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Gene expression level of toll-like receptor 4 and insulin receptor substrate 1 in type II diabetic Malay patients and their first-degree relatives.

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Abstract

Background: Type II diabetes mellitus (T2DM) is a polygenic disorder that can be prevented or delayed in case of the adoption of proper interventions. The identification of susceptible genes and novel biomarkers of T2DM could be of great help in the early detection of high-risk individuals. First-degree relatives of T2DM patients have a high risk of this disease, even when they have no major abnormalities in glucose metabolism. The present study was conducted to examine the status of the expression of two genes, namely toll-like receptor 4 (TLR4) and insulin receptor substrate (IRS1), involved in glucose metabolism in peripheral blood, in individuals genetically predisposed to T2DM development.
Methods: Blood samples were collected from 54 participants in three research groups, including Malay subjects with T2DM, first-degree relatives of T2DM patients, and healthy controls. The measurement of gene expression was accomplished using a quantitative real-time polymerase chain reaction.
Result: The results were indicative of the significant upregulation and downregulation of TLR4 in patients with T2DM and their first-degree relatives, respectively ($P<0.05$). With regard to IRS1, the data revealed a decreased expression in T2DM patients as compared to that in the healthy controls ($p<0.05$).
Conclusion: The results indicated that TLR4 and IRS1 might be involved in the pathogenesis of T2DM. Moreover,the altered expression of TLR4 in the first-degree relatives of diabetic patients is an important marker showing a genetic predisposition to T2DM. Therefore, the two investigated genes could be used as a diagnostic tool for the prediction of T2DM in this population.

Keywords: Type 2 diabetes mellitus, Gene expression, TLR4, IRS1, First-degree relative.

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Introduction

Type II diabetes mellitus (T2DM) is a preventable chronic metabolic disorder characterized by peripheral insulin resistance in insulin target tissues and pancreatic β-cell dysfunction which results in protein and lipid metabolism disorders [1]. The number of people affected with diabetes has been estimated to increase from 382 million in 2013 to 552 million by 2030 [2]. The epidemiological data are indicative of the high prevalence of diabetes in South-East Asia. Accordingly, Malaysia which is a fast-developing country located in this area could not escape the clutches of diabetes epidemic. Based on the National Health Morbidity Survey performed in 2015, the prevalence of diabetes (known and undiagnosed) in Malaysia increased from 15.2% in 2011 to 17.5% in 2015. The proportion of "undiagnosed diabetes" has contributed to this growing prevalence since 2011. The development of T2DM is caused by the interaction between strong genetic components and environmental factors. Studies conducted on twins and first-degree relatives (FDR) of people with T2DM provide substantial evidence regarding genetic susceptibility to T2DM [3]. In addition, there is a considerable difference in T2DM prevalence across various ethnicities and races [3].

Family history has been recognized as a major risk factor for the development of T2DM [4,5]. In this regard, the risk of T2DM rises by 2-4 folds when one parent is diabetic. Moreover, this risk heightens up to 6 folds in those with a history of diabetes in both parents [6,7]. Studies performed on the offspring and siblings of diabetic subjects have provided considerable evidence regarding β-cell impairment and insulin resistance [8,9]. These studies also indicated the presence of metabolic abnormalities, such as impaired glucose metabolism [8], postprandial lipemia without excessive hyperglycemia [10], and hyperinsulinemia [11]. Furthermore, a number of particular genes have been linked to both insulin resistance and β-cell dysfunction in the FDRs of people affected with T2DM

[12]. Therefore, the identification of susceptible genes and reliable biomarkers may be of great help in the early diagnosis of at-risk FDR which undoubtedly causes a reduction in the numbers of affected cases [13].

