Original Article

Comparison of Insulin Expression Levels in White Blood Cells of infants with and without Family History of Type II Diabetes

Seyyed Reza Mazhari[1,2], Reza Mirfakhraie[1], Mojgan Asadi[3,4], Nasrin Alipour Olyaei[2], Hamidreza Kheiri[5], Elham Moslemi[6], Mahnaz Khanmohamadi[7], Elham Tohidnejad[8], Vahid RezaYasaee[1,2*]

[1] Department of Medical Genetic, Faculty of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran.
[2] Genomic Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran.
[3] Osteoporosis Research Center, Endocrinology and Metabolism Clinical Sciences Institute, Tehran University of Medical Sciences, Tehran, Iran
[4] Endocrinology and Metabolism Research Center, Endocrinology and Metabolism Clinical Sciences Institute, Tehran University of Medical Sciences, Tehran, Iran
[5] Department of Pharmaceutical Nanotechnology, School of Pharmacy, Zanjan University of Medical Sciences, Zanjan, Tehran, Iran
[6] Department of Biology, School of Basic Sciences, Islamic Azad University, East Tehran Branch, Tehran, Iran.
[7] Department of Cellular Biology, Faculty of Pharmaceutical Sciences, Islamic Azad University, Tehran, Iran.
[8] Department of Biostatisistic, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran

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Abstract

Background: Type II diabetes is known as one of the most important, prevalent, and expensive diseases of mankind. Late diagnosis and subsequent delayed initiation of treatment or surveillance of patients create a variety of problems for affected individuals. This has raised increasing concerns for public health authorities throughout the world. In the current study, we aimed to find a new approach for early identification of high-risk individuals at initial months of their life. This allows us to take preventive measures as early as possible.

Materials and Methods: In our study, 102 infants - from one to six months - were selected and placed in two case and control groups. The case group contained 52 babies with at least one of their parents identified as a type II diabetic patient. The control group comprised 50 babies with no family history of type II diabetes in paternal and maternal first-degree relatives. Afterwards, the expression level of insulin gene was analyzed in white blood cells of both groups. Information related to infants - referred to outpatient and inpatient wards of three main pediatric hospitals placed in Tehran - and their parents were collected through questionnaires within a two-year period. The study inclusion criteria for infants were confirmed type II diabetes in at least one of their parents, the absence of any metabolic disorder, and the absence of any disturbing vital signs. After drawing 2 ml of babies' peripheral blood, total RNA of white blood cells (WBC) was extracted, and used for cDNA synthesis. Real-Time PCR was then applied to quantitatively evaluate the expression levels of insulin gene. The results of Real-Time PCR were statistically analyzed by non-parametric tests of Mann-Whitney and Kruskal-Wallis.

Results: The expression of insulin gene was observed in white blood cells of all samples. However, there was a significant difference in expression levels between case and control groups ($p<0.05$). There was a statistically significant difference in mean levels of gene expression among babies with diabetic mother, and healthy groups (RQ=0.5, P-value=0.002), but this value wasn't significant for babies with diabetic father (RQ=0.78, $P>0.05$).

Conclusion: Numerous genes contribute to the development of diabetes and novel disease-causing genes are increasingly being discovered. Identification of disease-prone individuals through examining merely one

underlying gene is complicated and challenging. Interestingly, all of these abnormally functioning genes finally manifest themselves in the altered expression levels of insulin gene. The expression status of insulin gene in WBCs could be suggested as a useful approach for identification of individuals at high risk for developing diabetes. This paves the way for taking appropriate measures at infancy period in order to prevent the disease as well as inhibit its various side effects in the following years of patient's life.

