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A novel association between *IL1-Ra* (receptor antagonist) gene polymorphism and T1DM in Al-Madina Al-Mounawra

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Abstract. – OBJECTIVE: The Interleukin-1 receptor antagonist *(IL1-Ra)* is initiated to terminate the acute pro-inflammatory event and prevent chronic inflammation from damaging healthy cells. We aim to draw the attention of *IL1-Ra* (VNTR) gene polymorphism and determine whether *IL1-Ra* confer susceptibility to type 1 diabetes mellitus (T1DM) and evaluate the genotype and allele distribution of *IL1-Ra* gene in a Saudi population.

PATIENTS AND METHODS: Case control study included (100) T1DM Saudi children, plus 102 healthy unrelated individuals as control group. They were evaluated for variable number of tandem repeat (VNTR) of *IL1-Ra* gene polymorphism. Polymerase chain reaction amplification of VNTR of 86bp in intron 2 of *IL1-Ra* was performed.

RESULTS: A1A1 and A1A2 genotypes with alleles A1 and A2 frequency were the most common both in cases and controls (healthy population); prevalence (28%, 56% & 57.8%, 39.2% respectively) and (58%, 38% and 77.5%, 22.5% respectively). In addition *IL1-Ra* gene polymorphism had higher risk significantly different between diabetic children and controls. (A1/A2) genotype had higher frequency statistically significant in DM patients than controls [56% *vs*. 39.2%, $p < 0.02$] and had twice time risk [OR = 1.97, 95% CI = 1.1-3.4, $p < 0.02$]. With further stratification, there was strong association between diabetic patients carriage *IL1-Ra* (A2) allele and controls [38% *vs*. 22.5%, $p = 0.001$] which had higher risk [OR = 2.11, 95% CI = 1.4-3.2, $p = 0.001$] for susceptibility of diabetes.

CONCLUSIONS: This study emphasizes a positive association between *IL1-Ra* (VNTR) polymorphism and DM among Saudi children. This may suggest that (A2) allele may play important role in disease susceptibility.

Key Words:
Diabetes, IL1-Ra, Gene polymorphism, VNTR, T1DM.

Introduction

Type 1 diabetes mellitus is an autoimmune disease occurring in the pancreatic islets. It accounts for 90% of diabetes in children and adolescents[1]. It is known that T1DM is a multi-factorial disease, with genetic and environmental factors that could explain the incidence rates that have been found in different ethnic groups and countries[2-3]. Genes involved in the metabolic pathway of insulin have been regarded as good candidates for both type 1 and type 2 diabetes pathogenesis[3-4].

IL1RN (VNTR) has being studied in the development of inflammatory disorders for several years[5-6]. On the other hand pro-inflammatory cytokines stimulate adaptive immunity and attenuate T-cell regulation and tolerance induction. They also profoundly impair β-cell function, proliferation, and viability, activities of similar importance in the context of type1 diabetes mellitus[7]. Moreover, *IL1-Ra* is initiated to terminate the acute pro-inflammatory event and prevent chronic inflammation from damaging healthy cells. The level of cytokine production might be influenced by occurring functional polymorphism in cytokine genes. Altered levels of *IL1-Ra* have been described in the pathogenesis of several diseases where inflammatory or auto-immune processes are involved[8-9].

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The IL-1 family has three well-studied members, two agonists, IL-1α and IL-1β, and the antagonist *IL1-Ra* . The *IL1-Ra* is an important immunologic regulator that competes with other IL-1 family members for the IL-1 receptor in target cells and acting as its negative regulator with anti-inflammatory effects[10]. *IL1-Ra* inhibits IL-1 induced inflammation action by blocking the binding of IL-1 to IL-1 Type-I Receptor (IL-1RI)[11]. It has been shown that pancreatic islet beta cells require unexpectedly high concentrations of *IL1-Ra* in order to block the IL-1 induced changes in functional activity[12].