Recent studies have suggested inflammation as an important etiological factor for the development of insulin resistance, obesity, and T2DM [14,15]. Toll-like receptors (TLRs) are a family of pattern recognition receptors. They recognize pathogen-associated molecular patterns expressed by microbial products or endogenous signals of tissue injury, including necrotic cells, oligosaccharides, heat-shock proteins, and nucleic acid fragments. The TLRs are transmembrane glycoprotein receptors that regulate the expression of pro inflammatory cytokines and initiate an adaptive immune response [16]. The most widely known member of TLR family is TLR4 which greatly contributes to the pathogenesis of insulin resistance, diabetes, and atherosclerosis in both clinical and experimental conditions [17]. The TLR4 is a signaling receptor for the lipopolysaccharide of Gram-negative bacterial cell walls, which bind to specific ligands, such as free fatty acids [18]. Upon activation, the nuclear factor kappa B (NF-κB) pathway triggers the transcription of pro-inflammatory cytokines genes. Overexpression of these inflammatory genes is associated with inflammation and insulin resistance. This indicates that TLR4 could be linked to impaired glucose metabolism and diabetes. These cytokines inhibit insulin action in insulin target tissues [19]. As indicated in the literature, TLR4 expression undergoes an increase in the animal model of diabetes [20], as well as in T1DM and T2DM patients [21,22]. The enhanced expression of TLR4 has also been reported in the peripheral blood mononuclear cell (PBMC) and adipose tissue of insulin-resistant subjects [23]. Furthermore, previous genetic studies have linked TLR4 polymorphisms to T2D, which suggests a relationship between TLRs function and diabetes [24].

Insulin receptor substrate 1 (IRS1), an endogenous substrate of insulin receptor, plays a key role in the insulin-stimulated signaling pathway [25]. In fact, IRS1 functions as the main docking protein for signaling proteins with Src homology-2 domains, such as phosphatidylinositol 3-kinase (PI3-kinase). Although tyrosine phosphorylation of IRS1 is critical for the regulation of its function and downstream metabolic effects (e.g., insulin-stimulated glucose transport), its Sr/Thr phosphorylation attenuates insulin signaling and IRS protein expression through the separation of IRS from insulin receptor [26,27]. Reduced tyrosine phosphorylation of IRS1, PI3-kinase activation, and IRS protein expression in diabetes have been reported in both human and mouse models [25,28,29]. In the same vein, in a study, IRS1 knockout mice demonstrated mild insulin resistance and glucose intolerance [30]. Furthermore, in another study performed on glucose-intolerant relatives of T2D patients, IRS1 expression was reported to reduce in the skeletal muscle resulting in decreased IRS1-associated PI3-kinase activity and insulin resistance [31]. Decreased IRS1 expression was also indicated in the adipose tissue of 30% of individuals at high risk for T2DM (i.e., FDR and obese subjects) [32,33]. Moreover, these subjects were diagnosed with some metabolic abnormality, such as higher fasting glucose and insulin levels [33]. These findings suggest that the expression of IRS1 is a main regulatory step that greatly contributes to the development of insulin resistance. Therefore, these observations led us to select IRS1 as a suitable candidate gene for the investigation of the genetic basis of T2DM in subjects that are likely to have the inherited defect of insulin action. Genetic studies have mainly focused on gene expression in diabetic patients, while studies on the FDRs of these patients are not well established. Moreover, there is a paucity of data regarding the expression of the selected genes among the Malay population. With this background in mind, the present study was conducted to investigate these genes in the peripheral blood of T2DM patients and their relatives. To this end, the quantitative reverse transcription-polymerase chain reaction (qRT-PCR) experiments were performed to evaluate the potential role of the given genes as markers for the increased risk of developing diabetes in subjects with a family history of T2DM.

Materials and Methods

Study population

The present study was performed on three groups of T2DM patients (n=18; 10 males and 8 females, mean age=61 years), T2DM patients' FDRs (i.e., son, daughter, brother, or sister; n=18; 7 males and 11 females, mean age=44 years), and healthy controls (n=18; 10 males and 8 females, mean age=56 years). The study population was selected from the Outpatients Endocrinology Department of the Serdang Hospital, Faculty of Medicine, University Putra, Malaysia.

