Gene Expression Pattern of *PI3K/AKT* Pathway Based on Insulin Resistance and Vitamin D Toward the Understanding of T2DM Pathogenesis

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Abstract

Background Insulin-stimulated glucose transport occurs via *PI3K/AKT*-dependent pathway which results in *GLUT4* translocation from intracellular vesicles to plasma membrane and glucose uptake. *PTEN*, as a phosphatase, is the main antagonist of the *PI3K/AKT* pathway's kinases. Present study was performed to investigate underlying mechanism responsible for defects in insulin signaling and the plausible role of vitamin D in pathogenesis of T2DM through the comparison of serum vitamin D and expression of genes involved in insulin signaling in diabetics and non-diabetics.

Results RT-PCR was employed to investigate mRNA expression level of *IRS1/PI3K/PDK1/AKT2/GLUT4/PTEN* in diabetic and non-diabetic participants and serum vitamin D was measured by HPLC. Findings provide evidence that *IRS1* expression was preserved while *PI3K/AKT2/GLUT4* were expressed significantly lower and *PDK1* was expressed significantly higher in diabetics compared to non-diabetics. Albeit there was no significant difference in *PTEN* expression between groups, it was up-regulated by the years of having diabetes. No significant correlation between serum vitamin D and gene expression of GOIs was observed in either group.

Conclusion Since *PTEN* has been identified as a negative regulator of insulin signaling, it has generated great interest in new therapeutic approaches. This study reveals that insulin resistance is not caused through an alteration in *PTEN* expression as a primary defect but may be caused by decreased *PI3K/AKT2/GLUT4* signalling and dysregulation of feedback loops. Particularly, *PTEN* expression showed a significant relation with duration of diabetes, suggesting that *PTEN* may not be the cause of the reduced expression of *PI3K/AKT* pathway in diabetics while it can be the effect of that. Therefore, the future investigations should be focused on regulators of pathway instead of *PTEN*. No significant correlation between serum vitamin D and gene expression of GOIs could be due to the non-linearity relationships as insulin signaling is a cascade with amplifying properties.

Background

Glucose homeostasis is an essential aspect of insulin action, which impaired insulin regulation of glucose transport through *PI3K/AKT* pathway results in insulin resistance and Type II diabetes [1]. Insulin initiates its function by interacting with insulin receptors (*IRS*) and phosphorylated/activated *IRS* can be reversely dephosphorylated/deactivated by *PTEN* phosphatase and *SHIP2* [2], [3]. Phosphorylated *IRS1* binds and activates *PI3K* which causes to phosphorylation of PIP2 to form PIP3 (*PTEN* and *SHIP2* dephosphorylate PIP3 back to PIP2), then PIP3 interact allosterically with *PDK1* and leads to *AKT/PKB* and *PKC* phosphorylation to activate *GLUT4* translocation to the plasma membrane from intracellular vesicular compartment [4], [5]. Hence, *PTEN* inactivation leads to PIP3 accumulation and consequently, to the hyper activation of *AKT*, which leads to decrease serum glucose level [6]. This suggests that higher level of *PTEN* may make individuals more susceptible to the development of Type II diabetes by modulating insulin sensitivity [7]. Overexpression of *PTEN* in T2DM, results in inhibition of *AKT* signalling pathway and *GLUT4* translocation to the cell membrane, hence decreases glucose uptake. In contrast,

decreasing of *PTEN* expression, enhances insulin stimulated *AKT* phosphorylation [8]. Therefore, regulation of insulin function is performed by the balance between phosphorylation and dephosphorylation of the *PI3K/AKT* pathway components.

The insulin signalling pathway includes multiple feedback loops [9], as phosphorylated/activated *AKT* phosphorylates and negatively regulates *PTEN*. This phosphorylation impairs the function of *PTEN* to dephosphorylate *IR* and *IRS1* [10]. This reveals positive feedback loop (*AKT* inhibits signal attenuation *PTEN* hence inhibits dephosphorylation of the receptor and *IRS1*) that consists of a double negative feedback loop (phosphorylated kinase (*AKT*) negatively regulates the phosphatase (*PTEN*) that dephosphorylates it) (Fig. 1).

Based on tremendous results of some recent studies, there is a relationship between vitamin D and insulin sensitivity not only in both *in vitro* and *in vivo* studies but also in epidemiological and clinical studies [11]. However, the mechanism underlying the effects of vitamin D on insulin sensitivity has not been clarified yet. Vitamin D may modulate *PTEN* and/or other components of the *PI3K/AKT* pathway hence, affects insulin sensitivity and glucose homeostasis [12], [13]. In our previous study, the effects of vitamins D on an insulin-resistant model of neuronal cells was investigated which caused significant increase in *IR*, *PI3K* and *GLUT4* expression levels, as well as *AKT* phosphorylation and glucose uptake [14].