Keywords: insulin, gene expression, diabetes screening, type II diabetes prevention

***Corresponding Author:** Vahid Reza Yassaee, MD, PhD; 1- Genomic Research Center, Taleghani Hospital, Aarabi St., Yaman Ave., Evin, Velenjak, Shahid Beheshti University of Medical Sciences, Tehran-1966645643, Iran
2- Dept. of Medical Genetic, Faculty of Medicine, Shahid Beheshti University of Medical Sciences,Tehran-1966645643, Iran. Tel: (+98) 21 22439959. Fax: (+98) 21 22439961. Email: s.reza.mazhari@gmail.com

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Introduction

Type II diabetes has proven to be as one of the most important, prevalent, and money-consuming chronic diseases for human (1, 2). Late diagnosis and subsequent deferred initiation of treatment or surveillance of patients have provoked growing concerns for public health professionals (3). World health Organization (WHO) defines diabetes as a disorder in which the body cannot produce enough insulin or cannot appropriately use the insulin produced (4). In this condition, the capacity of cells to fully metabolize sugars is lowered which leads ultimately to the increase of blood sugar. In addition to sugars, extensive irregularities also occur in the metabolism of proteins and lipids (5). Therefore, diabetes in its strict definition is a metabolic syndrome. Diabetes is a significant, widespread, and high-cost disease in contemporary societies and urban development, modernization, and the change of life-style have highlighted its importance. The age of disease development has been reduced dramatically and this trend is growing in an alarming manner (6).

Type II diabetes is mostly associated with changes in the three-dimensional structure as well as function of certain proteins such as insulin receptor on the cell surface, and many agents known as molecular intermediates which function intracellularly after receptor activation. Malfunction of all of these proteins results from alterations in their encoding genes (7). Thus far, more than 100 genetic loci associated with diabetes have been identified (8). However, the list of novel genes linked to diabetes is continually expanding. Although type II diabetes has a genetic origin and some of its harmful effects appear from the very initial years of life, the first paraclinical signs, including increased FBS (Fast Blood Sugar) or abnormal glucose tolerance tests are usually seen during the fourth decade of life and thereafter (9).

Screening assays for early identification of diabetic patients mainly focus on measurement of FBS, CPG (causal plasma glucose), glucose tolerance test, and hemoglobin A1c. The major goal of these assays is to initiate therapy as early as possible and prevent side effects of the disease. These side effects include nephropathy, retinopathy, neuropathy, and a variety of other secondary disorders which if remain untreated, will lead to diverse complications for the patient. It is known that damages begin to appear long before emergence of hyperglycemia even from the early days of life and severe harms to the pancreas gradually result in the decrease of insulin production (10, 11).

Apart from considering a number of risk factors, there is not any systematic and rigorous approach for identification of patients before the above-mentioned symptoms occur. Screening programs for early diagnosis of the disease are performed after the age of 30 and are based on detecting alterations in the levels of blood sugar, analyzing glucose tolerance tests, etc. Although public health authorities, following guidelines recommended by WHO, suggest life-style change as the major strategy to inhibit chronic disorders such as diabetes, adhering to these guidelines is also influenced by patient phenotype and non-

specific risk factors. On the other hand, the complex genetic pattern of diabetes - as seen in many polygenic disorders - makes it difficult to exactly assess the risk of the disease.

The involvement of over 100 genes associated with diabetes (8), with a diverse range of possible alterations such as deletion, point mutation, and different polymorphisms not only causes difficulty for risk assessment, but also makes the procedure uneconomical and money-consuming. However, taking one important characteristic of diabetes into account may present a solution to this problem. In type II diabetes, minor and major changes in disease-related genes introduce alterations into the structure and function of proteins which in turn lead to the decline of glucose uptake from blood by cells. The feedback induced by increased blood glucose on beta cells of the islets of Langerhans, irrespective of its underlying type of genetic alteration, elevates insulin production (12). This raises the question whether it is possible to analyze the expression level of insulin in the pancreas and use this information in order to evaluate one of the primary molecular reactions of the body during the first days of a baby's life. The lack of accessibility of pancreas and problems of sampling through biopsy for screening purposes make it impossible to analyze the insulin gene expression in the target pancreatic tissue. Therefore, another question is raised: could the expression analysis of insulin in an easy-to-sample tissue such as white blood cells lead to satisfactory results? The current study aimed to address these questions. Answer to these questions provides an appropriate means for identification of diabetes-prone individuals at birth. Furthermore, as this information determines the chance of disease development, it facilitates programming for prevention of diabetes itself and not only treatment of its symptoms.