The T2DM cases were selected from patients with fasting plasma glucose of ≥ 7.0 mmol/l or 2-h plasma glucose of ≥ 11.1 mmol/l based on the World Health Organization criteria or from those already diagnosed with T2DM as confirmed in their medical records. With regard to the relatives of diabetic patients (e.g., sibling or offspring), those without any significant current or past medical illnesses were screened. Additionally, the individuals without a family history of diabetes or medical disease at the time of participation with a fasting blood glucose of <5.6 mmol/l were randomly enrolled from the local background population as the control group. On the other hand, pregnant women, individuals with T1DM, inflammatory disorders, cancer, late diabetic complications (e.g., heart disease, proliferative retinopathy, and consolidated nephropathy), and abnormal liver, renal, or thyroid function were excluded from the study. The present study was approved by the Ethics Committee of the Faculty of Medicine and Health Science, University Putra Malaysia and National Medical Research Register (ref number: KKM/NIHSEC/P15-795). Prior to the commencement of the study, written informed consent was obtained from all participants. Thereafter, peripheral blood was collected, and participants' demographic data, including age, height, weight, blood pressure, alcohol consumption, cigarette smoking, and family history, were recorded during blood sampling. All blood biochemical

factors, such as total cholesterol, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol (LDL), and hemoglobin A1C (HbA1c), were measured using standard kits. Table 1 presents the main clinical characteristics of all participants.

Table 1. *Clinical characteristics of the participants in the different groups.*

	Control (n=18)	T2DM (n=18)	FDR (n=18)
Age (year)	56.80 ± 4.12	61.86 ± 9.86	44.26 ± 12.71*
Gender (F/M)	10M,8F	7M,11F	10M,8F
BMI (kg/m²)	24.21 ± 1.74	25.31 ± 3.41	26.86 ± 5.81
FPG(mmol/L)	4.64 ± 0.60	7.98 ± 2.46*	5.05 ± 0.57
HbA1c (%)	5.10 ± 0.47	7.94 ± 1.81*	5.23 ± 0.45
Total cholesterol (mmol/L)	4.59 ± 0.55	5.14 ±.53	4.84 ± 0.12
Triglycerides (mmol/L)	0.95 ± 0.26	1.61 ±0.51*	0.94 ± 0.33
LDL-cholesterol (mmol/L)	2.42 ± 0.51	3.08 ±1.36	2.77 ± 0.64
HDL-cholesterol (mmol/L)	1.70 ± 0.19	1.11 ±0.36*	1.69 ± 0.22
SBP(mm Hg)	118.53 ± 11.66	133.80 ± 15.79*	117.80 ± 8.84
DBP(mm Hg)	77.00 ± 7.89	73.46 ± 26.79	74.66 ± 7.79

Average clinical data for all subjects in the three experimental groups. All subjects were Malay. Data are expressed as means ±S.D. n: number, F: female, M: male, BMI: body mass index, FPG: fasting blood glucose, LDL: low density lipoprotein, HDL: high density lipoprotein, SBP: systolic blood pressure, DBP: diastolic blood pressure. *indicates significantly different versus control ($P < 0.05$).

RNA isolation and cDNA synthesis

Peripheral blood (3 ml) was collected from all subjects and preserved in the Tempus™ Blood RNA tubes (Ambion, Austin, TX, USA), which were frozen at -20°C before use. Total RNA was extracted using a Tempus™ Spin RNA Isolation Kit (Applied Biosystems, USA) according to the manufacturer's protocol. The quantity of the extracted RNA was subsequently assessed using a Nanodrop ND-1000 Spectrophotometer (Thermo Scientific, USA), and its integrity was determined by the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA).

The RNA samples were used for complementary DNA (cDNA) synthesis if the A260/A280 ratio was more than 1.8 and RNA integrity number was ≥ 7. All samples were stored at -80°C until further use. First-strand cDNA was synthesized from total RNA (1 µg) using a QuantiTec Reverse Transcription kit (Qiagen, Germany) according to the manufacturer's guidelines. In brief, the samples were incubated at 42°C for 2 min in a wipe-out buffer to eliminate genomic DNA contamination. Thereafter, reverse transcriptase, RT buffer, and RT primer mix were added, and the samples were incubated at 42°C for 15 min. Incubation continued for 3 min at 95°C to inactivate reverse transcriptase. The presence of genomic DNA contamination was detected by performing a control reaction without reverse transcriptase.

