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Cell-free dna methylation evaluation in patients with thyroid diseases

Cell free Dna methylation in thyroid diseases

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Abstract
Aim: The main aim of the study was to evaluate the DNA 5-methylcytosine (m5C) level, measured in circulating cell-free DNA (ccfDNA) as a distinct feature of thyroid gland-related disorders, including thyroiditis, benign nodule, and malignant nodule.
Material and Methods: The study included 75 patients with 30 benign nodules, 30 thyroiditis, and 15 thyroid cancers; 19 subjects were evaluated as a control group. We collected peripheral blood samples from three disease groups and the controls, and then separated the plasma from the whole blood. We measured m5C in ccfDNA purified from plasma samples of patients and healthy individuals.
Results: The level of m5C, measured in thyroiditis patients was significantly different from those measured in the control group, malignant and benign patients. We observed hypomethylation in benign and malignant patients when compared with the control group. However, there was no significant difference between the malignant patients and the control group and between the benign patients and the control group. After comparison of disease groups, we observed that there was no statistically significant difference between benign and malignant patients. We observed a statistically significant difference between thyroiditis and malignant patients ($p<0.01$) and between thyroiditis and benign patients ($p=0.001$).
Discussion: Very few studies have reported that DNA methylation is an epigenetic mechanism in thyroiditis patients. Here, we reported that the level of m5C of ccfDNA could be used as a biomarker for thyroiditis. Further studies are required with the higher number of malign and benign patients to investigate the differences between patients with nodules and healthy individuals.

Keywords
Thyroid diseases, Epigenetic, DNA Methylation, Circulating Cell-Free DNA, Cancer, Thyroiditis

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Introduction

Thyroid gland-related disorders are among the most common endocrine disease. It was reported that such disorders affect 3-5% of the human population [1]. Recent studies indicated that there are undiagnosed individuals who are unaware of their condition in healthy populations. For example, studies performed with healthy individuals showed that approximately 5% and 1.5% of the population were diagnosed with hypothyroidism and hyperthyroidism [2, 3].

Thyroid nodules are common in the healthy population and are defined as lesions within the thyroid gland [4]. The nodules are clinically critical since they have the potential to become malignant, which are resulted from approximately 5-8% of benign nodules [4]. Thyroid malignancies are the most critical malignant tumors of the endocrine system and are observed at a rate of 1%. Except for lesions in the thyroid gland, thyroiditis is a state of inflammation of the thyroid gland due to several factors, including autoimmune diseases, infections, drugs, or fibrosis [5].

Currently, ultrasound and fine-needle aspiration (FNA) biopsy are mainly used to diagnose thyroid gland-related lesions. High-resolution ultrasound is a commonly used method to detect 19-68% of randomly selected individuals [6]. Despite the widespread use of needle biopsy, failure to obtain enough and specific tissue for the diagnosis, and even inadequate cytological techniques may result in approximately 20% failure of the results causing more repeating sampling [7]. When all these processes are considered, it is difficult to diagnose thyroid gland-related disorders and investigate large human populations in thyroid diseases. Screening of individuals with reliable molecular biomarkers might provide an opportunity to detect thyroid gland-related disorders in an early stage for individuals who are at risk. Furthermore, investigating such markers might potentially be a complementary function to available biomarkers to increase the accuracy of the diagnosis. In this context, early detection of thyroid gland-related disorders would enable us for enough time to make the possible diagnosis, observation, or treatment strategy that increases the overall survival rates.

