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The Expression of Activating Receptor Gene of Natural Killer Cells (*KLRC3*) in Patients with Type 1 Diabetes Mellitus (T1DM)

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ABSTRACT

Objectives: To identify the possible role of natural killer (NK) cells in the pathogenesis of type 1 diabetes mellitus (T1DM) through studying the expression of the *KLRC3* gene, which encodes the NK cell activating receptor (NKG2E). ***Methods***: This study was conducted at Alexandria University Children's Hospital from April to October 2015. The study was conducted with 30 newly diagnosed T1DM patients (15 males and 15 females), aged 7–13 years (10.6±1.8 years) and 20 non-diabetic subjects served as age- and sex-matched controls. The patients were further sub-divided into two groups; group I included patients who first presented with classical symptoms of DM (polyuria, polydipsia, and polyphagia) without diabetes ketoacidosis (DKA) and group II included patients who first presented with DKA. The expression of the *KLRC3* gene was measured in each group using the real-time polymerase chain reaction. ***Results:*** *KLRC3* gene expression was significantly downregulated in T1DM cases compared to healthy controls ($p = 0.001$). Expression was more downregulated in group I patients ($p = 0.008$). Moreover, there was higher mean value of glycated heamoglobin and lower C-peptide levels in group I than group II. Serum pancreatic amylase showed no significant difference between the two groups. ***Conclusions:*** *KLRC3* gene expression was downregulated in patients with T1DM compared to healthy controls. Downregulation of expression was greater in DKA patients compared to those who presented with classical symptoms. Expression of KLRC3 in T1DM might play a role in the pathogenesis of T1DM and could be a predictor of its severity.

Diabetes mellitus (DM) is one of the most common chronic metabolic disorders and is caused by either impaired insulin secretion or insulin action, or both.[1] The two broad categories of diabetes mellitus are titled as type 1 and type 2 diabetes. Unlike type 1, many type 2 diabetic patients need insulin for their well-being but not for survival.[2]

Type 1 diabetes mellitus (T1DM) is most often diagnosed in children and adolescents.[3] The classical symptoms are polydipsia, polyphagia, and polyuria, as well as hyperglycemia, which initiates the immediate need for exogenous insulin replacement.[3] According to the World Health Organization (WHO) classification, T1DM is further divided into two subtypes: autoimmune (type 1A) and idiopathic (type 1B) diabetes.[4]

The main pathology in T1DM is the destruction of pancreatic beta cells. Immune cells have a critical role in this process, but the precise mechanisms involved in the progression of the disease are still unclear.[5] A combination of autoimmune conditions, genetic susceptibility, and environmental factors have been suggested to affect disease development and progression.[6,7]

Many studies have focused on the role played by T cells in the development and progression of the T1DM as it is the main cell proved to be infiltrating the diabetic pancreas.[1] Unlike T lymphocytes, natural killer (NK) cells exert their tasks without the need for previous activation. This important characteristic makes them highly suited to mediate the first line of defense against infection, as part of the innate immune response.[8,9] There are many quantitative and qualitative changes in NK cells in diabetic patients.[10]

Previous studies have revealed a destructive action of the NK cells on pancreatic islet beta cells and their significant involvement in the development of the disease.[11,12]

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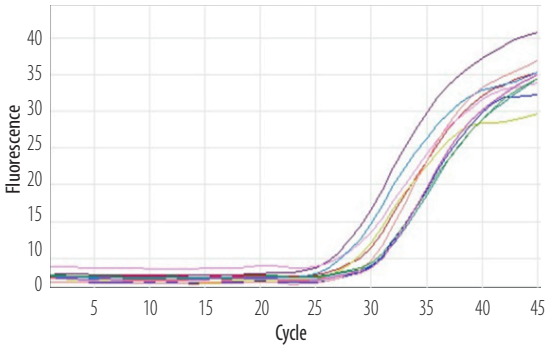

Figure 1: Amplification plot curves for *KLRC3* gene expression.