Methods

Demographic and biologic data on patients: 102 infants, from one to six months, were chosen for the study. These babies had been referred to outpatient, inpatient, and laboratory wards of three pediatric hospitals located in Tehran, including Mofid Children's Hospital, Children's Medical Center, and Vali-e-Asr Hospital. All selected babies fulfilled the inclusion criteria (Table 1).

The infants were selected based on face to face (directed) interviews with their parents and primary laboratory examinations. The same questionnaire was allocated to all selected individuals and based on information received. They were divided into case and control groups. Babies with no family history of diabetes (in first-degree and second-degree relatives) were placed in control group and babies with diabetes in at least one of their parents were placed in case group. Out of 1674 infants, 110 individuals were chosen for complementary examinations in order to be checked for inclusion in the case group. 52 of 110 babies met the inclusion criteria and the rest were excluded from the study. 1564 infants had non-diabetic parents or grand-parents among which 50 individuals were selected for the control group. The nonexistence of diabetes in voluntary parents was confirmed by using FBS (Fasting blood sugar) test. In contrast, only information received through interviews or taking family history was used for maternal and paternal grandparents and other second-degree relative variables related to selected samples are shown in Table 1.

After informed consent was obtained from parents, 1-2 ml of peripheral blood was drawn from babies and poured into special EDTA tubes. Maintaining cold chain, the tubes were transferred to the main storage place within less than 3 hours.

RNA extraction: RNA extraction was performed immediately after receiving the samples using standard protocol. 100µl of each sample was poured into separate sterile microtubes and then 500µl of ice cold RNX-PLUS was added to the solution, vortexed for 10 seconds, and incubated at room temperature for 5 minutes. 200µl of chloroform was added to the microtubes and vortexed for 15 seconds. The microtubes were placed on ice for 5 minutes in order to be prepared for centrifugation. After centrifugation at 12000 rpm for 15 minutes, the supernatant was transferred to a new microtube and incubated with isopropanol. The microtubes were again centrifuged at 12000 rpm for 15 minutes. The supernatant was removed and the precipitate was incubated with 1000µl of 75% ethanol. The suspension was centrifuged at 7500 rpm for 8 minutes and the supernatant was discarded. The pellet was kept at

Table 1: Different variables related to examination of sample individuals.

Number of cases	
1674	Total number of primarily examined individuals
542*	Number of individuals with ambiguous medical history*
430**	Number of individuals with disturbed vital signs **
181***	Number of individuals with other important background diseases***
937	Number of individuals not exclusively fed with mother's milk
124	Number of individuals with problems in laboratory procedures and tests
1572****	Total number of individuals excluded from the study ****
102	Total number of individuals included into the study
52	Number of case individuals
50	Number of control individuals

*Considering the necessity of obtaining information on grandparents, parents of this category of babies did not provide adequate information on grandparents.

** Many of those who were referred to Mofid Children's Hospital, Children's Medical Center, and Vali-e-Asr Hospital (particularly inpatient cases) had at least one disturbed vital sign.

*** Considering the fact that samples were selected from those who were referred to medical centers for diagnostic and therapeutic purposes, a considerable number of them had important background health-related problems.

**** Due to the presence of some common study exclusion factors, total number of excluded individuals due to different factors mentioned in the table is higher than the true number of those who were excluded from the study.

room temperature to dry. Finally, 50µl of DEPC-treated water was added to the pellet. The extracted RNA was stored at -20°C before being used for cDNA synthesis.

cDNA synthesis: cDNA was synthesized by using cDNA synthesis kit (Vivantis company) 10µl of RNA, 1µl of 10 mM dNTP, and 1 µl of random hexamer were mixed together. The mixture was incubated at 65°C for 5 minutes. Afterwards, 2µl of 10X MMULV and 0.5 ml of MMULV enzyme were added and the final volume was adjusted to 20µl by adding deionized water. The microtubes were incubated at 42°C for 1 hour.

