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CTLA-4 Polymorphism in Children with Down Syndrome and Autoimmune Thyroid Diseases

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Abstract

This study explored the association between the CTLA-4 polymorphism and thyroid function parameters in children with Down syndrome (DS). A cross-sectional study involving children aged 1 month to 5 years diagnosed with DS was conducted from February to November 2020 at Dr. Soetomo General Hospital, Surabaya, Indonesia. The subjects were recruited consecutively, and if they presented critical or severe illness, they were excluded. A medical history and physical examination was performed to obtain the sociodemographic and clinical characteristics of subjects. A blood sample was obtained to test thyroid function. This test consisted of anti-TPO, anti-TG, TSH, T3, and fT4. The CTLA-4 CT60 polymorphism was analyzed from peripheral blood mononuclear cells using polymerase chain reaction–restriction fragment length polymorphism. The majority of subjects were 1 to 5 years of age (81%); 83.3% suffered from central hypothyroidism, and 16.7% primary hypothyroidism. Almost two-thirds of subjects were positive for anti-TPO (65%), and more than four in five were positive for anti-TG (86%). Most had low TSH levels (88.1%). The CTLA-4 CT60 polymorphism significantly correlated with T3 ($p = 0.049$; $r = 0.480$ in CT-60; $p = 0.046$; $r = 0.29$ in CT-1822). A significant association was found between the CTLA-4 A/G and G/G genotypes with a higher proportion of low TSH levels among DS patients with hypothyroidism. This study showed the potential influence of CLTA-4 on thyroid function in children with DS.

Keywords: Cytotoxic T-lymphocyte-associated Protein 4, Hypothyroidism, Down Syndrome, Thyroid-Stimulating Hormone.

1. Introduction

Individuals with Down Syndrome (DS) have a higher risk for medical problems, including endocrine abnormalities. Thyroid dysfunction is the most prevalent. The spectrum of thyroid dysfunction in patients with DS ranges from congenital hypothyroidism, subclinical hypothyroidism, acquired hypothyroidism (autoimmune or non-autoimmune), to hyperthyroidism (Amr.2018).The prevalence of thyroid hormone abnormalities in DS ranges from 8 to 49%. A retrospective study reported that

50% of DS patients have a thyroid disorder by adulthood, with 20% of hypothyroidism diagnosed by 6 months of age and 50% testing positive for anti-thyroid antibodies (Pierce et al., 2017).

Cytotoxic T-Lymphocyte-Associated Protein 4 (CTLA-4) is located on human chromosome 2q33 and is one of the candidate gene markers for autoimmune diseases. It encodes a cell surface molecule that is expressed on the surface of activated T-lymphocytes and plays a role in the downregulation of the immune response (Patel et al.,2016). CTLA4 functions as a competitive CD28 inhibitor acting as a negative regulator of T cell activation. Single nucleotide proteins (SNPs) in CTLA-4 have been associated with many autoimmune diseases, including autoimmune thyroid disease (AITD**)** (Lee et al.,2015**)**. CTLA-4 has also been linked to autoimmune polyglandular syndrome (APS), which is defined by the presence of at least two autoimmune-induced endocrine diseases (Houcken et al.,2018).

2. Significance of The Study

Individuals with DS have a higher predisposition to autoimmunity, including autoimmune thyroid disorders. Several gene factors, such as altered thymic expression of the autoimmune regulator (AIRE) gene and the contribution of class II major histocompatibility complex (MHC) genes, have been described to explain this finding. A strong association between the MHC class II DQA 0301 allele and hypothyroid autoimmune thyroiditis was found in DS patients (Giménez-Barcons et al.,2014). In contrast, the association between CTLA-4 polymorphism and thyroid function among DS patients has not been studied.

3. Review of Related Studies

To the best of our knowledge this is the first study that explores the association between the CTLA-4 polymorphism and thyroid function in a DS population. But there are several studies about CTLA 4 and autoimmune thyroid diseases (AITD).