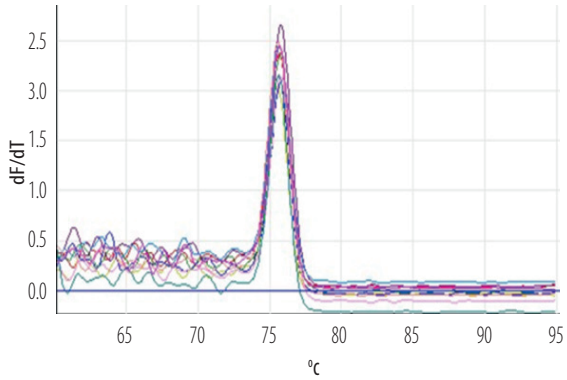

Figure 2: Melting curve for *KLRC3* gene.

The function of NK cells is achieved by a balance between activating and inhibiting receptors so as to sense their environment and differentiate between healthy and diseased cells. It is not known whether a specific receptor by itself is capable of triggering NK cell function. A balanced interaction between various receptors appears to be needed.[13]

These activating and inhibitory receptors are controlled by certain genes; one of them is *KLRC3*. *KLRC3* is a member of NKG2 family of genes. *KLRC3* encodes for NKG2E, which is a significant activating receptor of NK cells.[14]

The aim of this study was to identify the possible role played by NK cells in T1DM pathogenesis through comparing *KLRC3* gene expression between patients with T1DM and healthy controls.

METHOD

This study was conducted at the Alexandria University Children's Hospital from April to October 2015. The study was conducted on 30 patients newly diagnosed with T1DM and 20 non-diabetic subjects serving as a control group (age range: 7–13 years). The included patients were further sub-divided into two groups of 15 patients each; group I included patients who first presented with classical symptoms and group II included patients that first presented with diabetic ketoacidosis (DKA). The diagnosis of T1DM was based on the American Diabetes Association (ADA)[2] definition; either a fasting plasma glucose (FPG) ≥ 126 mg/dL or a random plasma glucose ≥ 200 mg/dL in a patient with classic symptoms of hyperglycemia, or glycated heamoglobin (HbA_{1C}) levels ≥ 6.5% and/or a two-hour oral glucose tolerance test (OGTT) > 200 mg/dL. Written informed consent was obtained from all subjects' guardians enrolled, and the study was approved by the Ethics Committee of Alexandria University.

All participants in the current study were subjected to full history taking, physical examination, and laboratory investigations for fasting serum glucose level, HbA_{1c}, fasting C-peptide, and serum pancreatic amylase. Liver and kidney function and a complete blood count (CBC) were also done. Finally, *KLRC3* gene expression was done using the real-time reverse transcriptase polymerase chain reaction (RT-PCR). Fresh peripheral blood samples were withdrawn from the patients in all groups under strict aseptic conditions in vacutainer EDTA tubes. Samples were processed within few hours of collection. Purification of total cellular RNA from human whole blood was done using miRNeasy Mini Kit (Qiagen, USA).[15] RNA samples were stored at -80 °C until processed. cDNA was synthesized from total RNA using QuantiTect Reverse Transcription Kit.[16] *KLRC3* gene expression was analyzed by real-time quantitative PCR using real-time cycler Rotor gene Q® (Qiagen, USA) using QuantiTect® SYBR® Green PCR kit.[17]

The gene expression level was normalized with the values of the housekeeping control gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

The results of real-time RT-PCR were represented by the parameter Ct (threshold cycle), Ct is the amplification cycle at which the signal crosses a detection threshold. The difference in Ct values between *KLRC3* and GAPDH reactions was given by Delta Ct (Δ Ct).

Amplification plot curve was used to calculate Ct for both *KLRC3* and GAPDH. A melting curve was done to increase sensitivity and specificity of the test [Figure 1 and Figure 2].

Table 1: Demographic data of the two diabetic study groups and the control group.