The present study was carried out to investigate the molecular mechanisms of the *PI3K/AKT* pathway responsible for insulin resistance in Type II diabetes. In order to verify whether the regulation of key genes of insulin action is altered in Type II diabetes, mRNA expression level of *IRS1*, *PI3K*, *PDK1*, *AKT2*, *GLUT4* and *PTEN* in non-diabetics and Type II diabetics were investigated. Also, in order to elucidate the plausible role of vitamin D in pathogenesis of T2DM, the relationship between serum vitamin D concentration and expression level of genes involved in insulin signaling were compared in diabetics and non-diabetics.

Results

Analysis of mRNA Gene Expression by RT-PCR

RT-PCR method was employed and optimized with two housekeeping genes (*GAPDH* and *β-ACTIN*) to identify the alterations of *IRS1*, *PI3K*, *PDK1*, *AKT2*, *GLUT4* and *PTEN* expression levels as GOIs (gene of interest) in diabetic participants in compared to non-diabetics.

The amplification curves and melting curves of GOIs and housekeeping genes indicated the absence of contaminating products in negative control. The specificity of all primers were evaluated by melt curve analysis and a single amplified product has been observed for all genes.

Relative Quantification Of Gois Expression Levels

Normalised expression levels of GOIs in non-diabetic and diabetic participants are shown in Table 1 and Fig. 2.

Table 1
Relative Quantification of GOIs Expression Levels between Diabetic and Non-Diabetic Participants

GOIs	Non-diabetics (n = 50) Mean ± SE	Diabetics (n = 50) Mean ± SE	df	t	P-value
IRS1	0.23 ± 0.01	0.21 ± 0.01	98	-0.819	0.415
PI3K	0.32 ± 0.02	0.46 ± 0.02	98	3.68	0.001*
PDK1	0.70 ± 0.04	0.57 ± 0.03	98	-2.00	0.014*
AKT2	0.68 ± 0.04	0.87 ± 0.08	98	1.92	0.050*
GLUT4	0.74 ± 0.05	0.95 ± 0.07	98	2.24	0.028*
PTEN	0.44 ± 0.03	0.42 ± 0.03	98	-0.436	0.664
*P < 0.05					
The expression levels of <i>PI3K</i> , <i>AKT2</i> and <i>GLUT4</i> were significantly lower whereas the expression level of <i>PDK1</i> was significantly higher in Type II diabetic participants as demonstrated in Table 1. Gene expression level of <i>IRS1</i> and <i>PTEN</i> were slightly higher in Type II diabetic participants compared to non-diabetics but it was statistically insignificant.					

Gene expression levels of *IRS1* and *PTEN* were higher in Type II diabetic in comparison to non-diabetic participants but there was no significant difference between two groups, whilst the expression of *PI3K*, *AKT2* and *GLUT4* were significantly lower and *PDK1* was higher in Type II diabetic participants.

Further analysis of variables was performed to find out the possible mechanisms responsible for insulin resistance in Type II diabetic participants. Since the time is a proposed new classification system for diabetes [17], the association of duration of diabetes and gene expression level of GOIs were investigated in diabetic participants.

Target Genes Expression in Relation to the Duration of Diabetes

One-way ANOVA was used to compare differences between each of the GOIs and duration of diabetes (three groups were considered: <5, 5–10, 10 < years) as an independent variable in diabetic participants and these results are shown in Table 2.

Table 2
Relation of GOIs with Duration of Diabetes in Diabetic Group

		Sum of Squares	df	Mean Square	F	Sig.
IRS1	Between Groups	0.067	2	0.033	3.677	0.033*
	Within Groups	0.443	47	0.009		
	Total	0.510	49			
PI3K	Between Groups	0.040	2	0.020	0.645	0.529
	Within Groups	1.476	47	0.031		
	Total	1.516	49			
PDK1	Between Groups	0.165	2	0.082	2.828	0.069
	Within Groups	1.407	47	0.029		
	Total	1.572	49			
AKT2	Between Groups	0.233	2	0.117	0.337	0.714
	Within Groups	16.301	47	0.346		
	Total	16.534	49			
GLUT4	Between Groups	0.086	2	0.043	0.190	0.827
	Within Groups	10.481	47	0.223		
	Total	10.568	49			
PTEN	Between Groups	0.282	2	0.141	4.025	0.024*
	Within Groups	1.649	47	0.035		
	Total	1.930	49			
* <i>P</i> < 0.05						
<i>PTEN</i> and <i>IRS1</i> expression level revealed a significant relation with duration of diabetes which indicates that duration of diabetes is the strongest predictor for increasing of <i>PTEN</i> and <i>IRS1</i> gene expression.						