Patel et al.,2016 conducted study on Hospital Vadodara, Gujarat, India about Association of Cytotoxic T-Lymphocyte Antigen 4 (CTLA4) and thyroglobulin (TG) genetic variants with autoimmune hypothyroidism. In their study reported that CTLA 49 A/G, CT49 G/G, CT 60 A/G, CT 60 G/G susceptible to autoimmune hypothyroidism. Pastuszak-Lewandoska et al.,2012 conducted study on 28 Hashimoto's thyroiditis (HT) patients in Military Hospital of Medical University of Lodz, Poland. In their study described a significantly higher frequency of the A allele of CTLA-4 CT60 in AITD patients compared with controls. Ting et al.,2016 found a significant association between the G/G genotype of CT60 and increased risk of pediatric HT.

4. Objective of The Studies

- To analyze the association between CTLA-4 polymorphism and autoimmune thyroid disease in DS.

5. Hypotheses of The Study

- There is association between CTLA-4 polymorphism and autoimmune thyroid disease in DS.

6. Population and Sample

This cross-sectional study involving children with DS attending Dr. Soetomo General Hospital in Surabaya, Indonesia, was conducted between February and November 2020. Children were recruited consecutively. Inclusion criteria were children aged 1 month to 5 years diagnosed with DS (confirmed by karyotyping). Children were excluded if they presented critical/severe illness or were receiving levothyroxine therapy. This study was previously approved by the Ethics Committee of Dr. Soetomo General Hospital, Ref. No. 1960/KEKP/IV/2020).

History and physical examination was performed on eligible subjects to obtain sociodemographic and clinical data. A blood sample was obtained to test karyotyping, autoimmune marker of thyroid disease and thyroid function. The evaluation consisted of anti-thyroid peroxidase antibody (anti-TPO), antithyroglobulin antibody (anti-TG), thyroid stimulating hormone (TSH), T3, and free T4 (fT4). Anti-TPO and anti-TG were measured by using an ELISA kit from ©RSRltDiagnostika, while T3 and fT4 were measured with a chemiluminescence immunoassay (CLIA) using reagents from ©MyBioAssay. Anti-TPO values were interpreted as negative (<35 U/mL) or positive (≥35 U/mL) (Styne.2016). Anti-TG values were classified as negative (<100 IU/mL), borderline (100 - 150 IU/mL) which in this study considered positive, or positive (≥150 IU/mL) based on a Demeditec kit (Demeditec Diagnostics GmbH). The normal range of TSH and FT4 was based on Reference Range Values for Pediatric Care by Sperling et al., 2020. Hypothyroidism was diagnosed as high TSH and low FT4 and T3. Subclinical hypothyroidism was defined as elevated serum TSH with normal FT4 level, subclinical hyperthyroidism as a low TSH with a normal, euthyroid was defined as normal FT4 and TSH levels (Cooper & Biondi, 2012).

The CTLA-4 CT60 polymorphism from peripheral blood mononuclear cells was analyzed using polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP). The PCR kit was the QIAmp DNA Mini kit (Qiagen©). The technique used Fnu4HI for enzyme restriction. The primer sequences that were used based on Pastuszak-Lewandoska et al.,2012 were CTLA-4 CT60 A/G with a forward primer (5′→3′) -GATTTCTTCACCACTATTTGGGATATTAC-and reverse primer (3′→5′) AGATCAAAATGGCTGCAAGG-. The annealing temperature was 58°C, and amplification was performed for 29 cycles. The result was divided into G/G, A/G, and A/A genotypes.

6.1 Statistical Techniques Used in the Present Study

Data analysis was performed with SPSS version 17. A descriptive analysis was conducted to explain the distribution of subjects' characteristics and bivariate analysis to define the association between the CTLA-4 polymorphism and thyroid function.

6.2 Data Analysis and Interpretation

Table 1. Participant Characteristics

Characteristic	Value
Age (year), median (min; max)	1.7 (0.5; 6.8)
Sex, n (%)	
Male	28 (73.7)
Female	10 (26.3)

Maternal Ethnic, n (%)	
Javanese	33 (86.8)
Madura	2 (5.3)
Chinese	1 (2.6)
Bugis	1 (2.6)
Batak	1 (2.6)
Paternal Ethnic, n (%)	
Javanese	33 (86.8)
Madura	3 (7.9)
Chinese	2 (5.3)
Autoimmune Marker, n (%)	
Positive Anti-TPO	25 (65.8)
Positive Anti-TG	34 (89.5)
Positive Both of Anti-TPO & Anti-TG	23 (60)
Cummulative Positive Autoimmune Marker	36 (94,74)
Cummulative Negative Autoimmune Marker	2 (5.26)
Diagnosis, n (%)	
Hypothyroidism	32 (84.2)
Subclinical hypothyroidism	4 (10.5)
Subclinical hyperthyroidism	2 (5.3)

Interpretation of Table-1.

