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Analysis of the ZNT8, GAD65, HLA-DQA1, HLA-DQB1, and C-peptides in Indonesian children with type 1 diabetes mellitus

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Abstract--Autoimmune reaction in Type 1 Diabetes Mellitus (T1DM) is caused by genetic and environmental factors. The genes HLA-DQA1 and HLA-DQB1 are strongly associated with T1DM. Other established markers of T1DM were Zinc Transporter 8 Antibody (ZnT8), Glutamic Acid Decarboxylase Antibody-65 (GAD65), and C-peptide. This study aims to analyze ZnT8, GAD65, HLA-DQA1, HLA-DQB1, and C-peptide in Indonesian children with T1DM. A cross-sectional study of T1DM children was conducted in Dr. Soetomo Hospital, Indonesia. ZnT8,

GAD65, HLA-DQA1, HLA-DQB1, and C-peptide were examined with an ELISA kit. Mann-Whitney test was performed with a significant value of $p < 0.05$. A total of 62 participants consisting of 31 children for each group were enrolled. The prevalence of ZnT8, GAD65, and C-peptide in T1DM patients was 77.4%, 90.3%, and 87.1%. There are 74% of T1DM children had two positive antibodies and 55% had two positive HLA peptides. There was no difference in HLA-DQA1 and HLA-DQB1 peptides between T1DM and control groups ($p = 0.12$; $p = 0.19$). Meanwhile, the comparison between ZnT8, GAD65, and C-Peptide values between T1DM and control groups shows a significant difference ($p < 0.05$). HLA-DQA1 peptide value was higher in T1DM children and HLA-DQB1 peptide value was higher in non-T1DM children.

Keywords---T1DM, peptide, children, antibody, diabetes

17

Introduction

The incidence of Type 1 Diabetes Mellitus (T1DM) is increasing worldwide. Every year, it is estimated that 96,000 children under the age of 15 years are diagnosed with T1DM (IDF, 2017). Furthermore, approximately 600,000 children below 15 are currently living with T1DM. This number almost doubles to 1.1 million for persons under 20 years (Patterson et al., 2019). According to the Indonesian Paediatrician Association, there were 1,220 Indonesian children diagnosed with T1DM in 2018 (Pulungan et al., 2019). Insulin deficiency due to the damage of beta cells pancreas underlies the development of T1DM. The causes of T1DM are complex and multifactorial, with a combination of genetic and environmental factors resulting in an autoimmune reaction (Robertson and Rich, 2018; Lete et al., 2016; Noble, 2015).

30

The major genetic determinants of T1DM are polymorphisms of Human Leukocyte Antigen (HLA) Class II, consisting of alpha and beta chains. The encoding process from different alleles generates high polymorphism characteristics in this locus. Furthermore, the genes encoding the HLA-DQ and HLA-DR proteins are the main risk factors associated with several autoimmune diseases (Carlsson et al., 2012; Stayoussef et al., 2009; Farina et al., 2019). Several studies demonstrated that specific alleles at the DRB1, DQA1, and DQB1 loci are strongly associated with T1DM (Noble, 2015 and Farina et al., 2019). According to the central dogma theory, the passage of genetic information from genes into RNA, which then then pass to amino acid (Costa et al., 2021). The HLA-DQA1 and HLA-DQB1 genes synthesize HLA-DQA1 and HLA-DQB1 peptides, respectively. The HLA genes encoding HLA class I and II proteins represent peptides that form protein degradation (Sperling, 2020; Dudek and Purcell, 2016; Van et al., 2016). Other established markers of the T1DM-associated autoimmune process are Islet Cell Autoantibody (ICA), Glutamic Acid Decarboxylase Antibody (GAD65), Zinc-Transporter 8 antibody (ZnT8), and Insulin Autoantibody (IAA) (Robertson and Rich, 2018; Koo et al., 2014; Miersch et al., 2013). Genetic polymorphism and antibody levels in individuals with T1DM varied by ethnicity (Robertson and Rich, 2018 and Noble, 2015). The autoimmune process was characterized by a positive

autoimmune marker followed by beta cell pancreas destruction. Examination of C-peptide defines residual pancreatic cells (Sperling, 2020).