Quantitative RT-PCR

Quantification of the cDNA target was performed using a QuantiFast SYBR Green PCR kit (Qiagen, Germany) in the Rotor Gene 6000 instrument (Corbett life Science, Valencia, CA and USA). PCR conditions were 5 min at 95°C as the initial incubation step, 40 PCR cycle at 95°C for 10s denaturation and a combined annealing/extension step at 60°C for 30s. Real-time quantitative PCR analyses were done using QuantiTect Primer Assay (200) [Hs_TLR4_1_SG QuantiTect Primer Assay (200) (Cat. QT 00035238) for TLR4 and Hs_IRS1_1_SG QuantiTect Primer Assay (200) (Cat. QT 00074144) for IRS1. For normalization, Gylceraldehyde-3-phosphate dehydrogenase (GAPDH) was used [Hs_GAPDH_1_SG QuantiTect Primer Assay (200) (Cat. QT00079247), Qiagen].

The specificity of the amplification products was confirmed by monitoring the melting curve. No amplification of unspecific product was observed. TLR4 and IRS1 quantities were analyzed in duplicate, normalized against GAPDH as an internal control, and expressed in relation to mRNA isolated from healthy peripheral blood as a calibrator.

Statistical analysis

The normality of variables was examined using the Shapiro-Wilk test. The comparison of the variables among the T2DM patients, FDRs, and controls was performed using ANOVA, followed by Tukey's posthoc analysis. The expression ratio was calculated using REST (Relative Expression Software Tool) 2009, based on qPCR efficiency and fold changes of the threshold cycle values as compared to the controls [34]. This software uses the pairwise fixed reallocation randomization test as its statistical model. Correlation between IRS1 and TLR4 expression clinical and metabolic characteristics was assessed using the Spearman's rank correlation. The data were expressed as mean ± standard deviation. P value <0.05 was considered statistically significant. Statistical analysis was performed in SPSS software (version 21).

Results

Clinical characteristics of the research groups

The clinical and laboratory characteristics of the research groups are displayed in Table 1. The subjects in the diabetic and control groups were closely matched in terms of body mass index and age. The aforementioned two groups were older than the FDR group. The results demonstrated significantly higher fasting plasma glucose (FPG) and HbA1c levels in the T2DM patients, as compared to those in the FDR and control groups. Moreover, the three groups were not significantly different in terms of the LDL level. After overnight fasting, the plasma glucose level was reported to be higher in the FDR group in comparison to that in the control group (5.05 ± 0.57 vs. 4.64 ± 0.60 mmol/l; $p<0.05$). Moreover, the HbA1c level tended to be higher in the FDR group than in the control group (5.23 ± 0.45 vs. 5.10 ± 0.47; $p<0.05$). Both

FDR and control groups were non-diabetic based on fasting blood glucose and HbA1c test. The majority of the diabetic patients in our study had hypertension and hyperlipidaemia; accordingly, they were prescribed with antihypertensive and cholesterol-lowering medications. The mean duration of diabetes was 11.93 ± 6.23 years, and all patients were prescribed T2DM medication. Moreover, nearly all study subjects were non-smokers, except for two cases in the controls and one case in the diabetic group.

Expression level of toll-like receptor 4 and insulin receptor substrate1 in subjects with type II diabetes and first-degree relatives

The expression level of the selected genes was examined by qRT-PCR as described in the material and method section. Table 2, Figures 1A and 1B present the expression ratio of IRS1 and TLR4. The IRS1 was significantly down-regulated in the T2DM patients as compared to that in the healthy subjects (P=0.001). However, no significant difference was observed between the FDR and healthy groups in terms of the expression level of this gene (P=0.34; Table 2 and Figure 1A).