Recent studies have indicated that DNA methylation is a crucial hallmark of diseases, especially for cancer [8], and can be a diagnostic tool in thyroid cancer [9]. Epigenetic mechanisms play an essential role in gene expression, which is changed due to the selective formation of chromatin structures in different epigenetic states of DNA, including DNA methylation of cytosine base, histone modifications, and miRNAs [10]. DNA 5-methylcytosine (m5C) occurs by covalently linking the methyl group to the 5th position of the cytosine ring in a CpG dinucleotide [11]. Most of the studies focus on solid tissue or blood samples to investigate DNA methylation in human population studies. However, bodily fluids such as plasma, serum, etc., provide a unique opportunity to explore the molecular alterations in diseases. In this context, we aimed to evaluate the level of global m5C level of circulating cell-free DNA (ccfDNA) samples collected from thyroid patients with thyroiditis, benign nodule, and malignant nodule. Then, we compared the patient groups' results with the health group to show the distinctive properties of ccfDNA methylation for different thyroid gland-related disorders.

Material and Methods

Our study consisted of four groups, including thyroiditis patients (n=30), benign nodule patients (n=30), malignant nodule patients (n=15), and a healthy control group (n=19). Individuals were registered in the otorhinolaryngology clinic. All the participants completed questionnaires to determine the general characteristics of the study participants, including demographic characteristics such as age, sex, smoking habits (smoker, non-smoker), alcohol consumption (yes/no). Patients were diagnosed with thyroiditis as a result of a blood analysis (THS, T4, and T3). The diagnosis of patients with benign and malignant nodules was performed by an experienced ear nose and throat physician and their FNA biopsies results from the pathology department. After diagnosis were analyzed, blood samples were collected from patients before any treatment. The control group was composed of individuals who were not exposed to physical and chemical agents or who were free of thyroid diseases and other systemic disorders. Additionally, the participants in the control group lived in the same region as the patients. We applied all procedures to the control individuals, including blood and ultrasound of the neck during the same periods with patients. In this context, 19 subjects were included in the control group. The study was approved by the ethics committee (2011-KAEK-27/2016-E.70096). Consent forms were obtained from all the participants and the study was conducted according to the Helsinki Declaration.

Five ml of peripheral blood was taken in sterilized tubes containing K3EDTA. After taking blood samples, we applied two sequential centrifugation steps to obtain the final plasma samples. At the first stage, blood samples were centrifuged at 3000 g for 10 min to remove debris and any cells. At the second stage, the plasma samples taken after the first centrifugation were transferred and centrifuged at 16000 g for 10 min. After the second centrifugation, the plasma samples were stored at -80°C for DNA purification. DNA isolation from plasma samples was conducted using a commercial kit (Qiagen, USA). The DNA content of the samples was measured with a fluorescence-based Quant-iT™ high-sensitivity DNA assay kit and a Qubit® fluorometer (Invitrogen, Carlsbad, CA, USA).

The total DNA methylation amount was quantified using the ZYMO m5C DNA Elisa (Zymo Research Corp., USA) according to the manufacturer's instructions. We generated a standard curve to quantify the percentage of m5C in the DNA sample by using negative control and positive control provided by the kit. We used the equation below to determine the m5C percentage of ccfDNA based on their absorbance.

% m5C = e {(Absorbance - y-intercept)/Slope}

Statistical analysis

We applied the Kolmogorov-Smirnov test and Levene's test to check the distribution and normality of the DNA m5C level. If the results are not normal, then we applied log transformation to the results of m5C. We compared the results of each group by applying One-way ANOVA and then Tukey HSD post-hoc test. We applied the Spearman correlation test to reveal the association between parameters. Besides, we used an univariate general linear model to evaluate the effects of different variables (age,

gender, smoking habit, and disease states) on global DNA methylation level. Data analyses were performed using SPSS 19.0.0. Graphs were plotted using GraphPad Prism.

Results

The characteristics of individuals are presented in Table 1. The control group consisted of 8 males and 11 females, and the mean age of the control group was 31.1 years. There were 27 females and three males in the thyroiditis group, and the mean age of the thyroiditis patients was 36.6 years. In the benign nodule group, there were 19 females and 11 males, and the mean age of the group was 54.1 years. The malignant group consisted of 11 females and seven males, and the mean age in the group was 43.26 years.

The level of DNA m5C in the control participants and thyroid diseases patients is given in Table 2.