IL1-Ra is expressing from IL1RN gene which has a length variation within intron caused by 86bp (VNTR)[13-14]. Genes encoding IL-1 are located on the 430kb region of chromosome 2q13. In intron 2 of the *IL1-Ra* gene, a polymorphism due to the presence of variable numbers of an 86-bp tandem repeat (VNTR) has been described. This polymorphism leads to the existence of five alleles, each corresponding to a different number of repeats[13].

Patients and Methods

This case control study was conducted on 100 Saudi children unrelated individuals (54 boys and 46 girls of mean age 10.33 ± 3.15 years with a minimum and maximum age of 2.3 and 17 years) with T1DM. Patients were registered according to the World Health Organization [WHO] multinational project for childhood diagnosis of diabetes[15]. Compared with control group consisted of 102 unrelated healthy adult volunteers (30 males and 72 females, ages above 35 years). They were recruited from Madina maternity and Children's Hospital; Al-Madina, a North-West province of Saudi Arabia. The local Ethics Committee of hospital approved the project. This work has been done in laboratories of Faculty of Applied Medical Science at Taibah University. The age of the onset of the disease and the first occurrence of symptoms were acquired from the patient's medical records. All patients received a standardized, well-accepted drug regimen. Exclusion criteria if they had a clinically significant nephropathy or other complication.

Analysis of IL1-Ra

DNA was extracted from peripheral blood which was collected in EDTA tube, and it was purified using the generation DNA purification capture column kits (Gentra-Systems, Minneapolis, MN, USA). Amplification of DNA by PCR technique was carried out to intron 2 of *IL1-Ra* which contained 86bp VNTR zone. Each PCR was carried out in 25 µl reaction volume, mixture containing 10 µl PCR Master Mix (2×) (Hotstart, ACE-BIOTECH, Otsu, Shiga, Japan, cat.# 11M001-A, 11M001-B, 11M001-C), 8 µl PCR distilled water, 2 µl of primer *IL1-Ra* forward (5'-CTCAGCAACACTCCTAT-3'), 2 µl of primer *IL1-Ra* reverse (5'-TCCTGGTCT-GCAGGTAA-3') (Bio-Basic Inc., Markham, Ontario, Canada) and 3 µl extracted DNA[16]. PCR conditions were as follows: initial-denaturation cycle of 95°C for 5 min followed by 35 cycles in the form of 94°C for 30 seconds (denaturation), 55°C for 30 seconds (annealing) and 72°C for 1 min (extension) with a final extension cycle of 5 min at 72°C[17]. PCR product was detected by using agarose gel (2%) electrophoresis and visualized under ultraviolet trans-illuminator. *IL1-Ra* gene polymorphism were designated Figure 1.

Statistics Analysis

Statistical analysis was done using the statistical package of social sciences (SPSS Inc., Chicago, IL, USA) software version (20) IBM Statistics. Comparisons of genotypes and allele frequencies between cases and control were performed using χ^2 test. Odds ratios with 95% confidence intervals were also assessed. p values < 0.05 were considered statistically significant. HWE (Hardy-Weinberg equilibrium) was calculated to evaluate the relationship between gene frequencies and genotype frequencies.

Results

This study represents 100 diabetic Saudi children (54 boys and 46 girls; means ages 10.33±3.15 years) the main demographic and clinical features of studied cases were summarized in (Table I), plus 102 healthy subjects unrelated individuals as control group (31.4% males and 68.6% females; ages above 35 years) were enrolled in the study.

According to the differences of 86-bp tandem repeat number, four types of alleles can be recognized through the study. *IL1-RN*A1* (4 repeats, 420-bp), *IL1RN*2* (2 repeats, 240-bp) are the most common, meanwhile *IL1-RN*A3* (5 repeats, 498-bp) and *IL1RN*4* (3 repeats, 326-bp)

