Serum 8-OHdG and HIF-1α levels: do they affect the development of malignancy in patients with hypoactive thyroid nodules?

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Aim of the study: This study aimed to evaluate 8-OHdG and hypoxia-inducible factor 1 (HIF-1α) levels in patients with hypoactive thyroid nodules (toxic multi-nodular goiter, Graves' disease, and Hashimoto's thyroiditis), as these parameters may be related to oxidative stress and the pathogenesis of cancer.
Material and methods: The study included patients diagnosed with Graves' disease ($n = 20$), toxic multinodular goiter ($n = 20$), and Hashimoto thyroiditis ($n = 20$), and 20 healthy controls. HIF-1α levels were measured in blood samples and 8-OHdG levels were measured in urine – both via ELISA.
Results: HIF-1α and 8-OHdG levels were significantly higher in the patient groups than in the control group ($p < 0.05$). In the Hashimoto's thyroiditis patients a correlation was observed between 8-OHdG and thyroglobulin antibodies ($p = 0.03$). A significant relation was found between 8-OHdG and HIF-1α in the patient group ($p < 0.01$). Carcinoma was detected in 7 of 43 female patients, but not in any of the male patients. No difference was observed in 8-OHdG or HIF-1α levels between the patients with and without papillary carcinoma ($p > 0.05$). There was no significant difference in 8-OHdG or HIF-1α levels between the patients with biopsy results that were benign, malignant, and non-diagnostic ($p > 0.05$).
Conclusions: Serum HIF-1α and urine 8-OHdG levels were significantly higher in the patients with thyroid diseases; however, a relationship with cancer was not observed.

Key words: hypoactive thyroid nodule, 8-OHdG, HIF-1α.

Introduction

Carbonic anhydrases, having potential importance for the life of the cell in an acidic environment, occurring with glycolysis and hypoxia, play a role in ensuring pH regulation in normal tissue. In the case of hypoxia, many genes including *KAIX* and *KAXII* are over-expressed through *HIF-1α*. Carbonic anhydrase IX regulates hydrogen flow and it is argued that it mediates one of the hypoxic adaptation mechanisms when hypoxia is encountered [1]. Because it is over-synthesized in tumors with poor prognosis, it is seen as a potential target in cancer treatment. *KAIX* plays a role in cell proliferation and transformation [1]. *KAIX* is produced in stomach, biliary tractus, pancreas, small intestines, and seminal ductal epithelium. The hypoxia response element (*HRE*) being the binding site for *HIF-1α* occurs in the promoter region of *KAIX*. In several studies, it has been shown that hypoxic induction is not present in cell lines having a disorder in the *HIF-1α* pathway [1]. Mutations present in the nucleus of *HRE* lead to lack of the response to hypoxia. Thus, the importance of the *HIF* pathway in terms of the response was detected [2].

Solid lesions are exposed to conditions such as reduction of oxygen diffusion, and food intake during tumor formation, which leads to new vein formation and adaptation to hypoxic conditions [3, 4]. Increased *HIF-1α* levels also lead to the expression of genes associated with glycolysis, erythropoiesis, and angiogenesis [5]. *Met* protein is a receptor having high affinity for *HGF*. Two regions where *HIF-1* can bind to the promoter region of *Met* have been determined and this suggested that it is responsible for increased gene transcription in some types of cancer. Obtained results showed that *Met* plays a key role in the pathogenesis of thyroid papillary carcinoma [6, 7].

The expression of hypoxia-inducible factor 1 in thyroid carcinomas was investigated in a recent study. After evaluation of the regulation of *HIF-1α* and target gene expression in primary thyroid carcinomas and thyroid carcinoma cell lines (*BcPAP, WRO, FTC-133* and *8505c*), it was found that *HIF-1α* was not detectable in normal tissue but was expressed in thyroid carcinomas. Beside these, dedifferentiated anaplastic tumors (anaplastic thyroid carcinomas – ATCs) exhibited high levels of nuclear *HIF-1α* staining. *In vitro* studies revealed a functionally active *HIF-1α* pathway in thyroid cells with transcriptional activation. *HIF-1* is functionally expressed in thyroid carcinomas and is regulated not only by hypoxia but also via growth factor signaling pathways and, in particular, the *PI3K* pathway. Given the strong association of *HIF-1α* with an aggressive disease phenotype and therapeutic resistance, it was predicted that this pathway could be an attractive target for improved therapy in thyroid carcinomas [8].