We also present the distribution of results of each participant in Figure 1. Accordingly, when DNA methylation percentage was taken into consideration, the mean of DNA methylation was 3.07% and 4.84%. We observed global hypermethylation in thyroiditis patients compared to the control group. When the Mann-Whitney U test was applied, the level of DNA m5C detected in the thyroiditis was statistically higher than in the control group ($p<0.05$).

We found that the global DNA m5C level in benign and malignant patients was 2.46 and 2.48%, respectively. When compared with the control, we observed global hypomethylation in benign and malign groups. When One-way ANOVA followed by Tukey HSD post-hoc test was applied, we found that there were no differences between the malignant patients and the control group and between the benign patients and the control group ($p>0.05$).

We compared the patients in terms of global DNA methylation by applying One-way ANOVA and then Tukey HSD post-hoc test. The methylation levels in both groups were similar. No significant difference was found between the malign and benign patient groups ($p>0.05$). In contrast, hypermethylation was observed in thyroiditis patients compared with the patients with nodules. We found that global DNA methylation was statistically higher than in benign and malign patients ($p=0.001$ for thyroiditis -benign, and $p=0.008$ for thyroiditis and malign).

Age is a critical factor in altering DNA methylation level. We investigated the correlation between ccfDNA methylation level and age in three disease groups. According to the results, it was found that the association between age and ccfDNA methylation tends to be significant in thyroiditis and benign groups ($r= -0.33$, $p=0.07$ for thyroiditis group and $r= 0.32$, $p=0.08$ for the benign group). We found that age was significantly associated with ccfDNA methylation in malign patients ($r=0.70$ and $p=0.004$).

The level of ccfDNA m5C in thyroiditis patients was compared in terms of gender. Accordingly, there was no difference between males and females in disease groups. Similarly, when all patient groups were evaluated together, ccfDNA m5C level was similar in both genders ($p>0.05$). In the patient groups, the ccfDNA m5C level was compared in terms of smoking habit with the Mann-Whitney U test. For all groups, no difference was found in terms of smoking and ccfDNA m5C level. No statistically significant difference was found between smoking and ccfDNA level($p>0.05$).

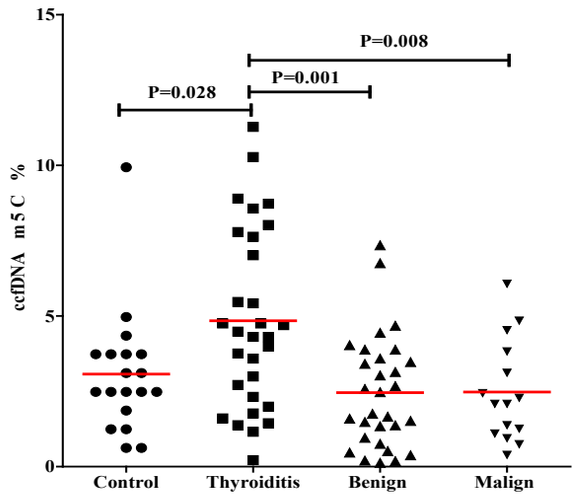

Figure 1. Distribution of DNA m5C level across thyroid gland-related diseases and in the control groups (Line shows the mean of the group)

Table 1. Main characteristics of the control and the patient groups

		Control Group		Thyroiditis Group		Benign Nodule Group		Malignant Nodule Group	
		n (%)	Mean ±SD	n (%)	Mean	n (%)	Mean	n (%)	Mean
Age		19	31.1±15.7	30	36.6±9.88	30	54.1±13.98	15	43.26±8.89
Age	Male	8 (42.0)	30.6±16.7	3 (11.1)	47.3±7.50	11 (36.7)	59.5±10.38	4 (26.7)	44.25±12.9
Age	Female	11 (58.0)	31.3±15.8	27 (89.9)	35.4±9.50	19 (63.3)	51.0±15.05	11 (73.3)	42.91±7.76
Smoker		8 (42.0)	-	8 (26.7)	-	5 (16.6)	-	7 (46.7)	-
Non-smoker		11 (58.0)	-	22 (73.3)	-	25 (83.3)	-	8 (53.3)	-
Alcohol	Yes	2 (10.5)	-	7 (23.3)	-	4 (13.3)	-	4 (26.7)	-
Alcohol	No	17 (89.5)	-	23 (76.3)	-	26 (86.7)	-	11 (73.3)	-