This study provides evidence that the gene expression levels of *IRS1* and *PTEN* were preserved under the conditions in which *PDK1* expression was higher and *PI3K*, *AKT2*, *GLUT4* expression were lower in Type II diabetic participants. These impairments are suggested to be the primary reason for insulin resistance and duration of diabetes is proposed to be the strongest predictor for increasing of *PTEN* and *IRS1* expression.

Assessment and Comparison of Serum Vitamin D Concentration in Relation to GOIs Expression Level

Serum vitamin D3 concentration (ng/mL) in diabetic participants were compared to non-diabetics. The average of serum vitamin D concentration was higher in non-diabetics (33.07 ± 0.97 ng/ml) compared to (30.47 ± 1.30 ng/ml) diabetics but the difference was statistically insignificant (T: -1.603, df: 98 and *P*-value: 0.112).

Pearson correlation test was performed to show the plausible relationship between expression levels of GOIs and serum vitamin D concentration which no significant correlation was observed in either group of participants (Table 3).

Table 3
Serum vitamin D level in relation to the gene expression level of GOIs in diabetic and non-diabetic participants

	Non-diabetic Participants		Diabetic Participants	
	Pearson Correlation (r)	P-value	Pearson Correlation (r)	P-value
IRS1	0.118	0.431	-0.031	0.828
PI3K	-0.252	0.083	-0.034	0.814
PDK1	-0.125	0.399	-0.030	0.839
AKT2	0.158	0.283	-0.214	0.136
GLUT4	-0.049	0.743	-0.266	0.065
PTEN	-0.210	0.162	0.001	0.993
**Correlation is significant at the 0.01 level (2-tailed).				
* Correlation is significant at the 0.05 level (2-tailed).				

Discussion

Although the necessity of the *PI3K/AKT* pathway in insulin signal transduction is documented [18], it has not attained sufficient *in vivo* and *in vitro* evidence to identify the underlying mechanisms of the pathway and contradictory findings have been reported through knockout and RNAi studies [19]–[21].

It is now commonly accepted that metabolic regulation relies on three types of control which involves; 1) Allosteric control of a key enzyme activity that triggers a metabolic pathway by binding to the activator, (mostly its substrate). 2) Posttranslational modifications such as phosphorylation, acetylation, glycosylation and proteolytic cleavage, which may affect the protein stability and/or equilibrium between

active and inactive enzyme. In these kinds of control, subsequent changes in protein-protein interaction may participate in generating the active/non-active enzymatic complex. 3) Transcriptional regulation such as DNA methylation, which affects the gene expression level of key enzymes and is considered as a longer time regulation scale. Most metabolic regulations rely on a collaboration of these various mechanisms. As the insulin signalling starts at the cell membrane and subsequent events occur via phosphorylation cascades, which mainly happen through the *PI3K/AKT* pathway, it is probable that a part of the insulin function results from posttranslational modifications of numerous transcription factors [1], [22]–[24].

Insulin resistance in Type II diabetes has been characterized by several defects in the insulin signalling cascade [1], [22]–[24]. These events are related to short-term post-translational regulation of specific protein functions and long-term transcriptional regulation of key genes of insulin signaling pathway [25]. This hypothesis is supported by findings of altered expression of genes encoding metabolic enzymes in Type II diabetic patients [26].

In this study, there was no alteration in insulin signalling at the level of *IRS1* and *PTEN* expression in diabetic participants despite the presence of reduced *PI3K*, *AKT2* and *GLUT4* expression levels and increased *PDK1* expression. These findings were in agreement with previous studies [27], [28] and suggesting that reduced expression levels of these genes may induce insulin resistance [8]. A contradiction in the results obtained from different investigations [1], [22]–[24] indicates several possible mechanisms of transcriptional regulation of the *PI3K/AKT* pathway. For instance, in the present study, no significant difference in gene expression of *IRS1* and *PTEN* in diabetic participants suggesting that defects in insulin signalling via *IRS1* and *PTEN* are unlikely to be the primary cause. Another possibility is that these genes exert their main role in the *PI3K/AKT* pathway, which, beyond a very narrow range of their changes, the homeostasis of the pathway will disappear. Thus, the insulin signalling is very sensitive to the alteration of these components. Otherwise, it should be considered that insignificantly higher level of *IRS1* in this study, might be due to the collaboration of various mechanisms including signal amplification as a compensatory mechanism and convergence of other signalling pathways. However, the role of negative feedback loops cannot be neglected, as control of insulin signalling can be achieved by autoregulation whereby downstream elements inhibit upstream components [29], [30]. For example, *AKT* negatively regulates *PTEN* and prevents dephosphorylation of *IRS1* by *PTEN* [31]. Therefore, it can be concluded that higher gene expression level of *IRS1* could be due to the higher amount of *PTEN* expression as well as lower *AKT2* expression as a compensatory mechanism. Alternatively, signals from other pathways can inhibit insulin signalling. The *IR* and the *IRS* are targets for such feedback control mechanisms. Phosphorylation of *IRS* on Serine residues could be a key step in these feedback control processes [32]–[35]. Most of the Serine/Threonine kinases that are stimulated by insulin, are downstream effectors of *IRS* and serve as negative modulators of its action. The blockage of these kinases by the *PI3K* pathway inhibitors, indicates that these kinases are downstream of *PI3K* as potential *IRS* kinases [33]. Also, insulin resistance inducers such as cellular stress, free fatty acids and tumor necrosis factor- α use similar mechanisms which activate some *IRS* kinases and inhibit their function by phosphorylation of Serine residues [33], [36]. Serine phosphorylation is considered as a short-term inhibitory mechanism,