Table 2. *Fold change of relative expression in blood obtained from type 2 diabetes mellitus (T2DM) and first-degree relative (FDR) in comparison with healthy control.*

	T2DM (n=18)		FDR (n=18)	
Gene	ExpR	P-value	ExpR	P-value
IRS1	0.25	0.001	1.267	0.349
TLR4	3.85	0.005	0.315	0.01

Differences between groups were calculated by REST Software. Relative gene expression of the related gene in the control group=1. ExpR, expression ratio; p < 0.05 means difference is significant.

Furthermore, the median expression level of IRS1 was lower in the FDR group than in the diabetic group (Figure 1A). A significant increase was detected in the expression of TLR4 in the peripheral blood of T2D patients (P=0.005). Moreover, reduced TLR4 expression was observed in the FDR group, compared to that in the control group (P=0.010; Table 2 and Figure 1B). In addition, the median expression level in the box-and-whisker plot was higher in the diabetic subjects, compared to that in the FDR group (Figure 1B).

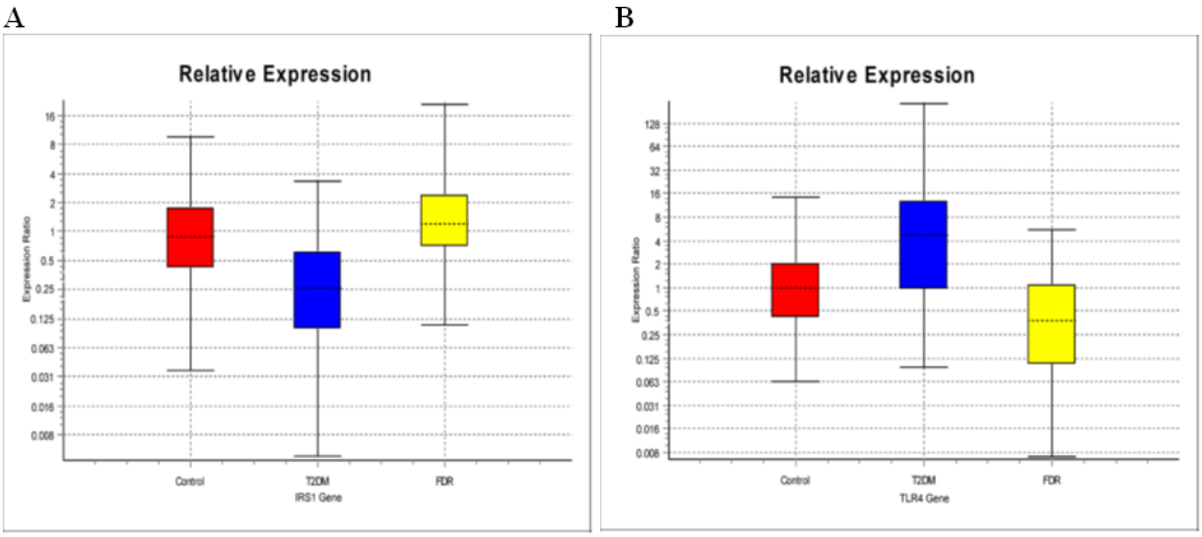

Figure 1. *Relative expression of TLR4 and IRS1 in the blood sample of type 2 diabetic patients (n=18) and first-degree relatives (FDR)(n=18) compared to control group (N=18); TLR4 and IRS1 gene expression was determined by quantitative real-time qRT-PCR as described in Material and Methods. The representative data are shown. (1A) IRS1 expression was found to be significantly downregulated in T2DM patients (P = 0.001) while it was not different in FDR (P=0.34) as compared with control group. (1B) TLR4 expression was significantly upregulated in T2DM patients (P=0.005) and downregulated in FDR (P=0.01) than the as compared to control group. The Boxes show 50% of all observation. The dotted line represents the sample median. The whisker shows lower and upper observation.*