Genetic and autoantibody variation at these loci may interfere with the peptide pool to initiate an immune response that plays a vital role in the pathogenesis of T1DM. However, studies about this concern of T1DM children in Indonesia remain limited. Therefore, this study aimed to analyze ZnT8, GAD65 antibody, HLA-DQA1, HLA-DQB1, and C-peptide of children with T1DM in Indonesia. Recognizing this relationship should be essential in determining pediatric susceptibility to T1DM.

Method

This cross-sectional study was conducted in the pediatric outpatient clinic of Dr. Soetomo General Hospital Surabaya, Indonesia, and has been approved by the Ethical Board Committee (approval no. 1889/KEPK/III/2020). Informed consent was obtained from the participants and their guardians. This study was performed following The Declaration of Helsinki. A total of 31 patients, aged 4-18 years old, diagnosed with T1DM according to American Diabetes (ADA, 2011), and routinely controlled were included. Comparatively, as controls, this study included 31 healthy children that also visited the outpatient clinic and had no history of T1DM, autoimmune disease, allergy, malignancy, or ongoing infection. The sample size was based on the calculation formula in a cross-sectional study. Participants' clinical characteristics such as sex, age, ethnicity, age of onset, duration of T1DM, weight, and height were obtained.

Sample Collection and Laboratory Assay

Peripheral blood mononuclear cells (PBMC) samples were drawn from each participant for plasma protein examination, including ZnT8, GAD65, HLA-DQA1, HLA-DQB1, and C-peptide. This examination was performed with an Enzyme-linked Immunosorbent Assay (ELISA) kit. The GAD65 antibody was assessed with Human GAD 1 ELISA kit from Merck, while the ZnT8 antibody was examined with Human ZnT8 ELISA Kit from Bioassay Technology Laboratory. The C-peptide was analyzed using Maglumi C-peptide (CLIA) from Bioassay Technology Laboratory. Analysis of the HLA-DQA1 peptide was conducted utilizing the HLA Class 2 Histocompatibility Antigen-DQ Alpha 1 Chain ELISA Kit from My BioSource (Cat.NO MBS1603573). Meanwhile, the HLA-DQB1 peptide was examined with HLA Class 2 Histocompatibility Antigen-DQ Beta 1 Chain ELISA Kit from My BioSource (Cat.NO MBS1603563). The interpretation of these examinations was a numeric number with units of ng/ml.

Statistical Analysis

The data were analyzed with Statistical Package for Social Sciences ver. 17.0 (SPSS Inc., Chicago, USA). Description analysis was performed to evaluate the distribution of the participants' characteristics. Furthermore, nominal variables are expressed by numbers (percentages), while values were described using mean \pm standard deviation or median (minimum-maximum) based on their Kolmogorov-

Smirnov normality test. The differences in nonparametric data were determined using Mann-Whitney Test, with $p < 0.05$ considered statistically significant.

Results

There were 31 subjects in the T1DM group, which included 19 males and 12 females, aged 15.04 ± 3.88 years. Furthermore, the control group consists of 31 healthy children, including 16 males and 15 females, aged 10.57 ± 2.39 years. The mean duration of illness in the T1DM group was 7.06 ± 4.18 years. Table 1 summarizes the baseline characteristics of the subjects. There was no significant difference in HLA-DQA1 and HLA-DQB1 peptide levels between T1DM and control groups ($p = 0.12$; $p = 0.19$). Meanwhile, the values of ZnT8, GAD65, and C-Peptide between T1DM and control groups showed a significant difference ($p < 0.05$). The results of the peptide examination are shown in Table 2. Furthermore, 74% of T1DM children had two autoantibodies, and 55% had two HLA peptides. Table 3 shows no correlation between the number of antibodies and clinical characteristics in patients with T1DM.