while regulation of *IRS* expression might promote long-term insulin resistance. Also, it should be considered that as *PTEN* antagonizes *PI3K*, it may cause the activation of a feedback loop involving *IRS1* by upregulating signalling through *PI3K* [37].

Insulin induces *PI3K*-mediated activation of *PDK1* and produces PIP3 that regulates *AKT* activity and its plasma membrane translocation. Interaction between *PDK1* and *PKC* may be required for insulin-induced phosphorylation of *AKT* [38]. Moreover, *PDK1* activates *PKC* which stimulates gluconeogenesis and contributes to insulin resistance through activation of pyruvate carboxylase [39]. Though, this study did not examine the gene expression level of *PKC*, contribution of endogenous glucose production along with impaired *PI3K/AKT* pathway is proposed in insulin resistance as a result of higher gene expression level of *PDK1* in diabetics.

It has been revealed that insulin's signal being mediated by protein phosphatases such as *PTEN* and *SHIP*. Knockout and RNAi studies can induce diabetes by up-regulating *PTEN*. These phosphatases which have different biological functions *in vivo*, can induce insulin resistance through attenuating the *PI3K/AKT* pathway [40]. Overexpression of *PTEN* decreases insulin-stimulated *PI3K/AKT* pathway, *GLUT4* translocation and glucose uptake into the cells [41], [42]. Microinjection of anti-*PTEN* antibody increases insulin-stimulated *GLUT4* translocation to the cell membrane and glucose uptake [41]. Therefore, *PTEN* reduces insulin sensitivity [43], as it is increased by inhibition of *PTEN* [44]–[46]. Although numerous phosphatases could be considered to be significant players in insulin signal transduction, only *PTEN* has been considered in this study. Changes in the abundance of PTPases and their collaboration or interaction may be involved in the pathogenesis of insulin resistance. Therefore, further *ex vivo* studies are required to assess the underlying mechanisms of *PTEN* function as well as other phosphatases and differentiate their roles, interaction and collaboration in antagonizing *PI3K/AKT* pathway and induction of diabetes. Understanding of mechanisms underlying the regulation of *PTEN* is important to identify its roles in diabetes. Regulation of *PTEN* is controlled at three steps; transcriptional regulation, post-translational mechanisms and membrane recruitment [18], [47], [48]. Initially it was assumed that *PTEN* expression is constitutively until numerous transcription factors have been observed that binding directly to *PTEN* promoter and regulating its expression [47], [49], [50]. Localization of *PTEN* plays an important role in the regulation of its activity in order to dephosphorylate PIP3 back to PIP2 at the cell membrane [48], [51], [52]. Since, *PTEN* acts as the main antagonist of the *PI3K/AKT* signalling pathway by converting PIP3 into PIP2 [53], directly reversing the effects of *PI3K* and deactivating/dephosphorylating *AKT* through a decrease in PIP3 levels [54], [55]. Reduced concentration of cellular PIP3 has been reported in Type II diabetic participants [56]. Hence, *PTEN* inactivation leads to PIP3 accumulation and consequently to hyper-activation of *AKT*, which leads to decreased serum glucose level [6]. Therefore, the intracellular concentration of PIP3 and PIP2 is regulated by the *PI3K/PTEN* equilibrium and dysregulation of *PI3K/AKT* pathway or no equilibrium between the *PI3K* and *PTEN* concentration might be implicated in Type II diabetes [57].

The findings of the present study showed reduced expression level of *PI3K*, *AKT2* and *GLUT4* in diabetic participants compared to non-diabetics, confirming previous studies [27], [28]. Nevertheless, there was no

significant difference in gene expression level of *PTEN* and *IRS1* in diabetic participants that it was in consistent with the findings of some studies [58]–[60]. This may lead one to the hypothesis that localization of *PTEN* plays an important role in the regulation of its activity [61]–[64]. It means that the main role of *PTEN* in regulation of insulin function is performed by dephosphorylating the active form (insulin-stimulated) of insulin receptor and by modulating post-receptor signalling through antagonizing *PI3K/AKT* pathway [19]–[21]. These findings indicate that *PTEN*'s transmembrane function is probably more imperative than its intracellular function in insulin signal attenuation.

