

Evaluation of SCUBE-1 and sCD40L biomarkers in patients with hypothyroidism due to Hashimoto's thyroiditis: a single-blind, controlled clinical study

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Abstract. – OBJECTIVE: This study was performed to investigate the levels of platelet activation marker SCUBE-1 and sCD40L which plays some role in the progression of atherosclerosis, in patients with hypothyroidism due to Hashimoto's thyroiditis.

PATIENTS AND METHODS: The cohort of this study includes 90 patients and 35 healthy volunteers, who admitted to the internal medicine outpatient. Simultaneously in the same samples; SCUBE-1 and sCD40L measurements were accomplished by enzyme-linked immunosorbent assay (ELISA). The data obtained were analyzed statistically.

RESULTS: Hypothyroidism due to Hashimoto's thyroiditis in comparison to the control group that includes healthy volunteers; SCUBE-1 and sCD40L, were increased. And these results were statistically significant ($p < 0.05$).

CONCLUSIONS: SCUBE-1 and sCD40L levels were increased in Hashimoto's hypothyroidism patients. This may indicate an increased endothelial dysfunction, platelet activation and cardiovascular risk in hypothyroidism. SCUBE-1 and sCD40L may be helpful in cardiovascular risk assessment of hypothyroid patients.

Key Words:

Cardiovascular risk, sCD40L, Hypothyroidism, Hashimoto's thyroiditis, SCUBE-1.

Introduction

As is known, thyroid hormones are taking office in somatic and the mental development,

basal metabolism and calorogenic effect formation¹, the cholesterol synthesis², glucose metabolism³ and the myocardium⁴.

Diseases causing such as Hashimoto's thyroiditis, are known to occur with environmental and genetic factors⁵. Although the predisposing genes like HLA-DR genes⁶, CTLA-4, cluster of differentiation 40 (CD40), protein kinase phosphokinase (PTPN)-22, non-MHC genes such as thyroglobulin and thyroid stimulating hormone (TSH) have been reported to be important in the terms of disease⁷.

Also, there are researches available in the literature that these kind of autoimmune diseases are influenced by many environmental factors such as iodine, medicines, infections caused by staphylococcal/streptococcal or viral pathogens, smoking history and stress^{8,9}.

Hypothyroidism is known to cause hypercholesterolemia, elevated low-density lipoprotein (LDL-C), and hypertriglyceridemia¹⁰.

In the recent studies, thyroid indicated platelet activation, elevated lipid profile, endothelial dysfunction, metabolic and hemodynamic disorders were suggested as a risk factor for cardiovascular diseases in hypothyroid patients¹¹⁻¹⁴. Several researches conducted with cardiovascular diseases showed that platelet α granules and cell proteins such as Signal peptide-CUB (complement C1is / C1s, Uegf, and Bmp1) – EGF (epidermal growth factor) – domain-containing protein 1 (SCUBE -

1)¹⁵ levels were increased during an acute ischemic stroke and/or an acute coronary syndrome^{16,17}.

In the research, a soluble isoform of CD40 ligand (sCD40L) which plays major role in the CD40 / CD40L system acting by binding to CD40 receptor has been reported to play a role in the progression of atherosclerosis¹⁸⁻²⁰.

To our knowledge there is no study with both SCUBE-1 and sCD40L in this field. In this study, the levels of SCUBE-1 – *one of the platelet activation marker* – and sCD40L levels which play role in atherosclerosis, endothelial dysfunction and cardiovascular diseases, were aimed to evaluate in patients with hypothyroidism due to Hashimoto's thyroiditis.

Patients and Methods

Ethical Permission

This research project was performed with Namik Kemal University Faculty of Medicine Ethics Committee permission (27/11/2014-112) and informed consent from the patients were taken.

Materials

SCUBE-1 [Cat # CSB-E15005h, Cusabio, Wuhan, Hubei Province (430206) China] and sCD40L [Cat # EK0573, Boster, Pleasanton, CA, (94566) USA] commercial kits were used. Anti-thyroid peroxidase (anti-TPO) antibody which is an anti-thyroid microsomal antibody (Cat # 06368690190); anti-thyroglobulin (anti-TG) antibody (Cat # 06368697190), thyroid stimulating hormone (TSH, Cat # 154906-01) and free tetraiodothyronine (fT4, Cat # 06437281190) were obtained from Roche Company (Tokyo-Japan)

Free Triiodothyronine (fT3) commercial kit (Cat # 06437206190) that occurs after deiodination in liver with 5'-deiodinase enzymes was purchased from Roche Company (Tokyo-Japan). High density lipoprotein cholesterol (HDL-C) (Cat # 04399603190), total cholesterol (Cat # 03039773190), and TG (Cat # 29767107-322) kits are also Roche (Tokyo-Japan) brand but Low density lipoprotein (LDL-C) values were calculated manually using the Friedewald formula²¹.