Table 2. Level of DNA m5C in the control and the patients with thyroid diseases

m5C (%)	Control (n=19)	Thyroiditis (n=30)	Benign (n=30)	Malignant (n=15)
Mean	3.7	4.84	2.46	2.48
Min	0.62	0.22	0.13	0.39
Max	9.94	11.28	7.35	6.7
SD	2.5	2.96	1.86	1.68

SD: Standard Deviation

We applied a univariate general linear model to investigate the factors affecting the ccfDNA m5C level in thyroid gland-related disorders. Firstly, we adjusted the model with the age, gender, smoking habit, and disease states of thyroiditis, malign and benign patients. When ccfDNA m5C level was a dependent variable, we found that age and thyroid disease states were significantly associated with ccfDNA m5C level.

Discussion

We observed that the ccfDNA m5C level in thyroiditis patients was significantly different from those obtained for the control group. Concordantly, ccfDNA m5C level observed for malignant and benign patients was significantly lower than those measured in thyroiditis patients.

Nucleic acids have been released from the cells with several mechanisms, including apoptosis and necrosis, and circulated in the peripheral blood [12]. ccfDNA in plasma and serum samples have been investigated in DNA content, tumor-specific loss of heterozygosity, gene mutations, and DNA integrity. Cancer cells-derived DNA is also available in the plasma, resulting from the apoptosis and necrosis of cancer cells [13]. Specifically, ccfDNA has been investigated in different cancer types, including breast, colorectal, pancreas, ovarian, brain, and melanoma cancers in the last 20 years [14-16]. Gene-specific or global changes of DNA m5C are a hallmark of cancer and are also associated with non-malign diseases. DNA m5C level of ccfDNA has been investigated in several cancer types [13]. However, similar studies on thyroid gland-related disorders are limited.

Methylation pattern or level are tissue-specific, which enable to distinguish differences among cancers and also among subtypes of cancers. In this context, hypo-, hypermethylation, gene-specific, or global methylation patterns could be specific for diseases indicating the potential of DNA methylation as a biomarker of diseases. DNA methylation in tumor suppressor, oncogenes, and thyroid-specific genes have been previously investigated in different types of thyroid cancer including papillary thyroid cancer, follicular thyroid cancer, medullary thyroid cancer, anaplastic thyroid cancer [17]. Most of the studies have been performed with tissue samples, and cell lines indicated the aberrant methylation patterns `[18-20]. It has recently been reported that specific DNA methylation sites were determined in benign nodules and malign nodules in a collection of surgical thyroid specimens, including benign nodules, malign nodules and adjacent normal thyroid tissues. Hypermethylation pattern was observed in benign nodules when compared with malignant nodules and adjacent thyroid tissue, which indicates the different methylation patterns between nodule types [21].