From another aspect it can be concluded that, although the *PTEN* level was higher in diabetic participants than in non-diabetics, the difference was not enough to be statistically significant while it was enough to affect *GLUT4* expression. It means that insulin sensitivity is impaired by reduced expression of components that amplify the insulin signalling such as *PI3K* and *AKT* [65]–[71]. Even though presence of bistable response has not been proved in insulin signalling pathway and we are waiting for more verifications of this property, there are indications that this pathway includes the required components to exhibit bistable behaviour [65]–[71]. Bistability can be generated due to the non-linearity in positive feedback loops or double negative feedback loops [72]. The non-linearity is due to the ultrasensitive response that is usually obtained through enzymatic cascades [73]. Bistable systems display hysteresis, which means that the signalling system switches between two separate steady states without resting in a transitional state and the required amount of stimulatory input for transition from one state to another is completely different from that required for the reverse transition [74]. The insulin signalling pathway includes multiple feedback loops [9], such as phosphorylated/activated *AKT* phosphorylates and negatively regulates *PTEN*. This phosphorylation impairs the function of *PTEN* to dephosphorylate *IR* and *IRS1* [10] and reveals a positive feedback loop. In other words, *AKT* inhibits *PTEN* as a signal attenuation, hence, it inhibits dephosphorylating of *IR* and *IRS*. This phenomenon consists of a double negative feedback loop (phosphorylated *AKT* negatively regulates *PTEN* that in turn dephosphorylates *AKT*). In except of this positive feedback loops considered in *PI3K/AKT* pathway, it is also known that many feedback loops have not been entirely characterized [9]. Thus, this pathway has the potential to convert stimulatory inputs into bistable responses. Therefore, we cannot rule out the hypothesis that bistability might exist in insulin-induced glucose absorption due to the ultrasensitivity of *GLUT4* expression level in response to the *PTEN* expression at this study. Our findings indicate that the *PI3K/AKT* pathway losses bistability beyond a very narrow range of *PTEN* expression levels in addition to impaired insulin sensitivity by reduced expression of components that amplify the insulin signalling such as *PI3K* and *AKT*. These results are in accord with the literature on the existence of bistability in insulin signal transduction [65]–[71]. Consequently, *PTEN/PI3K* could be a phosphatase-kinase couple that controls the transition of the signalling molecule between two phosphorylation states.

Since, the regulation of insulin function is performed by the balance between phosphorylation and dephosphorylation of the *PI3K/AKT* pathway components and *PTEN* has been identified as a negative regulator of this pathway, it has generated great interest in new therapeutic approaches. Nevertheless, this study reveals that insulin resistance is caused through reduced *PI3K/AKT2/GLUT4* signalling and not through alteration in *PTEN* expression. Furthermore, significant positive correlation between *PTEN*

expression level and duration of diabetes indicates that *PTEN* expression increases by the years of having diabetes. As the time is a proposed new classification system for diabetes [17], *PTEN* may not be the cause of the reduced expression of *PI3K/AKT* pathway in diabetics while it can be the effect of that.

According to the several studies [75], vitamin D is required for normal insulin function. A number of studies [76], [77] revealed that vitamin D level is positively correlated with insulin sensitivity and lower risk of impaired glucose tolerance and T2DM. The modulatory action of vitamin D in insulin receptor gene expression and insulin secretion may point to its role in the pathogenesis and development of T2DM [78]. Vitamin D deficiency causes reduced insulin secretion in rats and humans, whereas its replenishment increases glucose tolerance through improvements in β -cell function [79]. In addition, certain allelic variations in the vitamin D-binding protein (DBP) and vitamin D receptor (VDR) might affect glucose tolerance and insulin secretion [78], [80] thus contributing to the occurrence of T2DM. Furthermore, vitamin D has been reported to contribute to normalization of extracellular calcium which determines the normal intracellular calcium pool. Increased intracellular calcium impairs phosphorylation of insulin receptors, leading to decreased *GLUT4* activity and impaired insulin signal transduction [81], [82]. Also, it has been documented that vitamin D deficiency and obesity in adult C57BL/6 mice entailed hyperinsulinemia and impaired expression level of the *PI3K/AKT* pathway components which caused impaired glucose homeostasis and insulin resistance [12]. Furthermore, it has been demonstrated that vitamin D-induced activation of *PI3K/AKT* pathway is through *PTEN* down regulation and *AKT* up regulation [13]. Insulin controls glucose and lipid metabolism through the *PI3K/AKT* pathway and *PTEN* is a negative regulator of this pathway, hence, down-regulation of *PTEN* enhances the metabolic effects of insulin [83], [84] and reverses insulin resistance [85], [86].