Correlation analysis

The Spearman's rank correlation was performed to investigate the association of TLR4 and IRS1 expression levels with the clinical data displayed in Table 1. Correlation analysis in all groups, namely T2DM, FDR, and controls, demonstrated a significantly negative correlation between IRS1 expression and HbA1c (r=-0.379; p<0.01) and FPG (r=-0.279; p<0.05). On the other hand, a positive correlation was observed between TLR4 expression and HbA1c (r=0.309; p<0.05). It is

noteworthy that several associations that were observed in the entire population were not confirmed when the correlations were run separately in each group, except for IRS1. In this regard, IRS1 was negatively correlated with HbA1c (r=-0.656; p<0.01) in the diabetic group (Figure 2) but not in the FDR and control groups, which can be ascribed to reduced sample size.

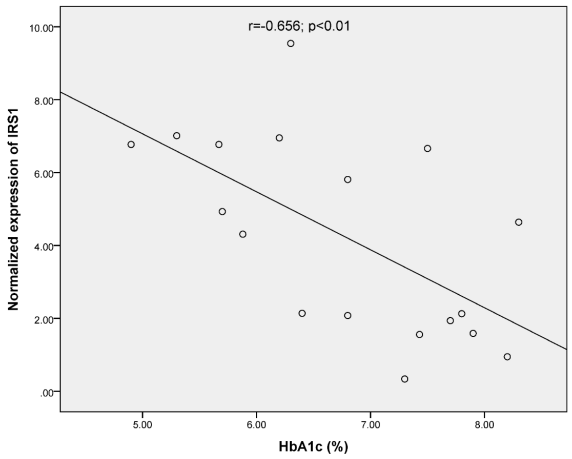

Figure 2. *The spearmans rank correlation between expression of IRS1 and HbA1c level. The expression of IRS1 is negatively correlated with HbA1c level in type 2 diabetic patients (n=18, r=-0.656, p<0.01).*

Discussion

Family history has been recognized as an independent predictor of the risk of developing diabetes [35]. Therefore, studies performed on the FDR of T2DM patients can be of great help for the detection of susceptible genes and candidate biomarkers of this disease. Previous studies have indicated the involvement of selected genes in T2D development. These studies have mostly focused on the expression of genes in other tissues, such as adipose tissue, skeletal muscle, and pancreas. It has been indicated that 80% of genes expressed in other tissues were also expressed in the peripheral blood cells. Therefore, the gene profiling analysis of peripheral blood cells could reflect the pathophysiological condition of the disease demonstrating the potential of peripheral blood cells as a suitable tissue source for genetic studies [36]. Moreover, it provides substantial benefits, such as a readily accessible source and a less invasive method. The present study involved the assessment of the expression of two genes (i.e., IRS1 and TLR4) in the peripheral blood of T2DM patients and their relatives among the Malay population. The aim of this research was to open up new horizons for the prevention and delay of diabetes.

The obtained results revealed the increase of TLR4 expression in the blood of T2DM patients having a significant correlation with the HbA1c level. In addition, our findings significantly support the role of TLRs in diabetes, which is consistent with other studies [21,23,37]. The TLRs are key mediators for innate immune response suggesting that the expression of these receptors is necessary for the regulation of the inflammatory cascade. Therefore, the activation of the innate immune system via TLRs, particularly TLR4, leads to the development of various types of inflammatory disorders, such as T2DM [23]. The TLR4 can activate NF-B pathway, which is an important regulator in insulin response, and glucose metabolism. The activation of NF-B pathway leads to the release of pro-inflammatory cytokines (e.g., TNF-α and IL-6) and chemokines [19]. In addition, increased TLR4 expression was reported in adipose tissue [20], PBMCs, and subcutaneous adipose tissue of db/db mice. This elevated expression was reported to significantly correlate with HbA1c, FPG, and inflammatory cytokines, such as TNF-α and IL-6 [23]. Increased TLR4 and TLR2 expression, ligands, and intracellular signaling are demonstrated in monocytes showing a significant correlation with HbA1c (glycated hemoglobin) levels in patients with T1DM and T2DM [15,22].