Oxidative stress affects the induction of DNA damage and DNA is the most biologically important target of oxidative attack by reactive species including free radicals [9]. Continuous oxidative DNA damage and cellular injury are caused by alteration of membrane permeability due to lipid peroxidation by reactive oxygen species. Reactive oxygen species are significant contributors to the age-related development of tumors and chemical carcinogenesis [10]. Free radicals may directly affect DNA and produce structural changes. Many free radicals are attacked by hydroxyl groups. Thus, DNA has a relation with genotoxic carcinogens [11]. 8-OHdG is a marker for oxidative DNA damage. It may be associated with lesions [12].

There are studies on the relations among oxidative DNA damage, cancer and inflammatory diseases. DNA being a stable molecule may suffer from spontaneous chemical oxidative damage. Major factors causing oxidative damage in DNA are ionized radiation, high oxygen concentration, chemicals, xanthine oxidase, and tumor necrosis factor α (TNF-α) [13]. The use of 8-OHdG levels in the urine and leukocyte DNA as an indicator of oxidative DNA damage is helpful in the evaluation of situations carrying carcinogenic risk [14]. Similarly, increased DNA damage indicators were determined in the lymphocytes obtained from individuals with autoimmune disease. 8-OHdG levels were found to be higher in autoimmune thyroid disorders, such as Graves' disease and Hashimoto's thyroiditis [15, 16].

Iodine deficiencies, nutrition with goitrogenic foods, or autoimmune events lead to diffuse thyroid hyperplasia. Some of these spontaneous mutations simulate growth by ensuring activation of the *cAMP* cascade. As a result, the gene expression of some growth factors (insulin-like growth factor 1 – IGF-1, transforming growth factor β1 – TGF-β1, epidermal growth factor – EGF) increase. The cells form small clones by splitting. Small clones start proliferation by self simulation. Thus, small focuses turn into nodule formation. Adenomas secreting TSH can reveal nodular formation in the thyroid tissue through similar pathways. There are the same mechanisms in some diseases such as Graves' disease and acromegaly [17].

We aimed to evaluate the markers of oxidative stress and cancer in patients with toxic multinodular goiter, Graves' disease, and Hashimoto's thyroiditis. Furthermore, we investigated the correlation between serum markers and other data (clinical status, the presence of autoantibodies, cytological and histopathological results).

Material and methods

The study included 60 patients (43 females and 17 males; mean age: 50.93 ±13.68 years) with hypoactive thyroid nodules (single or multiple) who were diagnosed with Graves' disease (*n* = 20, GD group), multinodular toxic goiter (*n* = 20, TG group), and Hashimoto's thyroiditis (*n* = 20, HT group), and 20 healthy controls (control group). The study protocol was approved by the Ege University, School of Medicine Ethics Committee. Patients were screened for other possible autoimmune diseases that may be associated with thyroid autoimmunity. The patients and controls were chosen from among non-smokers. All of the participants provided written informed consent to participate. Pregnant women and individuals in whom fine needle aspiration biopsy (FNAB) was contraindicated due to such risk factors as bleeding diathesis and anti-aging drug use were excluded from the study.

Thyroid function and thyroid autoantibodies in the patients were investigated, and parenchyma structure of the thyroid gland, the number, size, echogenicity, and activity of their nodules were determined via ultrasonographic and scintigraphic methods. Free T3, free T4, and TSH levels were measured using the electrochemiluminescence method: the reported reference ranges were as follows: free T3: 3.1–6.8 pmol l^{-1} (2.0–4.4 pg ml^{-1}); free T4: 12–22 pmol l^{-1} (0.93–1.7 ng dl^{-1}); TSH: 0.274.2 μIUml^{-1} (Roche Diagnostics, GmbH D-68298 Mannheim, Germany). The reference ranges for Anti-Tg Ab and anti-TOB Ab were 40 IU ml^{-1} and 35 IU ml^{-1}, respectively, and these parameters were measured via Immulite Anti-Tg Ab and Anti-TPO Ab assay (Immulite 2000 Systems, Siemens, 2009). TSH receptor Ab levels were measured manually using the radioimmunoassay method; values < 1 U l^{-1} were accepted as negative, values of 1.1–1.5 U l^{-1} were accepted as positive at limits, and values > 1.5 U l^{-1} were accepted as positive (Immunotech, Beckman Coulter Company, France, 2008).