Nevertheless, it remains to be elucidated whether alterations in insulin signalling gene expression in T2DM are influenced by the regulatory transcriptional properties of vitamin D. Since the active form of vitamin D, 1,25-dehydroxyvitamin D₃, influences expression of various genes [87], [88], the relationship between serum vitamin D concentration and gene expression level of insulin signal transduction components were assessed.

In this study, there was no significant correlation between serum vitamin D concentration and gene expression level of GOIs in either group of participants. Data presented in current report is not in agreement with previous study on vitamin D-induced activation of *PI3K/AKT* pathway by down regulation of *PTEN* in mice [13]. Also, in our previous *in vitro* study, vitamins D increased the expression level of *IR*, *PI3K* and *GLUT4* and phosphorylation level of *AKT* which caused increased glucose uptake on insulin-resistant model of neuronal cells [14]. The reason of contradiction between present study and our previous *in vitro* study [14] could be due to the non-linearity relationships, as the Pearson correlation test shows a linear correlation while insulin signal transduction is a cascade with amplifying properties [65]–[71]. Also, it should be considered that Pearson correlation test does not reveal the cause and effect relationships. Furthermore, in this study, only five diabetic participants were vitamin D deficient, thus, it was impossible to compare gene expression level of GOIs based on vitamin D status.

Conclusion

The focus of current study was to ascertain the plausible mechanisms responsible for transcriptional regulation of insulin signalling in humans through the comparison of alterations in expression level of *PI3K/AKT* pathway genes in diabetic individuals. Also, the authors intended to determine the plausible transcriptional regulation of glucose metabolism through vitamin D.

Most reported studies of insulin resistance are based on cell lines and animal models which suggesting increased *PTEN* expression as a main cause of insulin resistance [89]–[93]. Since, the regulation of insulin function is performed by the balance between phosphorylation and dephosphorylation of the *PI3K/AKT* pathway components and *PTEN* has been identified as a negative regulator of this pathway, it has generated great interest in new therapeutic approaches. Nevertheless, this *ex vivo* study provides novel findings and insights to the insulin signalling pathways in humans and reveals that insulin resistance is caused through reduced *PI3K/AKT2/GLUT4* signalling and not through alteration in *PTEN* expression. Furthermore, significant positive correlation between *PTEN* expression level and duration of diabetes indicates that *PTEN* expression increases by the years of having diabetes. It seem that *PTEN* is not the cause of the reduced expression of *PI3K/AKT* pathway in diabetics while it is the effect of that. Therefore, the future investigations should be focused on regulators of insulin pathway instead of *PTEN*.

Vitamin D is involved in insulin resistance through genomic and non-genomic molecular actions related to insulin signaling as well as reduction of oxidative stress, inflammation and regulation of gene expression. In this study, no significant correlations between serum vitamin D concentration and gene expression level of GOIs were observed in either group of participants. This result could be due to the non-linearity relationship as the Pearson correlation test shows linear correlations and it does not reveal the cause and effect relationships. Therefore, the role of vitamin D in maintenance of insulin sensitivity or pathogenesis of insulin resistance is not rejected through this study.

Methods

Experimental Participants

This cross-sectional study was conducted at Hospital Serdang and Universiti Putra in Malaysia to investigate the gene expression pattern of *PI3K/AKT* pathway based on insulin resistance and vitamin D status.

The appropriate sample size has been calculated based on independent-samples t-test to compare the means between two groups by using statistical software package Gpower 3.17. The effect size has been used as $d = 0.5$, the α error probability considered as 0.05, and Power of $1-\beta$ error probability considered as 0.95 based on recommended values. The number of recruited individuals for the study was 100 in age group of 35–60 (50 Type II diabetic participants and 50 non-diabetics served as control group). Mean age was not significantly different between non-diabetic and diabetic participants and the number of men and women were equal in each group. Participants who had cancers, nephropathy complications, thyroid

or parathyroid diseases and smokers were excluded from this study. The necessary approval was obtained from UPM ethic committee and coordination with the Health Ministry of Malaysia was fulfilled based on the applicable Malaysian laws (National Medical Research Register, (NMRR)). The study was conducted in accordance to the Declaration of Helsinki in its currently applicable version, the guidelines of the International Conference on Harmonization of Good Clinical Practice (ICH-GCP). Consent has been obtained from each participant after full explanation of the purpose and nature of all the procedures.