Table 1. Participants' clinical characteristic

Characteristic	T1DM group (n = 31)	Control group (n = 31)
Sex		
Male	19 (61.3)	16 (51.6)
Female	12 (38.7)	15 (48.4)
Ethnicity		
Javanese	27 (87.1)	31 (100)
Maduranese	1 (3.2)	0 (0)
Chininese	1 (3.2)	0 (0)
Malay	2 (6.4)	0 (0)
Age (years)	15.04 ± 3.88	10.57 ± 2.39
Age of onset (years)	9 (1-16)	-
Duration of T1DM (years)	7.06 ± 4.18	-
Height (m)	1.45 ± 0.18	1.29 ± 0.15
Weight (kg)	45 (9-61)	23 (13-53)
BMI (kg/m^2)	20 (10.94-30.18)	17.7 (13.42-22.91)

T1DM, Type 1 Diabetes Mellitus; BMI, Body Mass Index

Table 2. Tested autoantibodies and HLA protein in T1DM and control groups

	T1DM group	Control group	p-value
ZnT8-ab (%)	24 (77.4)	2 (6.5)	0.00*
ZnT8-ab titre (ng/ml)	0.35 (0.18-5.48)	0.27 (0.12-3.68)	0.00*
GAD65-ab (%)	28 (90.3)	2 (6.5)	0.00*
GAD65-ab titre (ng/ml)	10.18 (3.83-24.88)	4.11 (3.95-5.00)	0.00*
C-peptide (%)	27 (87.1)	11 (35.5)	0.00*
C-peptide titre (ng/ml)	0.68 (0.61-2.42)	1.00 (0.70-4.92)	0.00*

HLA-DQA1 peptide (%)	30 (96.8)	29 (93.5)	0.50
HLA-DQA1 peptide titre (ng/ml)	2.12 (0.84-6.60)	1.89 (0.75-3.65)	0.12
HLA-DQB1 peptide (%)	17 (54.8)	19 (61.3)	0.80
HLA-DQB1 peptide titre (ng/ml)	21.72 (1.05-9.27)	2.12 (1.02-8.89)	0.19

*Statistically significant; T1DM, Type 1 Diabetes Mellitus; ZnT8, Zinc Transporter 8; GAD65, Glutamic Acid Decarboxylase-65; HLA-DQA1, Human Leukocyte Antigen-DQ Alpha chain 1; HLA-DQB1, Human Leukocyte Antigen-DQ Beta chain 1

Table 3. Associations of the number of autoantibodies with clinical characteristic T1DM patients

	Autoantibody			P-Value
	0 AAb	1 AAb	2 AAb	
Age (years)	13.79 ± 0.33	15.07 ± 4.93	15.15 ± 3.85	0.89 ^a
Age of onset (years)	3.00 ± 1.42	7.50 ± 3.51	8.74 ± 3.62	0.11
Duration of T1DM (years)	11.00 ± 1.41	7.50 ± 6.35	6.61 ± 3.60	0.36 ^a
Height (meter)	1.45 ± 0.12	1.48 ± 0.22	1.45 ± 0.19	0.94 ^a
Weight (kg)	48.50 ± 16.26	42.47 ± 15.34	42.15 ± 14.56	0.88
BMI (kg/m ²)	22.82 ± 3.98	19.05 ± 5.96	19.15 ± 4.14	0.39

a, One-way Anova; k, Kruskal-Wallis; AAb, auto-antibody; T1DM, Type 1 Diabetes Mellitus; BMI, Body Mass Index