	Group I (n = 15)		Group II (n = 15)		Control group (n = 20)		Test of significant	*p*-value
	n	%	n	%	n	%		
Gender								
Male	8	53.3	7	46.7	10	50.0	$\chi^2 = 0.133$	0.936
Female	7	46.7	8	53.3	10	50.0		
Age, years								
< 12	11	73.3	11	73.3	11	55.0	$\chi^2 = 4.022$	0.130[MC]
> 12	4	26.7	4	26.7	9	45.0		
Min–Max	7.0–13.0		7.0–13.0		7.0–13.0			
Mean ± SD	10.6±1.7		9.7±1.9		10.4±2.0		F = 4.408*	0.018*
Median	11.0		9.0		10.5			
***p*-value between groups**	$p_1 = 0.167^{KW}$		$p_2 = 0.973^{KW}$		$p_3 = 0.259^{KW}$			
Family history of diabetes								
Negative	8	53.3	6	40.0	20	100.0	18.749*	< 0.001*
Positive	7	46.7	9	60.0	0	0.0		
***p*-value between groups, ,**	$p_1 = 0.464$		$p_2 = 0.001^{*FE}$		$p_3 < 0.001^{*FE}$			
Admission to ICU								
Negative	14	93.3	0	0.0	20	100.0	47.909*	< 0.001*
Positive	1	6.7	15	100.0	0	0.0		
***p*-value between groups**	$p_1 < 0.001^*$		$p_2 = 0.429$		$p_3 < 0.001^*$			

**Statistically significant at p < 0.050; χ^2, p: χ^2 and p-values for chi-square test; [KW]p: KW and p-values for Kruskal-Wallis test; p_1: p-value for comparing between group I and group II; p_2: p-value for comparing between group I group and control; p_3: p-value for comparing between group II and control; Group I: outpatient clinic cases; Group II: pediatric intensive care unit (ICU) cases; [FE]: Fisher Exact test; [MC]: Mont Carlo test*

Statistical analysis was performed using unpaired chi-square, F-test (ANOVA), Mann-Whitney, and Kruskal-Wallis test. Results were considered significant if the *p*-value was less than 0.050. Statistical analysis was carried out using SPSS Statistics (IBM Corp. Released 2011. IBM SPSS Statistics for Windows, Version 20.0. Armonk, NY: IBM Corp).

RESULTS

Patients in the two diabetic groups included 15 males and 15 females, and the control group included 10 males and 10 females [Table 1]. Patients were aged between 7–13 years. The mean age of group I, II and the control group was 10.6±1.7, 9.7±1.9, and 10.4±2.0 years, respectively. The majority of patients were children (below the age of puberty) and only eight patients were >12 years old.

The three studied groups were matched for age, sex, and family history of diabetes. The most frequent symptoms were polyuria in both groups I and II (100.0% and 73.3%, respectively), followed by polydipsia, and polyphagia [Table 2]. Only five patients presented with fever and influenza-like symptoms before the onset of DKA. Results of routine blood tests were comparable in the three studied groups.

There was no significant difference between groups I and II regarding fasting blood sugar levels.

Table 2: Comparison between the two studied groups according to different clinical symptoms.

	Group I (n = 15)		Group II (n = 15)		χ^2	*p*-value
	n	%	n	%		
Polyphagia					15.000*	< 0.001*
Negative	5	33.3	15	100.0		
Positive	10	66.7	0	0.0		
Polyuria					4.615	0.100[FE]
Negative	0	0.0	4	26.7		
Positive	15	100.0	11	73.3		
Polydepsia					13.393*	< 0.001*
Negative	2	13.3	12	80.0		
Positive	13	86.7	3	20.0		

**Statistically significant at p < 0.050; [FE]: Fisher Exact test*

Table 3: Comparison of fasting blood sugar, HbA_{1C}, C-peptide, and serum pancreatic amylase levels between the study groups.