Blood Collection In The Paxgene Blood Rna Tube

For RNA isolation, 2.5 ml blood was collected from fasted volunteers in PAXgene Blood RNA Tubes (Qiagen; cat. no. 762165, Germany). The system requires combined use of PAXgene Blood RNA Tubes for blood collection and RNA stabilization, followed by PAXgene Blood RNA Kit (Qiagen; cat. no. 762174, Germany). PAXgene Blood RNA tubes containing blood samples were kept at room temperature for around 8 hours and then transferred to the refrigerator to extract RNA a day after blood collection. During the pilot study, it was concluded that the integrity and purity of RNA was improved by storage of tubes at room temperature for about 8hr and deferred extraction for 24hr after blood collection compared to the extracted RNA 2hr after blood collection. This is also based on the Kit protocol recommendation that postpone of extraction for at least 2hr after blood collection is compulsory to get satisfactory result.

Rna Extraction

Extraction of RNA was started with a centrifugation step to pellet nucleic acids. The pellet was washed, suspended and incubated in optimized buffers with proteinase K (PK) to bring about protein digestion. An additional centrifugation through the PAXgene Shredder spin column was carried out to homogenize the cell lysate and remove residual cell debris, before the supernatant of the flow-through fraction was transferred to a fresh Microcentrifuge tube. Binding conditions were adjusted with ethanol and the lysate was applied to a PAXgene RNA spin column for a brief centrifugation. RNA selectively binds to the PAXgene silica membrane as contaminants pass through during the centrifugation. Remaining contaminants were removed in several efficient wash steps. Between the first and second wash steps, the membrane was treated with DNase I (RNFD) to remove trace amounts of bounded DNA. After the wash steps, RNA was eluted in elution buffer and heat-denatured. Isolated RNA was measured at A260/A280 for purity control. The integrity and size distribution of RNA were checked by agarose gel electrophoresis and Ethidium Bromide staining. The respective ribosomal bands appeared sharp on the stained gel and 28S ribosomal RNA bands presented with an intensity approximately twice that of the 18S RNA band.

cDNA Synthesis

Purified RNA sample was briefly incubated in gDNA Wipeout Buffer at 42 °C for 2 minutes to effectively remove any contaminating genomic DNA and then it was used in reverse transcription to produce cDNA.

QuantiTect Rev.Transcription Kit (Qiagen; cat. no. 205313, Germany) was used and reverse-transcription master mix was prepared on ice according to the manufacturer protocol. Template RNA (14 µl) was thawed on ice and added to each tube containing reverse-transcription master mix. The tubes were incubated for 15 min at 42 °C and then incubated for 3 min at 95 °C to inactivate Quantiscript Reverse Transcriptase. Reverse-transcription reactions were stored on ice and transferred to the fridge at – 80 °C until using in RT-PCR.

Real-time Rt-pcr

Optimization and quantitation of all the markers of interest (*GAPDH*, *ACTIN*, *PTEN*, *AKT2*, *GLUT4*, *PDK1*, *PI3K* and *IRS1*) were carried out on real time-PCR (Qiagen; cat. no. 204054, Germany). In this study, all the primers were designed by Qigen Company and stored in TE buffer in small aliquots at – 20°C. A reaction mix include 2x QuantiFast SYBR Green PCR Master Mix, primers and RNase-free water was prepared according to the manufacturer instruction on ice and appropriate volumes dispensed into PCR vessels while programming the real-time cycler. Template cDNA was thawed on ice and added to the PCR vessels containing reaction mix (≤ 100 ng/reaction). For two-step RT-PCR, the volume of the cDNA added (from the undiluted RT reaction) should not exceed 10% of the final PCR volume (2 µl of the cDNA was added in this study, as the final PCR volume was 20 µl). The cycling conditions were as follows: 95 °C for 5 min (PCR initial activation step); 95 °C for 10 s (denaturation); 60 °C for 30 s (combined annealing/extension); followed by 40 cycles and it was performed on a Bio-Rad Real-Time PCR detection system (Bio-Rad; CFX96, USA).

An NTC containing all the components of the reaction except for the template was performed to enable detection of contamination. An RT-control including all the components of the reaction except for Quantiscript Reverse Transcriptase was performed to detect probable genomic DNA contamination.

Data Presentation And Calculations Through Relative Quantification

Target nucleic acids can be quantified using either absolute quantification or relative quantification. With relative quantification, the amounts of the target genes and the reference gene within the same sample were determined and ratios were calculated between each target gene and the reference gene. Then these normalized values were used to compare the differential gene expression in different samples. In this method, the amount of internal reference gene relative to a calibrator (fold change between two Ct values) is given by the equation [15]: Fold difference = $2^{-\Delta Ct}$.