The sociodemographic characteristics of the subjects are presented in Table 1. Their median age was 1.7 years (range 0.5–6.8); the majority were aged 1 to 5 years (81%). Mean age the subjects who had positive anti-TPO, anti-TG, and both were 2.36, 2, and 3 years old respectively. This finding contrast with a study by Aversa et al.,2015 in which the majority at the onset of autoimmune disease in DS was 3 years or even older. Also contrast study from Pierce et al., 2016, 50% DS subjects had thyroid diseases in adulthood. However, this finding indicated more prevalent hypothyroidism among older subjects. This result support the study that 2020 suggest an increased prevalence of thyroid dysfunction in DS with increasing age (Pierce et al., 2016;Ambike & Chopade 2020). Almost three-quarters of the subjects were boys (73.7%). In contrast, previous studies did not find a difference in gender among DS patients with thyroid dysfunction (Pastuszak-Lewandoska et al.,2012, Dultz et al.,2009). The median duration from diagnosis was 1.3 years, and most of the maternal and paternal ethnic were Javanese. More than four in five subjects suffered from central hypothyroidism (83.3%). All the subjects diagnosed with thyroid diseases, 94,74% had AITD, 60% of AITD subjects had both Anti-TPO and Anti-TG. 5.26% subjects did not have AITD.

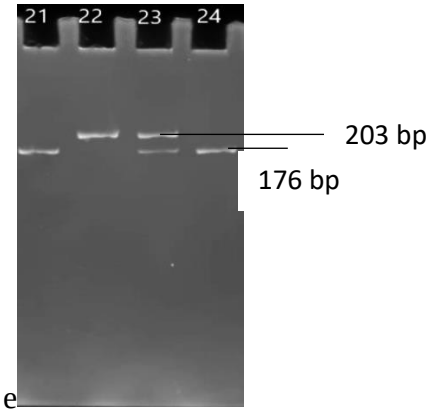

Figure 1. The Polymorphism of CTLA-4 CT 60 showed three types variants that can be seen in sample number 21-24.

Interpretation of Figure 1.

The three types of CTLA-4 CT 60 consist of : AA (Sample number 22), AG (Sample number 23), GG (Sample number 21 and 24)

Table 2. Association between Thyroid Function and Autoimmune Thyroid Marker

		Hypothyroid	Subclinical Hypothyroidism	Subclinical Hyperthyroidism	Total
Anti-TPO	Positive	21	3	1	25
	Negative	11	1	1	13
		32	4	2	38
Anti-TG	Positive	28	4	2	34
	Negative	4	0	0	4
Total		32	4	2	38

Interpretation of Table 2.

From this study we found that around two thirds of hypothyroidism subjects were positive for anti-TPO and around four in five was positive for anti TG. Three quarter of subclinical hypothyroidism was positive for anti TPO and all subjects were positive anti TG. A half of subclinical hyperthyroid subjects were positive anti TPO and all subjects were positive anti TG. Most had low TSH levels. All had low T3 levels, and more than half had low fT4 levels. All of the patients had thyroid function disorder from hypothyroidism, subclinical hypothyroidism, subclinical hyperthyroidism. We did not correlate between positivity of autoimmune marker with thyroid disorders. However, study from Koshie et al., 2107 in Iran found a significant association between anti TPO and subclinical hypothyroid (p=0,02).

In our study, autoimmune marker of DS population dominated with anti-TG, this study similar with Gentile et al, 2004 at Jacobson et al., 2007 found that >80% in Hashimoto's thyroiditis patients had anti-TG. Contrast with study from Patel et al., 2016 found that 59,52% hypothyroid population dominated with anti-TPO. Our study DS population dominated with hypothyroidism, but contrast with study from King et al., 2014 60% DS population dominated with subclinical hypothyroidism.