The enzyme linked immunosorbent assay (ELISA) device that was used during the measurements is BioTek Microplate Readers brand

and its origin is Winooski, (05404) USA. Biochemistry (Cobas e6000-E501) and hormone systems (Cobas e6000-e601) are Roche Diagnostics (Tokyo, Japan) origin.

Methods

To minimize the errors, the similar measurements were carried out by the same researchers. Measurements were repeated at least 3 times. ELISA analysis performed by the researchers, with the tubes that contained the blood samples which were coded with letter, in order to not know which blood belongs to which group. So researchers that performed the study analysis were blind.

Case Selection Criteria

As prospectively, the universe of this controlled, single-blind study were consisted from the people that admitted to the Internal Medicine Department between the 1st December 2014-1st June 2015, and which have Anti-TPO more than 35 mIU/L or anti-TG antibodies more than 40 IU/mL, TSH more than 4 mIU/L and fT4 lower than 0.8 ng/dL and fT3 lower than 1.57 pg/mL or within the normal range of 1.57-5.3 pg/mL. From these cases, the patients that have been diagnosed with hypothyroidism due to Hashimoto's thyroiditis and agreed to be volunteers were enrolled to this study (n = 90).

The diagnosis of Hashimoto's thyroiditis was performed according to circulating antibodies to thyroid antigens (Anti-TPO and anti-TG) and reduced echogenicity on thyroid sonogram in a patient with proper clinical features. Since cytological examination of thyroid aspirate is recommended in the presence of a concomitant thyroid nodule, we performed fine needle biopsy in patients with nodule²². According to the cytological results of fine needle biopsy, we did not observe false positive or negative diagnosis. Of these cases: patients with any malignancy, inflammation associated rheumatic diseases or acute/chronic infections, cases with diabetes mellitus; cases with cardiovascular or cerebrovascular diseases, pregnant women and those with chronic hepatic or renal diseases were excluded from the study²³⁻²⁶ (Figure 1).

55 cases who meet the criteria to be included and who heeded the call with hypothyroidism due to Hashimoto's thyroiditis were called Group I.

The control group of the study consisted of the volunteers who came for check-up to the Internal Medicine Clinic, and they were classified as

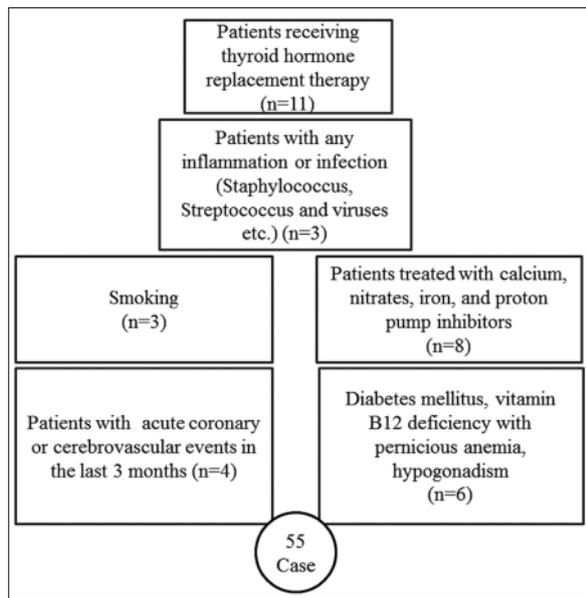


Figure 1. Exclusion criteria.

Group II. Group II was created from healthy volunteers ($n = 35$) with the similar age and sex with Group I.

LDL-C, HDL-C, TG and total cholesterol test results were recorded to standard form with demographic data of the Group I and Group II patients.

Then, both group's venous bloods SCUBE-1 and sCD40 ligand values were evaluated through ELISA microplate reader commercially available kits.

Measuring the Value of SCUBE-1 and sCD40L

Blood samples were obtained after a resting period of 30 min between 08⁰⁰ and 09⁰⁰ from the cannulated antecubital vein. Serum was obtained by centrifugation at 2000 g' for 15 minutes at +4°C. The plasma samples were stored at -80°C until analysis.