Serum Vitamin D (cholecalciferol) Analysis

In order to measure serum vitamin D3 concentration of participants, HPLC method [16] was employed. Agilent 1100 machine, quaternary pump and Kinetex 5u C18 100A 250 × 4.6 mm column (Phenomenex, USA) was used in reverse-phase condition. Mobile phase was in an isocratic gradient; acetonitrile and methanol with the ratio of solvent 88:12, filtered and degassed to avoid any air bubbles and contamination. The flow rate was set at 1 mL/min and 20 uL per injection sample at 40°C. The HPLC unit was an integrated system with a UV-detector at 265 nm and data analysis was run by using ChemStation Operation System. 500 uL of plasma was added to 350 uL of methanol and 2-propanol (80:20 by volume). The tubes were mixed in a vortex mixer for 30 s. Vitamin D3 was extracted by shaking three times (60 s each time) with 2 mL of hexane. The phases were separated by centrifugation and the upper organic phase was transferred to a conical tube and dried under nitrogen gas. The residue was dissolved in 100 uL of water and methanol (with the ratio of 76:4). The sample was filtered by using non-sterile polyvinylidene difluoride (PVDF) filter with the pore size of 0.22 µm and diameter of 13 mm (AMTEC brand, Malaysia) and eluent was collected in 1.5 mL amber vial. Calibration curves were constructed using six different concentrations of vitamin D3 (3.125, 6.25, 12.5, 25, 50 and 100 ng/mL) as a reference standard. Concentration of vitamin D of each participants were stratified into three groups according to their serum vitamin D levels as: Optimal (20–80 ng/mL), Deficiency (< 20 ng/mL) and Toxicity (> 80 ng/mL). Coefficient variation in this study was less than 10%. The correlation coefficient in Standard Curve was $r^2 = 0.98$ while the equation gradient was $y = 0.112x$.

Statistical Analysis

Each experiment was performed three times and all data were expressed as means ± SE. Statistical analysis was performed by using SPSS 21.0 statistical software package (SPSS Inc., Chicago, IL, USA). Shapiro-Wilk test was performed to normalize the data. Independent-samples t-test was applied to compare the means between two groups and threshold of significance was defined as a $P < 0.05$. Pearson correlation test was used as appropriate to analyze the relationships between serum vitamin D concentration and expression level of genes. One-way ANOVA was used to compare differences between the groups based on duration of diabetes in diabetic participants. Levene's test was used to check significant differences ($P < 0.05$) revealed by ANOVA.

Abbreviations

AKT (Protein Kinase B); GLUT4 (Glucose Transporter 4); GS (Glycogen Synthase); IR (Insulin receptor); IRS (Insulin-receptor substrate); PDK (Phosphoinositide-Dependent Kinase); PI3K (Phosphatidylinositol 3-kinase); PIP2 (Phosphatidylinositol-4,5-bisphosphate); PIP3 (Phosphatidylinositol-3,4,5-triphosphate); PKC (Protein Kinase C); PTPs (Protein Tyrosine Phosphatases); PTEN (Phosphatase and TENsin homolog deleted on chromosome 10)

Declarations

Ethics approval and consent to participate

The necessary approval was obtained from UPM ethic committee. The study was conducted in accordance to the Declaration of Helsinki in its currently applicable version, the guidelines of the International Conference on Harmonization of Good Clinical Practice (ICH-GCP) and coordination with the Health Ministry of Malaysia was fulfilled based on the applicable Malaysian laws (National Medical Research Register, (NMRR)). Written consent has been obtained from each participant after full explanation of the purpose and nature of all procedures used. Ref. no: UPM/TNCPI/RMC/JKEUPM /1.4.18.1/F1

Consent for publication

Not applicable

Availability of data and materials

The data that support the findings of this study are available.

Competing interests

The authors declare that they have no competing interests.

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The funding bodies played no role in design of the study, sample collection, analysis, interpretation of data and writing of manuscript.

Authors' contributions

SAHK¹, **MSAM¹** and **HK⁶** were responsible for the study concept and design. **SAHK¹** and **JAA³** contributed to data acquisition. **MFS²** assisted **SAHK¹** with data analysis and interpretation of findings. **SAHK¹** drafted the manuscript. **MSAM¹**, **HK⁶**, **ZR⁴** and **RMA⁵** provided critical revision of the manuscript for important intellectual content and approved final version for publication. All authors read and approved the final manuscript.

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Figures

Figure 1

Insulin Signalling Pathway Insulin initiates its function by interacting with the insulin receptor which can be reversely dephosphorylated by PTEN phosphatase. Phosphorylated IRS1 binds and activates PI3K which causes conversion of PIP2 to PIP3 (PTEN dephosphorylates PIP3 back to PIP2). Allosteric interaction of PIP3 with PDK1 leads to AKT/PKB and PKC phosphorylation to activate GLUT4 translocation from intracellular vesicular compartment to plasma membrane. The insulin signalling pathway includes multiple feedback loops as phosphorylated/activated AKT phosphorylates and

negatively regulates PTEN. This phosphorylation impairs the function of PTEN to dephosphorylate IR and IRS1.