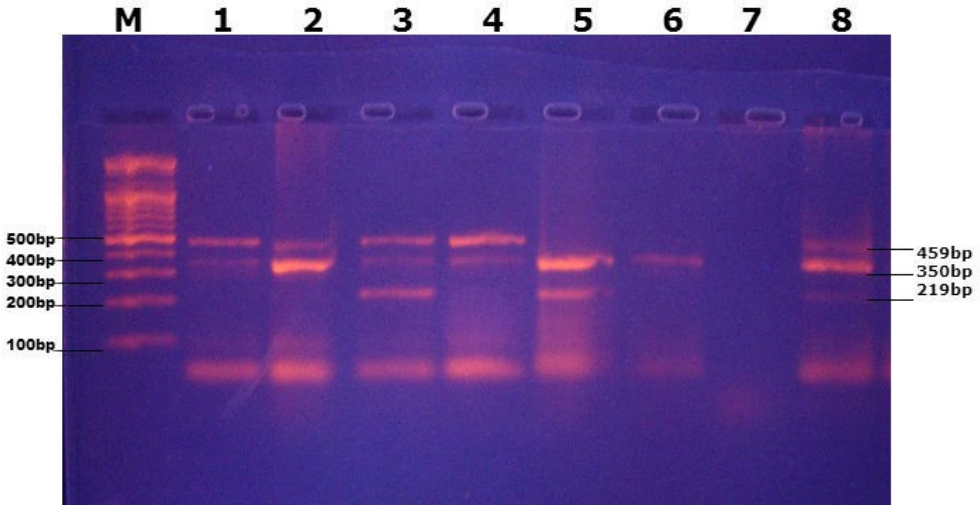


Figure.2 PCR products analyzed on 2% agarose gel



The presence of *GSTT1* and *GSTM1* genes were detected by presence of bands at 459bp and at 219bp, respectively. Absence of these bands indicated to null genotype. *ALBUM1N* was considered an internal control (350bp). M : 100bp ladder; *GSTM1* null : Lanes 1,2,4; *GSTT1* null : Lane 5; *GSTM1* & *GSTT1* null : Lane 6; *GSTM1* & *GSTT1* wild type : Lanes 3,8; and Lane 7 : negative control.

Table.2 Distribution of *GSTM1* and *GSTT1* genes among T2D patients and control

Genotype	Control	Cases	OR	95% CI
***GSTT1* (+)**	89(83.2%)	94(75.2%)	1.0	-
***GSTT1* (-)**	18(16.8%)	31(24.8%)	**1.63**	0.8 – 3
***GSTM1* (+)**	77(72%)	73(58.4%)	1.0	-
***GSTM1* (-)**	30 (28%)	52(41.6%)	**1.82**	1 - 3.1
***GSTM1 & GSTTI* (+,+)**	65(60.7%)	54(43.2%)	1.0	-
***GSTM1 & GSTT1* (- ,-)**	7(6.5%)	12(9.6%)	**2.0**	0.7- 5.6

Table.3 Effect of age of clinical onset on *GSTT1* null genotype in diabetics

Age group	Control	Cases	OR	95%CI
24-36 (+)	31(88.6%)	19(76%)	**2.4**	0.61-9.8
(-)	4(11.4%)	6(24%)		
37-47(+)	31(79.5%)	35(68.6%)	**1.8**	0.66-4.77
(-)	8(20.5%)	16(31.4%)		
48-58(+)	17(85%)	26(78.8%)	1.5	0.34-6.73
(-)	3(15%)	7(21.2%)		
59-69(+)	10(76.9%)	14(87.5%)	0.4	0.06-3.4
(-)	3(23.1%)	2(12.5%)		

Table.4 Effect of age of clinical onset on *GSTM1* null genotype in diabetics

Age group	Control	Cases	OR	95%CI
24-36 (+)	29(82.9%)	10(40%)	**7.3**	2.2-23.8
(-)	6(17.1%)	15(60%)		
37-47(+)	27(69.2%)	31(60.8%)	1.45	0.6-3.5
(-)	12(30.8%)	20(40%)		
48-58(+)	13(65%)	22(66.7%)	0.9	0.29-3
(-)	7(35%)	11(33.3%)		
59-69(+)	8(61.5%)	10(62.5%)	0.8	0.18-3.5
(-)	5(38.5%)	6(37.5%)		

Table.5 Effect of age of clinical onset on both *GSTT1/GSTM1* null genotype in diabetics

Age group	Control	Cases	OR	95%CI
24-36 (+)	25(71.4%)	7(28%)	**23.8**	1.1-514.3
(-)	0	3(12%)		
37-47(+)	21(53.8%)	22(43.1%)	**3.3**	0.62-17.9
(-)	2(5.1%)	7(13.7%)		
48-58(+)	12(60%)	16(48.5%)	0.4	0.03-4.63
(-)	2(10%)	1(3%)		
59-69(+)	7(53.8%)	9(56.3%)	0.3	0.02-3
(-)	3(23%)	1(6.3%)		

Table.6 Correlation between gender and *GSTT1/GSTM1* null genotype in diabetics

Genotype	Female(n=78)	Male (n=47)	OR	95%CI
***GSTT1*(+)**	60 (76.9%)	34 (72.3%)	1.0	-
***GSTT1*(-)**	18 (23.1%)	13 (27.7%)	1.3	0.56-2.9
***GSTM1*(+)**	51 (65.3%)	22 (46.8%)	1.0	-
***GSTM1* (-)**	27 (34.7%)	25 (53.2%)	**2.14**	1-4.5
***GSTT1&GSTM1* (+ , +)**	39 (50%)	15 (31.9%)	1.0	-
***GSTT1&GSTM1* (- , -)**	6 (7.7%)	6 (13%)	**2.6**	0.72-9.33

Table.7 Effect of psychological stress on *GSTT1 & GSTM1* null genotype in diabetics

Genotype	Non- stressed (n= 36)	Stressed (n=89)	OR	95%CI
***GSTT1* (+)**	32 (88.9%)	62 (69.7%)	1.0	-
***GSTT1* (-)**	4(21.1%)	27(33.3%)	**3.5**	1.1-10.8
***GSTM1* (+)**	21(58.4%)	52(58.4%)	1.0	-
***GSTM1* (-)**	15(41.6%)	37 (41.6%)	0.99	0.4- 2.1
***GSTT1&GSTM1* (+ , +)**	15(41.7%)	16 (18%)	1.0	-
***GSTT1&GSTM1* (- , -)**	1(2.8%)	11(12.3%)	**10.3**	1.1- 89.8

Table.8 Impact of smoking on *GSTT1/GSTM1* null genotype in diabetics

Genotype	Non-smokers (n=104)	Smokers (n=21)	OR	95%CI
***GSTT1*(+)**	82 (78.8%)	12 (57.1%)	1.0	-
***GSTT1*(-)**	22 (21.2%)	9 (2.9%)	**2.3**	1.04-2.48
***GSTM1* (+)**	60 (57.7%)	13 (61.9%)	1.0	-
***GSTM1* (-)**	44 (42.3%)	8 (39.1%)	0.8	0.28-2
***GSTT1&GSTM1* (+, +)**	46 (42.2%)	8 (30%)	1.0	-
***GSTT1&GSTM1* (- , -)**	8 (7.6%)	4 (19%)	**2.9**	0.7-11.8

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