Table 3. CTLA-4 CT 1822 Polymorphism and Autoimmune Thyroid Marker

		Anti-TPO			Anti-TG		
		Positive	Negative	Total	Positive	Negative	Total
Polymorphism of CTLA-4 CT 1822	CC	6	2	8	6	2	8
	TT	6	5	11	10	1	11
	CT	13	6	19	18	1	19
	Total	25	13	38	34	4	38

Table 4. CTLA-4 CT 60 Polymorphism and Autoimmune Thyroid Marker

		Anti-TPO			Anti-TG		
		Positive	Negative	Total	Positive	Negative	Total
Polymorphism of CTLA-4 CT 60	GG	11	4	15	14	1	15
	AA	4	3	7	6	1	7
	AG	12	4	16	13	3	16
	Total	27	11	38	33	5	38

Interpretation of Table 3 and 4.

In this study, around two-thirds of the subjects with CT60 and CT1822 Polymorphism were positive for anti-TPO, and more than four in five were positive for anti-TG. In this study only found a correlation between CT60 and anti-TPO (p=0,025).

In our study, the homozygous CTLA-4 CT60 (39.5% G/G; 18.5% A/A) variant was higher than the heterozygous variant (A/G genotype 42%) among DS subjects with hypothyroidism. Similar to several previous studies; a study by Patel et al.,2016, in Gujarat, India showed that more than two-thirds of subjects diagnosed with autoimmune hypothyroidism had homozygous CTLA-4 CT60 (61% G/G; 8% A/A; 31% A/G). Another study in Taiwan also reported that the rate of homozygous CTLA-4 CT60 in pediatric subjects with Hashimoto's thyroiditis was higher than the heterozygous genotype (67.7% G/G; 3.1% A/A; 29.3% A/G) (Ting et al., 2016). Study by Bicek et al.,2009 in Slovenia with Caucasian population showed the rate of homozygous CTLA-4 CT60 in pediatric subjects with Hashimoto's thyroiditis was higher than the heterozygous genotype (33% GG ; 20,6%AA ; 46,4%AG). Study by Kavvoura et al., 2017 showed that the frequency of homozygous haplotype was more prevalent than the heterozygous (47,9% G/G; 14,3% A/G; 36,2% A/A;1,6% G/A). The G/G haplotype consist of 63,1 % in Asian descent, 38,4% in Caucasian descent, and 29,5% Iranian descent). So the frequency of G/G haploptype in Asian is higher than Caucasian and Iranian descent Similarly, Pastuszak-Lewandoska et al.,2012 in Poland reported that homozygous CTLA-4 CT60 was more prevalent than the heterozygous genotype (5% G/G; 69% A/A; 26% A/G) among subjects with AITD. A study from Inoue et al., 2012 on a Japanese population stated that at the frequency of CTLA4 CT 60 homozygous genotypes in Hashimoto's thyroiditis patients were found more than heterozygous genotypes.

Table 5. Association between CTLA-4 Polymorphism and T3,FT4,TSH

Outcome Parameters	T3	FT4	TSH
CT-60	p = 0.049*	p = 0.469	p = 0.156
CT-1822	p = 0.046*	p = 0,041*	p = 0,294

*p-value <0.05 is considered significant. All data was statistically analyzed with spearman's correlation.

Interpretation of table 5. The CTLA-4 polymorphism and thyroid function.

We found significant correlations between both CTLA-4 CT 60 & CT 1822 with T3 and CTLA-4 CT 1822 with FT4.The disruption of CTLA-4 has a role in the pathogenesis of autoimmune thyroid disease by preventing apoptosis of activated T cells, thus producing autoantibodies that interfere the thyroid hormone synthesis (Chistiakov & Turakulov 2003). This disruption might contribute to the correlation with T3 and FT4 (polymorphism of CTLA-4 CT 1822) in this study. Although it should be mentioned that there was no control group in this study and all of the participants had low T3 levels. Further research is needed to assess the correlation between CTLA-4 and T3.

We found no significant association between anti-TG, TSH and fT4 and the CTLA-4 polymorphism. This finding agrees with a previous study that reported no correlation between the CTLA-4 polymorphism and thyroid function or thyroid autoantibodies. This conclusion might be because the CTLA-4 concentration is not influenced by environmental or hormonal factors but by genetic factors. (Daroszewski et al., 2009). Other study suggests that the role of CTLA-4 in AITD is not specific since it contributes only to autoimmunity in general and is not organ-specific (Chistiakov & Turakulov 2003).