Primer design: Specific primers were designed to amplify sequences encoding insulin and GAPDH genes. To inhibit the amplification of genomic DNA, primers were designed in a manner that could bind to and amplify exon-exon junctions. Both primers in a primer set were capable of annealing to neighboring exons. As a result, PCR reaction could favor the amplification of short fragment derived form template cDNA, not genomic DNA or primers could bind to exon-exon junction and only amplified cDNA. Table 2 shows characteristics of the primers. The optimum annealing temperature of all primers was set at 60°C.

The quality and quantity of RNA were examined using UV spectrophotometry and agarose gel electrophoresis.

Real-Time PCR: qPCR reactions were performed in 96-well plates with a final volume of 20µl for each well. PCR master mix solutions for target and reference genes were prepared separately. Each reaction contained 18µl of master mix and 2µl of synthesized cDNA. Analyses of amplification and melt curves were performed based on Applied Biosystems 7500 using SYBR Premix Ex Taq Kit (Perfect Real Time) (Takara, Japan).

The quality of PCR products was analyzed by using 1.5% agarose gel electrophoresis. To evaluate the expression of insulin gene in infants with a family history of diabetes and control babies, the expression analysis of insulin gene in both groups was performed through RNA extraction, cDNA synthesis, and Real-

Table 2: Sequence of the primers used in Real-Time PCR reaction.

Primer Name	Primer Sequence	PCR Product Size
GAPDH F	5' ATGGAGAAGGCTGGGGCT 3'	124 bp
GAPDH R	5' ATCTTGAGGCTGTTGTCATACTTCTC 3'	
INSULIN F	5' TTCTTCTACACACCCAAGACCC 3'	148 bp
INSULIN R	5' TACAGCATTGTTCCACAATGC 3'	

Table 3: Program used for Real-Time PCR reaction.

Step	Program
1	Initial denaturation: 95 ˚C, 10 seconds, 1 cycle
2	Denaturation: 95 ˚C, 15 seconds. 45 cycles
3	Extension: 60 ˚C, 60 seconds

Table 4: Evaluation of the expression levels of insulin gene in case and control groups.

102	Total number of individuals/nursing babies selected for the study
52	Number of case individuals
50	Number of control individuals
20	Number of case individuals with diabetic father
32	Number of case individuals with diabetic mother
0.63	Mean expression levels of insulin in case individuals compared with control individuals
0.80	Mean expression levels of insulin in case individuals with diabetic father compared with control individuals
0.52	Mean expression levels of insulin in case individuals with diabetic mother compared with control individuals

Time PCR. After the reaction was completed, data were extracted from the thermal cycler as CT and gene expression was quantified by using ΔΔCT method. The graphs were then drawn by GraphPad software.

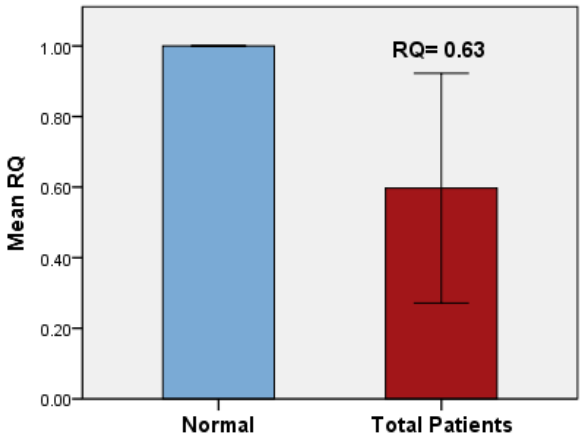
Results

To evaluate the specificity of primers, be assured of amplifying specific fragments, and examine the lack of generation of nonspecific PCR products, melt curves were drawn separately for insulin and GAPDH genes by Real-Time PCR machine. The graphs confirmed the specific annealing of the primers to the sequence of insulin gene and its subsequent amplification.