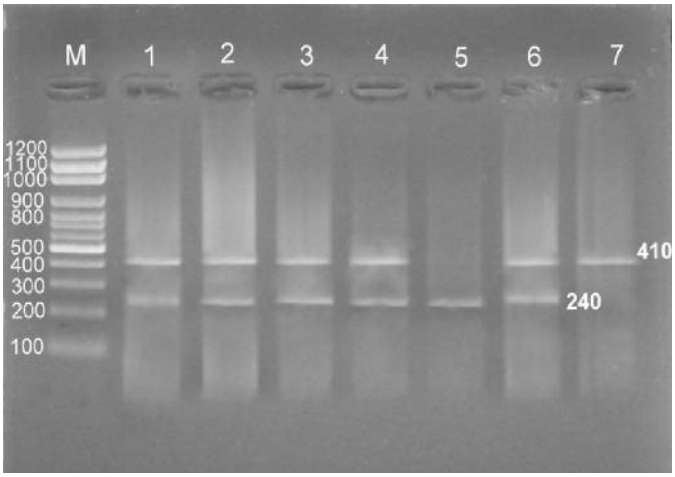

Figure 1. Agarose gel shows PCR product for *IL1-Ra* gene intron 2 polymorphism. Lane (M) shows DNA ladder (100-1200 bp). Lanes (1, 2, 3, 4, and 6) show A1/A2 heterozygote polymorphism of *IL1-Ra* where A1 at 410 bp and A2 at 240 bp. Lane (5) shows homozygote polymorphism of IL-1Ra A2/A2 genotype where A2 at 240 bp. Lane (7) shows homozygote polymorphism of *IL1-Ra* A1/A1 genotype where A1 at 410 bp.

were rare. The comparative distribution of genotype in diabetes versus control showed that overall genotype distribution for most common alleles either homozygous (A1A1) or heterozygous (A1A2) genotypes of *IL1-Ra* (VENTR) were highly significant, meanwhile there was a significant lower frequency of wild genotype (A1A1) among cases compared to control [28% *vs.* 57.8%, OR = 0.28, 95% CI = 0.2-0.5, $p < 0.001$]. On the other hand they showed a significant higher frequency of heterozygous carriage mutant allele (A1A2) among cases than controls [56% *vs.* 39.2%, OR = 1.97, 95% CI = 1.1-3.4, $p < 0.02$] respectively. Thus, both homozygous (*A2A2*) combined heterozygous (A1A2) (mutant) genotypes frequencies was significantly higher among cases compared to control [65% *vs.* 42.2%, OR = 2.55, 95% CI = 1.5-4.5, $p < 0.001$]. Regarding allelic frequencies, the mutant *A2* allele was significant higher in cases compared to control [38% *vs.* 22.5%, OR = 2.11, 95% CI = 1.4-3.2, $p = 0.001$]; the revers was noted with the wild type allele *A1* which was statistically significant lower among cases compared to controls [58% *vs.* 77.5%, OR = 0.4, 95% CI = 0.3-0.6, $p < 0.001$] (Table II).

Table I. Clinical data (means ± SD) of studied cases.

Subjects characteristics	Cases N=100 (100%)
Age (yrs.)	
Range	(2.3-17)
Mean ± SD	10.33 ± 3.15
BMI (kg/m^2)	18.12%
Z score of BMI	
Mean ± SD	-0.17 ± 1.59
Underweight (%)	12%
Normal weight (%)	63%
Overweight (%)	12%
Obese (%)	13%
Boys/Girls	54/46
HbA1c (%)	
Mean ± SD	10.33 ± 1.95
Duration of disease	
Range (yrs.)	(0.8-5)
Mean ± SD	2.97 ± 1.13

Discussion

Diabetes mellitus is one of major health problem in the kingdom of Saudi Arabia (KSA); its prevalence is the third in the world[18]. KSA prevalence was found 109/100.000, with marked variation between different regions[19]. Another study stated that Al-Madinah city has the highest incidence of childhood T1DM in the Middle East and North Africa region[20]. The wide variation in the clinical presentation of T1DM may reflect a possible heterogeneity in the pathogenesis of the disease[21]. There are clear differences in immune-genetic predisposition to T1DM between countries, and disease incidence seems to vary along with these differences in predisposition[22].

Identification of appropriate markers of T1DM for recognizing genetic influence upon initiation and progression of the disease might assist the

clinicians in adopting a more precise approach for the identification of "high-risk" T1DM patients and in the development of personal medicine strategies for targeting inflammatory components; thus meeting a crucial medical need, as well as enabling planning therapeutic interventions. Genetic polymorphisms studied so far in T1DM with TNF-α and IL-6 has also revealed no or only marginal association.