SCUBE-1 and sCD40L were performed at 450 nm absorbance measurement. Detection range and sensitivity of SCUBE-1 and sCD40L commercial kits's intra-assay CV and inter assay CV values are, respectively, < 5.9%/ < 6.8% / - / < 15 pg/mL and < 8%/ < 10%/ 0,625 to 40 ng/ml/ 0.156 ng/ml.

Statistical Analysis

Results for normally distributed continuous variables are expressed as mean \pm standard deviation, and continuous variables with non-normal

distribution are presented as median values and interquartile range. Analyses of normality in the continuous variables were performed using the Shapiro-Wilk test, histograms and Q-Q plots. Categorical variables were expressed as numbers and percentage. The chi-square test was used to compare proportions in different groups. Student's *t*-test or Mann-Whitney U-test was used to compare the two independent groups according to distribution state. Correlations between levels of circulating SCUBE-1 and other variables were evaluated with Pearson correlation. All statistical analyses were performed with SPSS software (version 21.0; IBM Corporation, Armonk, NY, USA). A two-sided $p < 0.05$ was considered significant.

Results

Demographic data are expressed as mean or median with standard deviation or percentages (%). The groups are age, gender and body mass index [BMI; The BMI was calculated from the formula of (weight/height squared, kg/m²)] matched. TSH, anti-TG, and anti-TPO levels were higher in Hashimoto's thyroiditis group than healthy control group (Table I).

We found higher plasma SCUBE-1 and sCD40L levels in patients with Hashimoto's thyroiditis compared to healthy controls and these differences were statistically significant ($p = 0.005$ and $p = 0.009$ respectively) (Figure 2, Table II).

SCUBE-1 and sCD40L levels of Hashimoto's thyroiditis group were statistically correlated to lipids (except HDL-C), TSH, ft4, anti-TPO and anti-TG levels. Statistically significant positive correlation was found between total cholesterol, TG and LDL-C levels and SCUBE-1 and sCD40L. Negative correlation observed between HDL-C and SCUBE-1 and CD40L was not statistically significant (Table III).

Statistically significant positive correlation was found between TSH, anti-TPO and SCUBE-1 and sCD40L levels. Statistically significant negative correlation of ft4 to these two biomarker levels was found.

But however the correlations of ft3 to SCUBE-1 and sCD40L were both negative, only the correlation of ft3 to sCD40L level reached statistical significance ($p < 0.05$). SCUBE-1 and sCD40L parameters were positively correlated with each other (Figure 3).

Table I. Demographic data and biochemical measurements.

| Parameters evaluated | Group I (n = 55) | Group II (n = 35) |
|--------------------------------------|-------------------------------|--------------------------------|
| Age (mean ± SD) | 46.87 ± 13.26 | 47.26 ± 14.07 |
| Gender (percentage) | Female (92.73) Male (7.27) | Female (77.14) Male (22.86) |
| BMI (kg/m ²) (mean ± SD) | 25.7 ± 1.9 | 25.4 ± 1.3 |
| LDL-C (mg/dL) | 148.67 ± 47.79 | 122.63 ± 33.09 |
| HDL-C (mg/dL) | 44.78 ± 11.75 | 54.32 ± 12.12 |
| TG (mg/dL) | 150.75 ± 58.98 | 94.34 ± 44.15 |
| TSH (mIU/L) | 28.08 ± 25.56 | 1.74 ± 0.92 |
| ft3 (pg/mL) | 2.69 ± 0.67 | 3.21 ± 0.37 |
| ft4 (ng/dL) | 0.58 ± 0.16 | 1.26 ± 3.25 |
| Anti-TPO (mIU/L) | 511.60 ± 421.99 | 12.85 ± 3.25 |
| Anti-TG (IU/mL) | 132.71 ± 439.98 | 13.97 ± 1.78 |

SD: Standard deviation, BMI: body mass index, LDL-C: low density lipoprotein cholesterol, HDL: high density lipoprotein cholesterol, TG: triglyceride, TSH: thyroid stimulant hormone, ft3: free Triiodothyronine, ft4: free tetraiodothyronine, Anti TPO: antithyroid peroxidase, Anti-TG: anti-thyroglobulin.