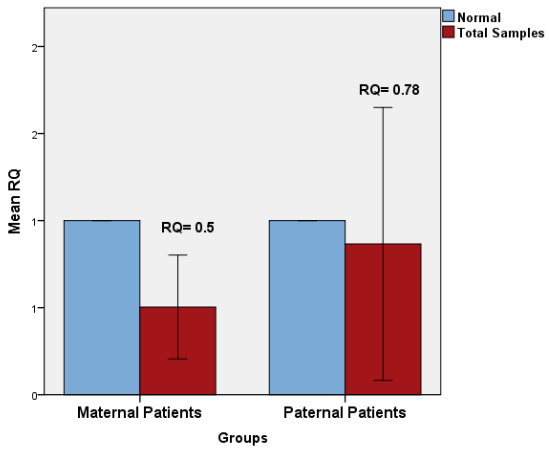
The identity of PCR product was confirmed by performing electrophoresis on 1.5% agarose gel The agarose gel electrophoresis of PCR product revealed the amplification of a single band size matched the molecular weight of the amplified insulin-coding sequence.

Expression analysis of insulin gene: The results of Real-Time PCR exhibited the expression of insulin gene in WBCs of both case and control groups. Table 4 indicates the results of insulin gene (INS) expression analysis in both case and control categories.

The comparative expression analysis of GAPDH and insulin genes was performed using ΔΔCT method and RQ calculation. Detailed analysis of the results of gene expression in case group highlighted the fact that despite overall reduced expression of insulin in case versus the control group, some case individuals indicated increased levels of insulin expression. This enhanced expression is found in 9 of 52 individuals in case group (17.3%). But, as previously mentioned, a significant number of infants with diabetic father or mother showed decreased levels of insulin expression compared with control group (82.7%). According to Kolmogorov-smirnov statistical test, gene expression



Graph 1. Comparison of mean RQ between case and control group.



Graph 2. Comparison of mean RQ among case individuals with diabetic father, case individuals with diabetic mother, and control individuals.

data have a normal distribution (P-value=0.05). The expression level of insulin in diseased group was 0.63 fold relative to healthy one. Thus, the parametric T-test was used for comparison of both groups. Analysis based on this parametric test suggested a significant difference between case and control groups (P-value =0.017) (Graph 1).

Furthermore, independent sample T-test revealed that there is a statistically significant difference in mean levels of gene expression among babies with diabetic mother, and healthy groups (RQ=0.5, P-value=0.002), but this value wasn't significant for babies with diabetic father(RQ=0.78, P-value>0.05) (Graph 2). Compare means between babies with diabetic mother and babies with diabetic father showed 0.64 fold expression in first group relative second one but this difference was statistically insignificant.

Discussion

Decreased binding of insulin to its cognate receptor on the cell surface and abnormalities induced by structural changes of intracellular signal-transducing proteins, leading to reduce of the glucose take up by cells from the blood (13). This increases the blood glucose level and subsequently laboratory signs of diabetes appear. In type II diabetes with abnormal structures of cellular proteins, compensatory elevated production of insulin by pancreas controls the blood glucose level before hypoglycemia occur (12). Thus, it is expected the expression of insulin gene in the pancreatic beta cells is enhanced before disease onset or even a long time after emergence of paraclinical signs. This could be used as a molecular method for early diagnosis of the disease when paraclinical signs have not yet been observed. More ever, biopsy samples cannot be taken from the pancreatic tissue for screening purposes. As a result, it is importance to find and use the patient cells which are available and easy-to-sample, express insulin gene, and similar to beta cells of the islets of langerhans are influenced by changes resulting from decreased uptake of glucose by cells.