The association between CTLA-4 and autoimmune hypothyroidism in DS has not been studied before. However, the association between CTLA-4 and autoimmune hypothyroidism has been well documented. A study from Nithiyananthan et al.,2002 in United Kingdom showed that relation between A-G polymorphism and autoimmune hypothyroidism(p=0,001) and CTLA-4 gene region on chromosome 2q33 is acting as susceptible locus for autoimmune hypothyroidism and indeed acting as susceptibility locus for autoimmune disease process. Similarly study by Patel et al.,2016 in Gujarat, India reported the association of CT49A/G (OR 4.319; 95% CI 1.408–13.249), CT49G/G (OR 4.309; 95% CI 1.102–16.855), CT60A/G (OR 7.096; 95% CI 1.616–31.171), and CT60G/G (OR 5.855; 95% CI 1.467–23.368) with susceptibility to autoimmune hypothyroidism. Overall, the G allele in CT60 and CT49 was associated with a higher frequency of autoimmune hypothyroidism. Another study by Ting et al.,2016 in Taiwan found a significant association between the G/G genotype of CT60 and increased risk of pediatric Hashimoto's thyroiditis (HT) (OR 1.42; 95% CI 1.05–1.93) In contrast. Pastuszak-Lewandoska et al.,2012 in Poland described a significantly higher frequency of the A allele of CTLA-4 CT60 in AITD patients compared with controls (OR 2.48; 95% CI 1.05–5.83). In our findings, the G allele (A/G and G/G genotypes) demonstrated a larger proportion of low TSH and a smaller proportion of high TSH than the A/A genotype. High TSH is a feature of primary hypothyroidism, including autoimmune hypothyroidism. Studies have demonstrated the role of CTLA-4 in the risk of Autoimmune polyglandular syndrome (APS). One possible explanation is a link between the G allele and the risk of APS. Dultz et al.,2009 reported study from German University Center, a 1.63-fold higher prevalence of APS in the presence of the G

allele in the CTLA-4 CT60 gene (95% CI 1.03–2.55). A more recent study by Houcken et al., 2018 in Gutenberg University Medical Center, Germany also found the G-allele in CTLA-4 CT60 to be more frequent in APS than controls (OR 1.55; 95% CI 0.81–2.99). in Contrast Study from Sakai et al., 2001 in 123 Japanese people showed that CTLA 4 gene is not linked to Hashimoto thyroiditis because susceptible at chromosome 8q23-q24 not at 2q33 . Similar study with Inoue et al.,2012 found that all of CTLA4 polymorphisms were not associated with disease severity or intractability. The history of treatment with levothyroxine may have also altered the TSH level so that it does not represent the initial TSH. Nevertheless, this study has shown that CTLA-4 may have a role in thyroid function among DS patients. Further study is recommended to define the effect of the CTLA-4 polymorphism on the development of thyroid dysfunction in DS.

Only a small proportion of subjects in this study were diagnosed with primary hypothyroidism, a feature of autoimmune hypothyroidism. Interestingly, the percentage of positive subjects for anti-TPO and anti-TG, which are autoimmune thyroid disorder markers, was significantly larger than that of negative ones (65.8% and 89.5%, respectively).

7. Recommendations

- This study can provide scientific information regarding the CTLA-4 gene polymorphism as a risk factor for autoimmune thyroid disease in Down syndrome children.
- This study can be used as an early detection of autoimmune thyroid disease in children with Down syndrome through examination of the CTLA-4 gene polymorphism, anti-thyroid peroxidase activity, anti-thyroglobulin levels, T3 and FT4 levels
- This study can be used to improve management of Down syndrome children towards the risk of autoimmune thyroid disease.
- Further research is needed to assess the correlation between CTLA-4 and T3.
- Further study is recommended to define the effect of the CTLA-4 polymorphism on the development of thyroid dysfunction in DS.

8. Conclusion

To the best of our knowledge, this is the first study that explores the association between the CTLA-4 polymorphism and thyroid function in a DS population. A significant association was found between CTLA-4 A/G and G/G genotypes with a larger proportion of low TSH levels among DS patients with hypothyroidism, contradictory to previous studies that linked the G allele to autoimmune hypothyroidism characterized by a high TSH level. While the reason is yet to be explored, this study showed the potential influence of the CLTA-4 polymorphism on thyroid function in DS. Further study is needed to identify the impact of the CTLA-4 polymorphism on thyroid function and autoimmune thyroid abnormalities in DS and the underlying mechanism.

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