It is not surprising that most of the known cytokines have partaken in the pathogenesis of T1D**.** Levels of *IL-1ra* are strongly correlated to insulin resistance[23]. On the other hand, in normal monocytes in culture the allele *IL1RN*2* was associated with increased production of *IL1-Ra*[24]. The level of cytokine production might be influenced by occurring functional polymorphism in cytokine genes. Altered levels of *IL1-Ra* have been described in the pathogenesis of several diseases where inflammatory or auto-immune processes are involved[25]. Persons homozygous for allele 2 of the *IL-1RA* gene (*IL1RN*2*) have a more prolonged and more severe pro-inflammatory immune response than persons with other *IL-1RA* genotypes[6].

This study showed potentially meaningful diagnostic and therapeutic implications in the field of diabetes; *IL1-Ra* gene polymorphism could have an important genetic contribution to T1DM. The genotype (A1/A2) and (A2) allele was carried more frequently and highly significant in our diabetic patients, in addition they twice times more likely to have risk than non-carriage (Table II).

However, to the best of our knowledge, the latter finding have not been previously reported to evaluate whether these cytokines polymorphism *IL1-Ra* (VNTR) have functional influence on the susceptibility to T1DM in the Saudi children population or other population, so require more elaborate investigations. Clinical testing of cytokine antagonists in T1DM is in its infancy, with few reported studies and even fewer ongoing trials.

One investigation indicated that *IL1RN*2* appeared to be a marker of disease severity (diabetic nephropathy) rather than susceptibility for diabetes[26]. Similar study in T2DM reported a significant association between coronary artery disease and carrier of allelic variants of the *IL1- Ra* gene, leading to reduced serum *IL1-Ra* levels[27]. Another study serves as susceptibility indicators for T2DM[28].

IL1RN*2 gene polymorphism is associated with increased plasma levels of *IL1-Ra*, led to hypothesis that *IL1-Ra* might be linked to acquired resistance to insulin and leptin, the hallmarks of obesity and T2DM[23,29]. In agreement study[30] reported that the anti-inflammatory *IL1-Ra* is the most highly elevated known cytokine of serum in diabetic patients T2DM. In another work there was 30% lower level of plasma *IL1-Ra* in subjects with T1DM carrying (A1/A1) genotype compared with level in those carrying (A1/A2) genotype ($p = 0.025$)[31].

In contrast to our results, other authors have described an association of *IL1RN*2* with T1DM in a Danish cohort[32], but other study provided no firm support for a major diabetes susceptibility marker in the IL-1 gene region[33]. The differences in findings between studies may reflect differences in the level of diabetic complications and in the groups analyzed.

There appears to be a balance between IL-1 and *IL1-Ra* protein, except in the case of autoimmune diseases. There are controversial results for the function of *IL1RN2* allele in *IL1-Ra* expression. *IL1RN2* allele is associated with increased *IL1-Ra* levels *in vitro*[24,34], decreased levels in ulcerative colitis[35] but at similar levels with inflammatory bowel disease[36]. Above researches imply that *IL1RN2* polymorphism function in affecting *IL1-Ra* protein expression depends on cell type and ethnic origin.

Meanwhile there are still debates regarding the association between *IL1-Ra* gene polymorphism and other disease. Similar to the association of *IL1-Ra* gene polymorphism with T1DM identified in our study, some studies[37,38] were consistent and confirming the association in other diseases. Important cancer investigations stated that cases carriage *A2* allele of *IL1-RA* polymorphism play prominent role in different solid tumors like cervical cancer[39], Bladder cancer[40] and lung cancer[41]. Similar finding was reported regarding autoimmune or chronic inflammatory disorders as inflammatory diseases[42,43], lupus erythematosus[44] and multiple sclerosis[45].