	Group I (n = 15)	Group II (n = 15)	Control (n = 20)	Test of sig.	*p*-value
FBS, mg/dL					
Min–Max	95.0–425.0	84.0–401.0	66.0–92.0	$^{KW}\chi 2 = 32.777^*$	< 0.001*
Mean ± SD	211.1 ± 87.5	230.5 ± 110.4	80.6 ± 7.7		
Median	190.0	219.0	81.0		
***p*-value between group**	**$p_1 = 0.663$**	**$p_2 < 0.001^*$**	**$p_3 < 0.001^*$**		
HbA_{1c}, %					
Min–Max	6.2–14.9	6.5–11.0	3.0–5.5	F = 62.642*	< 0.001*
Mean ± SD	10.2 ± 2.4	8.3 ± 1.4	4.4 ± 0.7		
***p*-value between groups**	**$p_1 = 0.003^*$**	**$p_2 < 0.001^*$**	**$p_3 < 0.001^*$**		
C-peptide, ng/mL					
Min–Max	0.01–0.93	0.01–0.13	1.2–4.4	$^{KW}\chi 2 = 38.481^*$	< 0.001*
Mean ± SD	0.2 ± 0.3	0.02 ± 0.03	2.9 ± 1.1		
Median	0.02	0.01	2.90		
***p*-value between groups**	**$p_1 = 0.046^*$**	**$p_2 < 0.001^*$**	**$p_3 < 0.001^*$**		
Serum pancreatic amylase, U/L					
Min–Max	32.0–67.0	34.0–64.0	26.0–59.0	F = 1.628	0.207
Mean ± SD	48.9 ± 10.2	48.5 ± 8.2	43.8 ± 9.5		

p_1: p-value for comparing between group I and group II; p_2: p-value for comparing between group I group and control; p_3: p-value for comparing between group II and control; Group I: outpatient clinic cases; Group II: pediatric intensive care unit cases; SD: standard deviation; HbA_{1c}: glycolated heamoglobin; $^{KW}\chi^2$: Kruskal-Wallis test; FBS: fasting blood sugar

Mean values of HbA_{1c} and C-peptide were higher in group I ($p = 0.003$ and $p = 0.046$, respectively). Levels of serum pancreatic amylase were not statistically different in the three groups [Table 3].

KLRC3 gene expression was compared between the two groups and against the control group [Figure 3 and Table 4].

The mean expression levels of *KLRC3* receptor for groups I, II, and control group were 0.32±0.33, 0.06±0.06, and 0.66±0.49, respectively. The level was significantly lowest in the group of patients who presented with DKA; group II ($p = 0.008$).

DISCUSSION

We found significant downregulation of the *KLRC3* gene in patients with diabetes compared to healthy controls. Moreover, *KLRC3* showed more downregulation in patients who presented with DKA.

NK cells could be involved in one or several steps of the immune-mediated attack that leads to T1DM.[8] Downregulation of the *KLRC3* gene may result in abnormal NKG2E receptor expression and, consequently, abnormal NK cells function. NKG2E forms heterodimeric complexes with CD94. These complexes interact with HLA-E, a major histocompatibility complex (MHC) class Ib protein, which is involved in pathogenesis of T1DM.[9]

In a previous study, *KLRC3* was found to be downregulated in patients with T1DM and fulminant type 1 diabetes compared to normal controls.[10] Fulminant type 1 diabetes is a clinical subtype of T1DM characterized by rapid onset of hyperglycemic symptoms, DKA at the time of

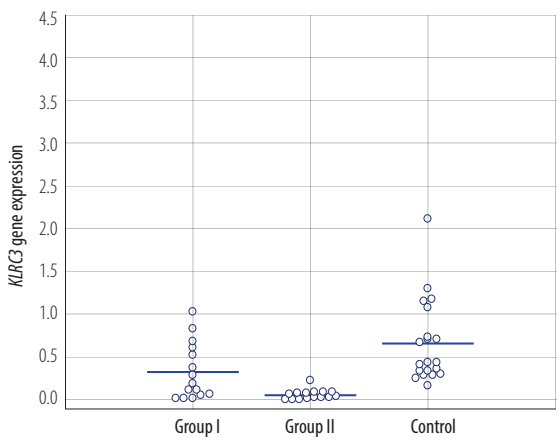

Figure 3: *KLRC3* gene expression in the three study groups.