In the current study, TLR4 expression in peripheral blood cells was in line with those reported in the skeletal muscle of insulin-resistant subjects [14], smooth muscle cells of coronary arteries [38], adipocytes [39], and human endothelial cells [40]. Moreover, the upregulation of other TLRs, including TLR2, TLR3, and TLR8, as well as inflammatory cytokine production, have been reported in previous human studies [23,41]. In addition, in animal models, the increased expression of TLR5, TLR8, TLR9, and TLR12 genes, as well as the downstream signaling molecules, was reported in diet-induced obese mice [42]. A few studies both on humans and mice demonstrated that hyperglycemic state could increase TLR4/TLR2 expression and activation via the induction of inflammatory cytokines, oxidative stress, protein kinase C, and NF-κB activity [16,41]. Nonetheless, it has been reported that mild postprandial hyperglycemia is not able to increase TLR4 expression [43]. A number of previous studies [44-47] have indicated that TLR4 is also activated by high concentrations of free fatty acids (FFAs). In fact, FFAs might initiate intracellular inflammatory signals by the activation of TLR4 signaling, and such signaling can be linked to insulin resistance [44]. Moreover, the serum concentrations of fatty acids have been documented to increase in patients with metabolic disorders, such as obesity and diabetes [48]. This might contribute to T2DM patients' susceptibility to the development of insulin resistance. Davis et al. [49] performed a study on TLR-4-deficient 10ScN mice which were fed on a high saturated fat diet. They found that TLR4 mutation protected the 10ScN mice against the obesogenic effects of saturated fatty acids (SFAs) and related inflammatory response. The stimulatory effects of hyperglycemia are exacerbated with FFAs by engaging TLR4 receptors which may accelerate the development of T2DM and insulin resistance [50].

The striking result of the present study was the down-regulation of TLR4 in the peripheral blood cells of T2DM patients' relatives. This finding can be of great help in understanding the genetic background of T2DM. As presented in previous studies, the FDRs of T2DM patients have a high risk of diabetes development as compared to the normal population [51]. Moreover, metabolic disorders have been frequently reported among the normal glucose-tolerant FDR of

T2DM patients [52]. There is a paucity of information regarding the expression of TLR4 in the FDR of diabetic patients. Pietraszek et al. [45] determined the effect of dietary fat on the expression of inflammatory genes in the muscle and adipose tissues of the FDRs of T2DM patients among the Caucasian population. They found that the basal expression of TLR4 gene was lower in the normoglycemic FDRs and was significantly upregulated in response to a meal high in medium-chain SFA as compared to that in the control group. Since hyperinsulinemia has been reported in the normoglycemic offspring of T2DM subjects [53], the down-regulation of TLR4 in the FDR group can be attributed to the anti-inflammatory effects of high plasma insulin. Decreased expression of TLR4 could jointly act with hyperinsulinemia to protect this group from the negative metabolic effects of TLR4 activation. However, this remains theoretical until further investigation.

The next finding of the present study was the down-regulation of IRS1 expression in the T2DM patients in comparison to that in the nondiabetic and healthy subjects. Nevertheless, no significant difference was detected in IRS1 expression level among their relatives. The IRS1 is the main substrate for the insulin receptor and other tyrosine kinases and plays a key role in the insulin-stimulated signal transduction pathway. Tyrosine phosphorylation of IRS1 triggers intracellular signaling pathway that ultimately regulates glucose metabolism [25]. The obtained results of the current research are supported by other studies reporting a significant down-regulation in IRS1 and a decrease in insulin-stimulated IRS1 phosphorylation and PI3-kinase activity in the skeletal muscles of obese nondiabetic and diabetic patients [54], as well as in the ob/ob mice [55] and Zucker fatty rats [56]. However, in a study conducted by Björnholm et al [57], IRS1 protein expression was not significantly different in the skeletal muscle of T2DM subjects and controls. In line with these results, Kovacs et al. [58] highlighted the relationship of between single nucleotide polymorphisms in the promoter region of IRS1 and decreased IRS1 mRNA expression in the muscle and adipocytes of Pima Indians in Arizona. This finding indicates the significant role of the gene in the pathogenesis of T2DM in this population. Likewise, Carvalho et al. [59] demonstrated decreased IRS1 mRNA and protein expression in the adipose tissue of T2D and non-diabetic insulin-resistant individuals. This decrease was associated with the reduced expression of GLUT4 and impaired insulin-stimulated glucose transport. On the other hand, correlational analysis revealed a negative association between IRS1 expression and HbA1c level.