Previous studies have demonstrated that WBCs express over 80% of identified genes (14). This suggests that white blood cells represent potential to be used as candidate cells for early diagnosis of diabetes. At the very beginning of life, these cells may provide some clues on type II diabetes. It was previously believed that insulin gene is exclusively expressed in the pancreatic beta cells (15). However, it has now become evidant that WBCs not only express insulin gene, but also show alterations in the expression levels of insulin during starvation (14). It was initially thought WBCs, like beta cells of the islets of langerhans, indicate higher expression levels of insulin in insulin-resistant individuals than normal ones. In normal condition, the pancreatic beta cells are responsive to elevated levels of blood glucose and release their own insulin reserves. Due to the short half-life of insulin, the release of insulin reserves and its consumption in the body necessitate further insulin production. Specific feedback mechanisms contribute to the increase of the insulin mRNA synthesis (15).

When insulin receptor or proteins related to glucose uptake pathway are structurally impaired, glucose uptake from the blood is decelerated, blood glucose levels remain high, and therefore insulin secretion continues. Production of excess insulin proceeds until reaching an appropriate concentration. The result of this compensatory production of insulin is reflected by decrease of blood glucose. This process, initiated from the very beginning of life, continues as long as pancreas maintains its capacity for producing excess insulin. It was previously postulated feedbacks which impact the pancreatic beta cells also affect white blood cells and lead to the increased expression of insulin.

Our findings indicate that the expression of insulin gene in infants with diabetic parents is significantly reduced versus babies with no family history of diabetes. In other words, the function of white blood cells presents a strike contrast to the function of beta cells. This difference could be explained by different hypotheses.

An interesting study by Choong Chin Liew et al. (2005) indicated that starvation and feeding can change the expression of insulin gene. They demonstrated that starvation and feeding lead to the increase and decrease of the insulin gene expression, respectively. These results support the observations of our study. One of the other aspects of our study is that alterations observed in the expression levels of insulin gene is in accordance with statistical findings obtained from epidemiological reports on diabetes. According to epidemiological findings, if one of the parents has type II diabetes their children will have approximately 15% chance for developing the disease (16). Assuming the correlation of decreased expression of insulin gene in white blood cells and diabetes, our results indicated the reduced insulin expression in 82.7% of babies with diabetic parents. Seven percent probability of disease development does not contradict the presence of at least a number of tens of impaired genes associated with type II diabetes in healthy children.

Development of diabetes and appearance of paraclinical signs arise from minor and major changes in disease-related genes. In this context, it is possible that total consequence of these changes in healthy children of a diabetic father or mother will not be high enough to create the disease. Therefore, it is not surprising that we saw different levels of decreased insulin gene expression in white blood cells derived from babies. In our study, 82.7% of babies with one diabetic parent showed a significant decline in the expression levels of insulin. Our results exhibited the expression levels of insulin gene vary from $1/10^6$ to 98.5%.

Further investigations as cohort studies with a larger sample size and future monitoring of babies will be helpful to fulfill the complementary goals of our study. Early identification of babies at risk of developing type II diabetes from birth, improvement of nutritional condition, and weight management programs play important roles in maintaining the functional capacity of the pancreas. If the pancreas is freed from pressures triggered by producing higher amounts of insulin, its longevity increases and therefore the beginning of diabetes symptoms is delayed. This delay reduces diabetes- mediated side effects in terms of both types of pathological signs and severity.

Conclusion

As a result, it seems that babies with higher decreased levels of insulin are more likely to develop the disease. In this study, we tried to use a homogenized condition for all babies. However, it cannot be ignored that uncontrollable condition could disturb homogeneity and introduce bias. To achieve a homogenized condition, all infants in the study were selected to be between one to six months. In this time period, babies exclusively fed with mother's milk are selected and thus error induced by variations in the nutritional condition of babies is dramatically lowered. Also, all babies were included into the study at least one-month after their birth. Elapse of one month from birth makes it possible to identify many important chronic diseases such as metabolic disorders. Babies with these types of diseases were excluded from the study. On the other hand, the one-month period of time counteracts the interfering effects of insulin which is produced in the mother's body and transferred to the baby's body via umbilical cord.

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Conflict of interest

The authors declare no financial or commercial conflict of interest.

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