Table 4: Comparison between the two studied groups and control according to the expression of *KLRC3*.

	Group I (n = 15)	Group II (n = 15)	Control` (n = 20)	$^{KW}\chi^2$	*p*-value
Threshold					
Min–Max	0.01–1.02	0.0–0.22	0.16–2.12	26.021*	< 0.001*
Mean ± SD	0.32 ± 0.33	0.06 ± 0.06	0.66 ± 0.49		
Median	0.20	0.04	0.43		
***p*-value between groups**	$p_1 = 0.008^*$	$p_2 = 0.012^*$	$p_3 < 0.001^*$		

*Significance between groups was done using Mann-Whitney test. *Statistically significant at p ≤ 0.050.*
$^{KW}\chi^2$: Kruskal-Wallis test; p_1: p-value for comparing between group I and group II; p_2: p-value for comparing between group I group and control; p_3: p-value for comparing between group II and control; Group I: outpatient clinic cases; Group II: pediatric intensive care unit cases; SD: standard deviation.

diagnosis, and a fasting serum C-peptide level < 0.3 ng/mL. However, when comparing T1DM to the fulminant type 1 diabetes, there was no significant difference in *KLRC3* gene expression. They also found no significant differences in the expression of the other *NKG2* family genes that code for NK cell receptors as *KLRC2* (NKG2C), *KLRC1* (NKG2A), and *KLRK1* (NKG2D) when comparing diabetic patients to normal controls suggesting a more important role of the *KLRC3* gene.

A more recent study found decreased numbers of NK cell subsets (CD56bright and CD56dim) and an impaired function of NK cells in patients suffering from T1DM.[18] There was a slightly decreased expression of NKG2D, an activating receptor belonging to the *NKG2* family. The decreased expression was found in all patients with T1DM relative to control subjects, independent of the duration of disease.

The incidence of T1DM peaks in puberty.[1,2] However, we did not find any positive correlation between *KLRC3* expression and older age approaching puberty.

In our study, the most prevalent symptom in the diabetes groups was polyuria. A previous report found that polydipsia was the most prevalent symptom in their cases;[19] however, patients with T1DM usually have both symptoms at presentation.

HbA_{1c} was lower in patients with DKA at presentation. Although their fasting blood sugar was higher, this low HbA_{1c} could be explained by their shorter duration of the disease compared to patients who presented with classical symptoms. However, we did not recognize any correlation between *KLRC3* expression and HbA_{1c}.

C-peptide was significantly lower in patients with DKA at presentation. C-peptide measurement assesses beta cell reserve and, hence, endogenous insulin secretion.[20] This lower level of C-peptide indicates the more acute onset of the disease. However, there was no correlation between *KLRC3* expression and C-peptide.

Serum pancreatic amylase was not affected in either diabetic group reflecting a normal exocrine pancreatic function. Previous studies[19,20] have reported higher levels of serum pancreatic amylase in patients presenting with DKA. This result can be explained by temporary exocrine dysfunction, which resolved after insulin treatment and/or lymphocytic infiltration of the exocrine pancreas.

Our study was limited by its single-center design and small patient number.

CONCLUSION

The *KLRC3* gene was downregulated in patients with T1DM. Greater downregulation was observed in patients with DKA compared to those who presented with classical symptoms. Expression of *KLRC3* in T1DM might play a role in the pathogenesis of T1DM and be a predictor of its severity.

Disclosure
The authors declared no conflicts of interest. No funding was received for this study.

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