The patients' thyroid glands were imaged ultrasonographically using a high-resolution broad-band linear probe (13–5 MHz frequency) and the nodular formations were measured across their long axes (Siemens, Antares, Germany, 2008). For scintigraphic evaluation of the thyroid gland a static image of 400 s 500 kcount^{-1} was obtained by focusing on the thyroid lodge using a pinhole collimator for 20 min after 5 mCi IV injection using pertechnetate Tc 99m (Elscint NM, Apex model SPX4, Israel, 2008). All patients were brought to the euthyroid state and FNAB of hypoactive thyroid nodules in the patients with Graves' disease and toxic multinodular goiter, accompanied by ultrasonography, was performed according to the ATA guide (2006), and the biopsy specimens were cytologically examined.

Fine needle biopsy was not done from hyperactive nodules of toxic multinodular goiter. Nodules were considered hypoactive because the patients had Graves' disease. As hypoactive nodules are associated with a high risk of malignancy (5–10%) and hyperactive nodules a much lower risk of malignancy (< 1%), hypoactive nodule samples were selected for both groups. All nodules in the Graves' disease patients were hypoactive. Taken biopsy was eligible from hypoactive nodules in the toxic multinodular goiter group.

Cytopathological interpretation of FNAB results was evaluated as malignant/benign/non-diagnostic. Patients with suspicious and malignant cytology results underwent surgery (total/near total thyroidectomy). Microcarcinoma was defined as ≤ 10 mm in any direction. Blood and urine samples (20 cc) were collected to measure indicators of DNA damage (*8-OHdG*).

In vitro quantitative determination of human HIF-α1 concentrations

Human HIF-1 ELISA Kit was applied to serum samples taken from the patient and the control groups. This test was

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Table 1. Demographic and clinical features of groups

Group	Mean age	Gender	Number of nodules (%)		Nodule size (%)		Nodule localization (%)		
	mean ± SD	(F/M ratio)	single	two or more	20 mm↓	20 mm↑	right	left	bilateral
A (n = 20)	45.1 ±12.34	12/8	40	60	70	30	25	15	60
B (n = 20)	57.9 ±13.57	14/6	–	100	25	75	–	–	100
C (n = 20)	49.70 ±12.49	17/3	45	55	60	40	20	25	55
A + B + C (n = 60)	50.93 ±13.68	43/17	28.30	71.70	51.70	48.30	15	13.30	71.70
control (n = 20)	48.05 ±11.91	15/5							

applied with the sandwich-based ELISA method. HIF-1α protein levels were quantified using the Human HIF-1α ELISA kit (Cusabio Biotech, Chine). The procedure was as described in the protocol provided by the manufacturer. Absorbance was measured at 450 nm using a Microplate Reader Multiscan FC (Thermo-Scientific). The HIF-1α protein concentration in each well was calculated using the equation of the HIF-1α standard.

Urinary 8-OHdG measurement via ELISA

Into each well of an 8-OHdG-coated microtiter plate, 50 μl of fresh urine sample and 50 μl of reconstituted primary antibody were placed, followed by incubation at 37°C for 1 h for ELISA (NWLSS™ Urine 8-OHdG ELISA Kit, Northwest Life Science Specialties, LLC). Secondary antibody was added to the plate, followed by incubation at 37°C for 1 h, the unbound enzyme-labeled sec ondary antibody was removed, and then the antibodies that were bound to the plate were identified using a substrate that contained 3,3',5,5'-tetramethylbenzidine. Absorbance was measured using a computer-controlled spectrophotometric plate reader (Multiscan FC-Thermo-Scientific) at a wavelength of 450 nm. The concentration of 8-OHdG in the urine samples was interpolated from a standard curve drawn with the assistance of logarithmic transformation. The detection range of the ELISA assay was 0.5–200 ng ml^{-1}. The urinary 8-OHdG level in each participant was normalized by the creatinine level in urine and was expressed as ng mg^{-1} of creatinine.