In contrast study[46] represented that the susceptibility to IgA-N seems to be associated with the presence of *IL1-Ra* 1/1 genotypes among nephropathy. Controversial study[47] reveled that *IL1RN* allele 2 has a protective effect on restenosis after Percutaneous Transluminal Coronary Angioplasty for individuals with single vessel disease. Other work[48] concluded that *IL1RN* polymorphism may not associate with the risk of pulmonary tuberculosis.

Regarding to influenced of osteoporosis, need carriage of at least one copy of the *IL1-RN*2* al-

Table II. Genotypes and allele frequency of *IL1-Ra* (VNTR) polymorphism among Saudi diabetic children compared to controls.

Genotype	Cases No. (%)	Controls No. (%)	*p*	OR (95%CI)	χ^2
***IL1-Ra* genotypes**	100 (100)	102 (100)			
IL1-Ra*					
A1A1	28 (28)	59 (57.8)	p < 0.001	0.28 (0.2-0.5)	17.14
A1A2	56 (56)	40 (39.2)	0.02	1.97 (1.1-3.4)	1.97
A2A2	9 (9)	3 (3)	0.12	3.26 (0.9-11.7)	2.32
A2A3	2 (2)	0 (0)	0.46		
A1A4	4 (4)	0 (0)	0.12		
A3A4	1 (1)	0 (0)	0.99		
Combined homozygous and heterozygous alleles					
A2A2+A1A2	65 (65)	43 (42.2)	0.001	2.55 (1.5-4.5)	9.69
Allele frequency	**N = 200**	**N = 204**			
A1	116 (58)	158 (77.5)	*p* < 0 .001	0.4 (0.3-0.6)	16.63
A2	76 (38)	46 (22.5)	0.001	2.11 (1.4-3.2)	10.72
A3	3 (1.5)	0 (0)	0.23		
A4	5 (2.5)	0 (0)	0.06		

IL1-Ra (Receptor antagonist); (VNTR) variable number of tandem repeats. Alleles A1, A2, A3, A4 are expressed in percentages, *p* = probability test to study the statistical difference between cases and control where $p < 0.05$ significant, *p*: probability, OR: Odds Ratio, CI: Confidence Intervals, χ^2: Chi square; $p < 0.001$ extremely significant, $p < 0.05$ significant.

lele[49]. Conversely, *IL1RN*1* allele has an increased risk of osteoporotic fractures[50]. Two genotypes (1/2 and 2/2) showed a trend toward susceptibility to knee osteoarthritis[51].

According to pregnancy there is a study proved that *IL1RN*2* homozygous association with idiopathic recurrent miscarriage[52], beside severe pre-eclampsia than who possessed other *IL1-RN* genotypes[53].

The frequency of the individual alleles also varies among different ethnic or geographic populations, but *IL1Ra* A1 allele, is always more common than *IL1Ra* A2[13]. One of our objectives of is to provide population genetic characterizations of VNTR polymorphism in Saudi Arabia. A comparison of the distribution of *IL1-Ra* polymorphism in our population revealed certain key variations. It was noted that the distribution of homozygous A1A1 and heterozygous A1A2 genotypes related to *IL1-Ra* gene polymorphism are the most frequent confirmed by the highest percentile of *A1* and *A2* alleles among controls group (Table II). In agreement comparison with other populations like Mediterranean [Egyptian[54], Turkish[55]], European [German[56], Berlin[57]], Russian [Scottish[58], Caucasian[59]], African [African American[59]], Asian [North Indian[60], Taiwan Chinese[61]].

Conclusions

IL-1RA (VNTR) has highly polymorphic content that may help in genetic association studies. Especially allele (A2) polymorphism may be used as useful markers for predicting susceptibility to type 1 diabetes mellitus; it may help to prevent complication of the disease. Conversely, diabetes is not associated with (A1) allele. These data suggest that cytokine genes may act as enhancers or attenuators of diabetes susceptibility. If so, therapy may play a potential role targeting cytokines to prevent or limit DM of children. Furthermore, the impact of other cytokine polymorphisms on the development to diabetes merits further study. Also we need further studies with large number of population to confirm importance of *IL-1RA* (VNTR).

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Conflict of Interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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