Although substantial evidence showed the distinct DNA methylation pattern in diseases-related thyroid tissues, it was reported that DNA methylation could not be measured in most of the thyroid tissues due to tissue contamination with cells other than follicular epithelial cells [21]. Similar contamination has been known for the failure of the cytological examination of tissues by FNA approach. Therefore, surrogate tissue such as ccfDNA provides more knowledge in terms of diseases. In this context, few studies investigated DNA methylation patterns in blood, plasma, and serum samples in thyroid gland-related diseases. For example, methylation analysis of five genes (CALCA, CDH1, TIMP3, DAPK, and RARβ2) was performed in DNA samples obtained from serum samples of thyroid cancer and patients with benign nodules. It was reported that methylation in these five genes was 95% specific and DNA methylation change was found to be significant in individuals who could not be detected cytologically or later diagnosed as cancer [22]. In another study, investigating the comparison of thyroid cancers with the healthy group, it was stated that ccfDNA samples taken from cancer individuals were important indicators with SLC5A8 and SLC26A4 hypermethylation [23]. Although we expect significant differences among the malignant patients, the benign patients, and the control group, we did not observe it in our study. Generally, global non-significant hypomethylation was observed in malignant and benign patients after comparison to the control group. In addition, the number of individuals in our patient groups was lower that may have contributed to obtaining significant differences among groups. Therefore, we suggest investigating the genome-wide ccfDNA methylation pattern in patients who have thyroid gland-related disorders.

Very few studies have reported that DNA methylation is an epigenetic mechanism in thyroiditis patients. It was recently reported that DNA methylation in pregnant women with Hashimoto's thyroiditis was significantly different compared with the non- thyroiditis group [24]. Another study has recently reported that PTPN22 gene promoter DNA methylation in thyroiditis was significantly different from those obtained in the control group [25]. As far as we know, no data regarding DNA methylation of ccfDNA in thyroiditis have been reported. Our results indicate that DNA methylation of ccfDNA obtained from thyroiditis patients has a unique potential compared to the control and thyroid nodule-related disorders. Since age has the potential to be a confounding factor, we estimated the regression lines that predict DNA methylation level from age for each group. Thus, we calculated the age-adjusted DNA methylation level for each participant in each group. In our age-adjusted model, we found that DNA methylation level in thyroiditis was statistically significantly different from those obtained in benign and malignant patients ($p<0.001$ for each). Furthermore, we observed that DNA methylation level was significantly different compared with the control group ($p<0.05$). Therefore, our multivariate general linear model suggested that confounding factors are also considered when evaluating DNA methylation in circulating cell-free DNA as a biomarker for thyroid diseases.

This study has several strengths. For example, the study investigated the DNA methylation in cell-free DNA in three thyroid gland-related disorders and the healthy control group. Besides, we utilized a bodily fluid from easy-to-obtain from blood samples; this approach should be widely adapted to clinical studies. Furthermore, we measured global DNA methylation level, one of the epigenetic mechanisms that substantially impact gene expression and diseases. It has been known that there are still no robust biomarkers to diagnose thyroid diseases. Our results regarding thyroiditis have unique since there are no data regarding methylation of ccfDNA in thyroiditis. Using the non-invasive, repeatable, and economical method, the analysis of ccfDNA in terms of different molecular mechanisms can be used as a source of biomarkers. DNA methylation, which is a

hallmark of cancer and other diseases in ccfDNA can provide a unique opportunity to assess thyroid diseases. On the other hand, we acknowledge several limitations in the present study. We are aware that the number of individuals in each group was low, which might affect the results. However, we believe that a higher number of individuals will clarify the differences between the groups.

Conclusion

In conclusion, this is the first study to evaluate DNA methylation of ccfDNA in three thyroid diseases and the healthy individuals. Our results suggested that thyroiditis patients have distinct methylation pattern compared with patients with nodules and the controls. Although we observed hypomethylation in nodules patients compared to the control group, our results suggested performing similar studies with the large number of patients and the control groups. Gene-specific approaches (Pyrosequencing and Illumina sequencing for DNA methylation) should be followed for analysis using cell free DNA samples.

Scientific Responsibility Statement

The authors declare that they are responsible for the article's scientific content including study design, data collection, analysis and interpretation, writing, some of the main line, or all of the preparation and scientific review of the contents and approval of the final version of the article.

Animal and human rights statement

All procedures performed in this study were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. No animal or human studies were carried out by the authors for this article.

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Conflict of interest

None of the authors received any type of financial support that could be considered potential conflict of interest regarding the manuscript or its submission.

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