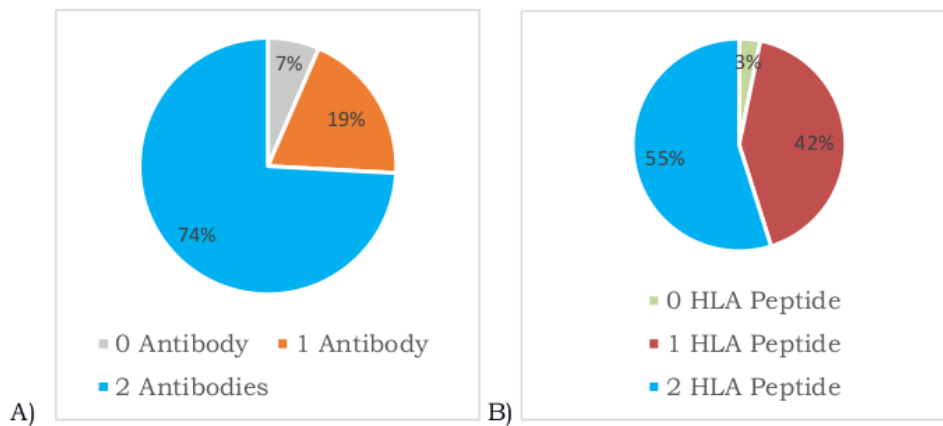


Figure 1. Summary of peptide examination on T1DM subjects. (A) Number of T1DM subjects with 0,1, and 2 autoantibodies (ZnT8 and GAD65). (B) Number of T1DM subjects with 0,1, and 2 HLA peptide (HLA-DQA1 and HLA-DQB1).

Discussion

This study showed that ZnT8 and GAD65 antibodies were more frequent in the T1DM group. Several studies also reported that ZnT8 and GAD65 were the most prevalent autoantibody in T1DM children (Robertson and Rich, 2018; Koo et al., 2014; Miersch et al., 2013). Another study also showed that GAD65, ZnT8, IA-2A, and IAA antibodies are reliable biomarkers for diagnosing T1DM in children and adults (Jahromi and Al Ozairi, 2019).

The Glutamic Acid Decarboxylase antibodies were detected in 90.3% of children with T1DM. This GAD65 prevalence was higher than the other studies in Saudi Arabia (84.4%) (Al-Alwan et al., 2012), Sudan (77.5%) (Mahdi et al., 2019), Qatar (59.7%) (Haris et al., 2021), and India (53%) (Vipin et al., 2021). Zinc Transporter-8 antibody prevalence in T1DM children in this study was 77.4%. This result was similar to another study in Indonesia, where 73.3% of T1DM patients were positive for ZnT8 antibody compared to the control group, which was 27.8% (Rochmah et al., 2020). The result of ZnT8 positivity in another country was reported to be 72% in Czechs (Petruzalkova et al., 2014), 65% in Argentinians (Faccinetti et al., 2016), 58.6% in Turkish (Elmaogullari et al., 2018), 29% in India (Vipin et al., 2021), 24.1% in Chinese (Yang et al., 2010), and 16.3% in Sudan (Mahdi et al., 2019).

The other results in this study showed no difference in HLA-DQA1 and HLA-DQB1 peptide values between the T1DM and control group. HLA-DQA1 peptide median was higher in T1DM children, while the HLA-DQB1 peptide median was higher in non-T1DM children. Currently, few studies describe HLA-DQA1 peptide levels on T1DM children. Most studies focused on the expression of HLA genes rather than their peptides. However, the central dogma theory could illustrate why children with T1DM have higher HLA-DQA1 peptide levels. Central dogma theory is the basis of cellular life, which fundamentally discusses the process of cell division through the information coded by DNA, transcribed into RNA, and then translated into proteins (Costa et al., 2021). Furthermore, the central dogma theory discusses the transfer of sequences during DNA replication, transcription, and translation into chains of amino acids to form proteins. According to this theory, higher levels of HLA-DQA1 peptide may be associated with increased levels of the HLA-DQA1 gene in children with T1DM.

HLA encoded by the significant MHC genes plays a crucial role in the antigen presentation to T lymphocytes. In conjunction with the inadequate function of regulatory T lymphocytes (TREG), some HLA genes cause an autoimmune disorder. In Caucasian populations, HLA-DR3-DQ2 and HLA-DR4-DQ8 haplotypes were related to T1DM (Chiarelli et al., 2019). Furthermore, polymorphisms of DQA1, DQB1, and DRB1 alleles were used to determine genetic susceptibility (Onengut-Gumuscu et al., 2015). Some studies showed that HLA class II dimer $\alpha\beta$ encoded by HLA-DQA1*0102/DQB1*0602(DQ0602) was a protective allele in T1DM (Ettinger et al., 2000). The HLA-DQB1 and HLA-DQA1 peptides were bonded to form a functional protein complex called antigen-binding DQ- $\alpha\beta$ heterodimer, presenting peptide non-self into the immune system (Yahya and Salisu, 2020). The most frequent HLA in Indonesian children with T1DM were HLA-DQA1 0101/0102 and HLA-DQB1 0301 (Soetjipto et al., 2022). Additionally,