A study performed by Cardellini et al. [60] failed to find any correlation between IRS1 expression and metabolic trait in the monocytes of insulin-resistant subjects. Nonetheless, the mentioned study demonstrated that IRS2 expression was inversely correlated with HbA1c and FPG. It has been suggested that elevated plasma FFA levels, chronic insulin exposure, and production of pro-inflammatory cytokines (e.g., TNFα) contribute to insulin resistance through the stimulation of Ser phosphorylation on IRS1, which reduces IRS1-associated PI3-kinase activity [61,62]. In addition, Ducluzeau et al. [63] reported about the impaired phosphorylation of IRS1 in response to insulin. They demonstrated the down-regulation of IRS1 mRNA in the skeletal muscle of T2DM patients after euglycaemic hyperinsulinaemic clamp. A few studies have also proposed that IRS1 protein expression is markedly reduced after chronic insulin treatment via proteasomal degradation [64,65], which may be one of the possible mechanisms which cause insulin resistance. Furthermore, it was found that the expression of IRS1 was not significantly different in the FDRs of T2DM patients as compared to that in the control group. Nonetheless, so far, a limited number of studies have addressed insulin signaling pathway in the offspring of T2DM patients. The obtained results of the current study are in agreement with those found in the cultured skeletal muscle cells and monocytes obtained from the relatives of T2DM patients [60,66]. Nevertheless, in contrast to our obtained results, Carvallho et al. [33] indicated low IRS1 protein expression in the adipose tissue of the FDR of T2DM patients and obese individuals. This finding revealed the role of impaired transcriptional activation in the pathogenesis of T2DM. Moreover, decreased mitochondrial function and increased IRS1 serine phosphorylation have been demonstrated in the muscle of the insulin-resistant offspring of T2DM patients before and during a hyperinsulinemic-euglycemic clamp [67]. In the mentioned study, it was hypothesized that impaired mitochondrial activity as the earliest defects of T2DM development causes insulin signaling defects and insulin resistance in the subjects who are genetically predisposed to T2DM development. This discrepancy between our results and those reported in the mentioned study can be ascribed to population and tissue-specific differences (i.e., blood versus skeletal muscle and adipose tissue). The simultaneous evaluation of gene expression in both T2DM patients and their relatives is one of the strengths of the present study. This point is of considerable importance since the majority of similar studies have included relatives without a diabetic group. However, every study has some limitations which should be addressed. In this regard, we acknowledge that the present study has some limitations. Firstly, the subjects in the FDR and control groups were from different age groups. In other words, the relatives were younger than the controls. This raises the possibility that insulin sensitivity may differ between controls and relatives due to age differences [68]. Secondly, most of the patients were under insulin therapy and received blood glucose-lowering medications; therefore, the effects of these medicines on the expression profiles could not be ruled out. Regarding this, it is suggested that large-scale studies be conducted to approve the validity of these findings. Moreover, it is recommended to measure insulin resistance and protein contents in peripheral blood.

Conclusion

In conclusion, we have shown increased TLR4 and IRS1 expression in T2DM patients in the Malay population, indicating that expression of these genes might be involved in T2DM in this population. We also detected down regulation of the TLR4 gene in first-degree relatives of T2D patients. This

new finding in FDR could be used as a good marker for early detection of populations at risk of the disease and may take advantage to reduce risk of diabetes in high-risk individuals by making changes in lifestyle and dietary habit. Moreover, further evaluation in the future will be required to clarify the potential role of these genes in T2DM pathogenesis.

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