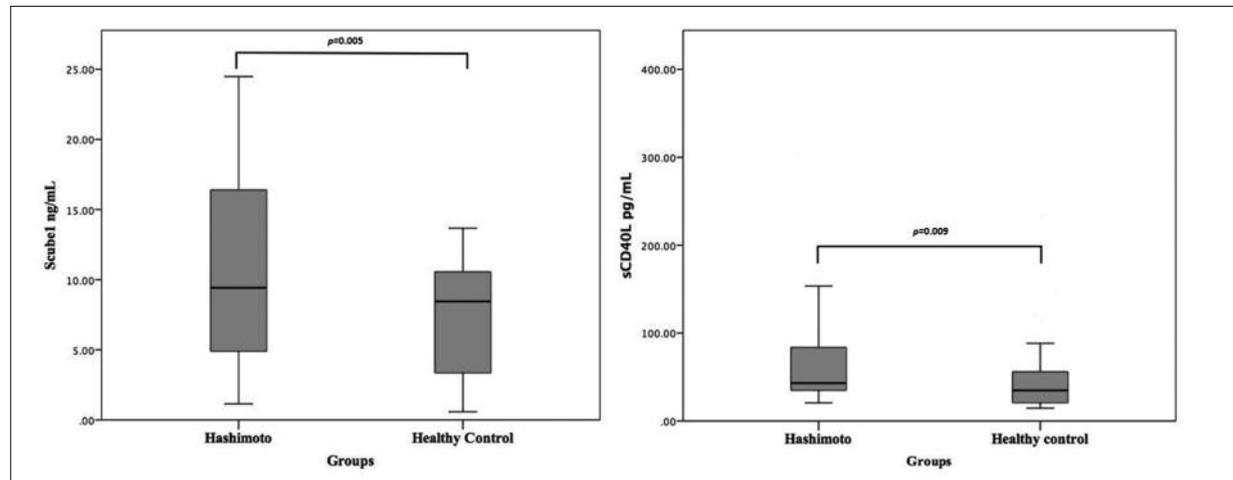


Figure 2. Laboratory measurements of Hashimoto's thyroiditis group.

Table II. Inter-group comparisons in terms of SCUBE-1 and sCD40L levels.

| | SCUBE-1 | sCD40L |
|--|--------------|---------------|
| Group I (mean ± standard deviation) | 10.53 ± 6.47 | 43.03 ± 48.88 |
| Group II (median ± standard deviation) | 7.34 ± 3.90 | 34.84 ± 38.83 |
| <i>p</i> | 0.005* | 0.009** |

*Student's *t*-test; **Mann-Whitney U-test.

Discussion

Early recognition of the patients at risk for acute thrombotic events myocardial necrosis, acute coronary syndrome, acute ischemic patients such as ischemic stroke, and risk classification prognosis is very important. Recent

studies have shown that combination of biomarkers of myocardial necrosis, inflammation, kidney and the myocardial function and neurohumoral activation may provide a better risk assessment with identifying patients at high risk for the cardiovascular events that may occur in the future^{27,28}.

Table III. Pearson correlation test between SCUBE-1, sCD40L, and all other characteristics evaluated.

| | SCUBE-1 | | sCD40L | |
|------------------|---------|-------|--------|-------|
| | r | P | r | P |
| Total-C (mg/dl) | 0.302 | 0.004 | 0.216 | 0.041 |
| LDL-C (mg/dL) | 0.284 | 0.007 | 0.214 | 0.043 |
| HDL-C (mg/dL) | -0.167 | 0.115 | -0.179 | 0.092 |
| TG (mg/dL) | 0.338 | 0.001 | 0.363 | 0.001 |
| TSH (mIU/L) | 0.224 | 0.034 | 0.222 | 0.036 |
| ft3 (pg/mL) | -0.184 | 0.83 | -0.268 | 0.011 |
| ft4 (ng/dL) | -0.278 | 0.008 | -0.325 | 0.002 |
| Anti-TPO (mIU/L) | 0.849 | 0.001 | 0.741 | 0.001 |
| Anti-TG (IU/mL) | 0.314 | 0.019 | -0.349 | 0.009 |

Total-C: total cholesterol, LDL-C: low density lipoprotein cholesterol, HDL: high density lipoprotein cholesterol, TG: triglyceride, TSH: thyroid stimulant hormone, ft3: free Triiodothyronine, ft4: free tetraiodothyronine, Anti TPO: antithyroid peroxidase, Anti-TG: anti-thyroglobulin.