HLA polymorphism was an adaptive mechanism with peptide antigens. Most nucleotide polymorphisms occur in exon 2 of the HLA class II (Mosaad, 2015). Post-translational modification of antigens affects the activation of immunogenic epitopes during antigen processing (Scally et al., 2013), thereby modifying the spectrum of presented peptides. Alterations of peptide antigens can influence their binding to specific HLA alleles (Purcell et al., 2019). Allele-encoding aspartic acid (Asp) at β 57 of HLA-DQB1 is associated with the resistance of T1DM. Whereas alleles encoding a neutral residue, such as Alanine (Ala) or Serine (Ser) at β 57 showed susceptibility of T1DM. The molecules Alanine and Serine are the risk factors for T1DM, specifically at the peptide-binding pocket nine (P9) of the DQB1-binding pocket that is involved in antigen presentation and T cell receptor interaction. The carboxylate group forms a salt bridge with Arginine (Arg) at α 57 of HLA-DQA1, stabilizing the heterodimer between the DQA1 and DQB1 chains. Aspartate in this position affects the molecule's stability and antigen presentation, making the molecules easier to bind with autoreactive antigen. Individuals with T-cells recognize peptides that present HLA. This phenomenon is called HLA restriction (Mosaad, 2015). The understanding of T-cell reactivity and the role of post-translational modifications lead to a new diagnostic and treatment of T1DM (Purcell et al., 2019).

In this study, 93% of T1DM children have one or more antibodies. This result is in accordance with a study in Sudan, which showed that 91,2% of children with T1DM have at least one autoantibody (Mahdi et al., 2019). A similar result was reported in a different countries, including Lithuania (92.5%) (Verkauskiene et al., 2016), India (72%) (Vipin et al., 2021), and United Arab Emirates (88%) (Al-Hassanni et al., 2014). Furthermore, this emphasizes the need for multiple antibody examinations before ruling out immune-mediated T1DM (Mahdi et al., 2019).

The mean age of onset in the autoantibody-positive group was higher than in the autoantibody-negative group. However, this result was not statistically significant ($p = 0.89$). Another study showed no difference in the mean age of T1DM diagnosis between the seropositive and seronegative groups (Mahdi et al., 2019). This is contrary to a study which stated that T1DM children with multiple antibodies had an earlier age of diagnosis compared to the single antibody group. Genetic susceptibility to HLA polymorphisms is associated with earlier age of diagnosis (Mrena et al., 2006).

There was no difference in the duration of T1DM between the negative and positive antibodies. This result was in accordance with the findings of a previous study (Mahdi et al., 2019 and Balasubramanian et al., 2003). The mean BMI was statistically insignificant between the three groups, with the negative autoantibody being higher (22.82 ± 3.98) than the one or two positive antibodies (19.05 ± 5.98 ; 19.15 ± 4.14). Furthermore, this was similar to a study in another country (Mahdi et al., 2019 and Balasubramanian et al., 2003). The limitation of this research is a single-centre study, which can lead to centre bias. Despite the limitation, the strength of this study was examined several peptides for T1DM where the study was still limited. The examination of peptides is less expensive and takes less time than genetic examination. Therefore, it is more applicable to use peptides examination for T1DM in children.

Conclusion

The most common antibody in the T1DM in this study was GAD65 antibody. HLA-DQA1 peptide value was higher in T1DM children and HLA-DQB1 peptide value was higher in non-T1DM children. Further research is required to identify another peptide of T1DM patients in Indonesia.

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Authors' contribution

NR and MF conceptualized, designed the study, analysed data, interpreted results, wrote the initial draft of the manuscript. YH and RKP analysed data, interpreted results, assisted in drafting the manuscript. SS and AE critically reviewed the manuscript, guided manuscript writing. TMN collected data, prepared the figure and tables, analysed data. All author agreed and give final approval to the submitted manuscript.

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Competing interest

No conflicts of interest related to this work

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PAGE 2

PAGE 3

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