There are studies in the literature which shows increased risk of cardiovascular disease and impaired vascular endothelial structure in the patients with subclinical hypothyroidism²⁹. In addition there are reports^{29,30} revealing increased oxidative stress and impaired platelet activation in overt hypothyroidism patients.

In our study we tried to evaluate the platelet activation and endothelial dysfunction also cardiovascular risk in hypothyroidism due to Hashimoto's thyroiditis with SCUBE-1 as it was suggested in the literature with sCD40L biomarkers. As a result, in the patients with hypothyroidism due to Hashimoto's thyroiditis; SCUBE-1 and sCD40L levels were found in high concentrations compared to healthy volunteers of the control group. There was a positive correlation between SCUBE-1 and sCD40 levels.

Recent studies³¹ indicate the pivotal role of accelerated thrombosis in the progression of ather-

osclerosis. Most studies about hemostasis in hypothyroidism have focused on coagulation and fibrinolysis. However, there are few reports³² about primary hemostasis in hypothyroidism.

Platelet activation and aggregation is known to play major role in the pathophysiology of atherothrombosis. In their research, Baldus et al³³ have reported a new biomarker in determining the risk of adverse cardiac events, sCD40L, which normally related to inflammation and thrombosis.

sCD40L, which causes destabilization of the plaque after binding to receptors found on atherosclerotic plaque's endothelial and smooth muscle cells to release the matrix metalloproteinase³³, has significantly higher levels in patients with acute coronary syndrome compared to the control group.

In addition, the same researchers supposed that sCD40L levels and future high risk of cardiovascular events had been associated and also it might provide better risk assessment for death in nonfatal myocardial infarction^{34,35}.

SCUBE-1 is a member of the SCUBE gene family, this is expressed from endothelial cells and activated platelets. SCUBE-1 was studied and has been found elevated in some acute thrombotic events such as acute coronary syndromes³⁶, ischemic stroke³⁷, pulmonary embolism³⁸, and acute mesenteric ischemia³⁹, SCUBE-1 levels were also found elevated in chronic conditions such as hypertension⁴⁰ and hemodialysis⁴¹.

SCUBE1 has been shown to increase in parallel with platelet activation in acute ischemic and thrombotic diseases. There has been no study evaluating SCUBE-1 levels in patients with Hashimoto's thyroiditis. Hypothyroidism is

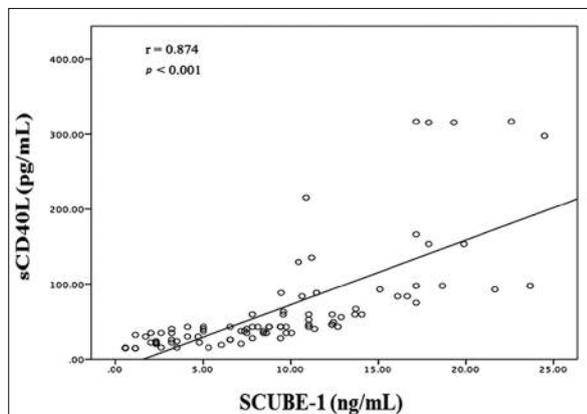


Figure 3. The correlation of SCUBE-1 and sCD40L.

known to cause hypercholesterolemia, elevated LDL-C, and hypertriglyceridemia. It was reported that high circulating TSH levels were associated with abnormally elevated serum lipids, increase in platelet activation, and increased oxidation of the LDL-C^{10-13,42-44}.

In such clinical researchs large number of study group and controls would be required. But the limitation of this study as small number of patients and controls involved.

In this study both SCUBE-1 and sCD40L levels positively correlated with total cholesterol, LDL-C and TG levels. A negative strong correlation with HDL-C levels was observed but it was not statistically significant. We found significantly higher SCUBE-1 and sCD40L levels in patients with Hashimoto's thyroiditis compared to healthy controls and statistically significant. TSH, anti-TG, and anti-TPO levels were higher in Hashimoto's thyroiditis group than healthy control group.

As a result, in the patients with hypothyroidism due to Hashimoto's thyroiditis, SCUBE-1 and sCD40L ligand levels were found in high concentration compared to healthy volunteers of the control group.

Conclusions

Early recognizing platelet activation and endothelial dysfunction, also cardiovascular risk factors with SCUBE-1 like sCD40L, may provide a better prognosis in cardiovascular diseases in hypothyroid patients.

Conflict of Interest

The Authors declare that there are no conflicts of interest.

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