Statistical analysis

After determining whether numeric values were normally distributed, Student's t-test was used to compare 2 groups and one-way ANOVA was used to compare ≥ 3 groups with normally distributed variables. If ANOVA test results were significant, Dunnett's test was then applied. The Mann-Whitney and Kruskal-Wallis tests were used for variables that were not normally distributed; however, χ^2 analysis was used for categorical variables by forming cross tables.

Results
Markers related to functional thyroid disorders

Mean ages and distributions according to the gender of the patient groups are given in Table 1. The distribution of the groups in terms of age and gender was similar. TSH receptor antibody levels of the patients with Graves' disease were significantly higher than other groups ($p < 0.05$). The autoan-

tibodies of patients were examined. They were detected as significantly lower in patients with toxic multinodular goiter. There was no difference in terms of Anti-Tg and Anti-TPO between cases with Hashimoto's thyroiditis and Graves' disease.

Patient groups (Group A/Group B/Group C) were evaluated in terms of some parameters (TSH receptor antibodies, thyroglobulin antibody, thyroperoxidase antibodies, HIF-1α, 8-OHdG).

A correlation between thyroglobulin in the Hashimoto thyroiditis group (Group C) was observed, supporting the existence of autoimmunity with *8-OHdG*, an indicator of DNA damage ($p = 0.03$).

Markers related to pathology

The evaluation of HIF-1α and 8-OHdG parameters between groups is given in Table 2. 8-OHdG and HIF-1α parameters were found to be significantly higher in the patient group ($p < 0.05$).

There was no difference in terms of 8-OHdG and HIF-1α among the patient groups (Figs. 1, 2). A correlation was not detected in terms of HIF-1α and 8-OHdG parameters among groups (Group A + C / B). In the patient group, there was a correlation between HIF-1α and 8-OHdG parameters ($p < 0.01$).

Biopsy results were positive for cancer in 7 of the 60 patients (11.6%) and were confirmed via histopathological examination, as follows: papillary carcinoma ($n = 5$ [8.3%]), papillary microcarcinoma ($n = 1$ [1.6%]), and papillary follicular variant ($n = 1$ [1.6%]). The distribution of the cytological findings in FNAB specimens is given in Table 3a.

Cytological distribution of FNAB specimens following repetition of non-diagnostics is shown in Table 3b. Pre-operative cytology and post-operative histology results are shown in Table 4.

All patients who were diagnosed with carcinoma were female (16.3%). Carcinoma was diagnosed in 5% of the

Table 2. Comparison of the patient and the control group in terms of HIF-α and 8-OHdG parameters

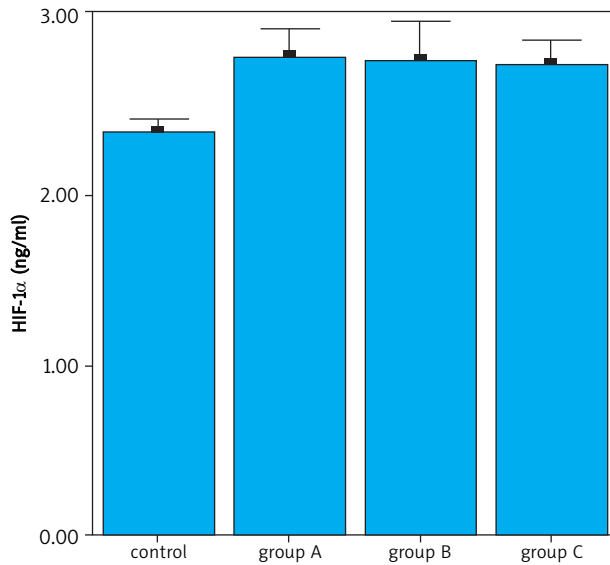|	Group A + B + C	Control	*p* value
8-OHdG (ng/ml) (mean ± SD)	22.26 ±12.41	5.11 ±2.44	< 0.05
HIF-1α (pmol/μl) (mean ± SD)	1.11 ±0.82	0.74 ±0.16	< 0.05
n – number of patients	60	20	–

Fig. 1. Examination of HIF-1α cytokine between the patient and the control groups. A difference is determined between the patients and the control group in terms of HIF-1α levels ($p < 0.05$). No difference was found between the patient groups in terms of HIF-1α levels ($p > 0.05$)
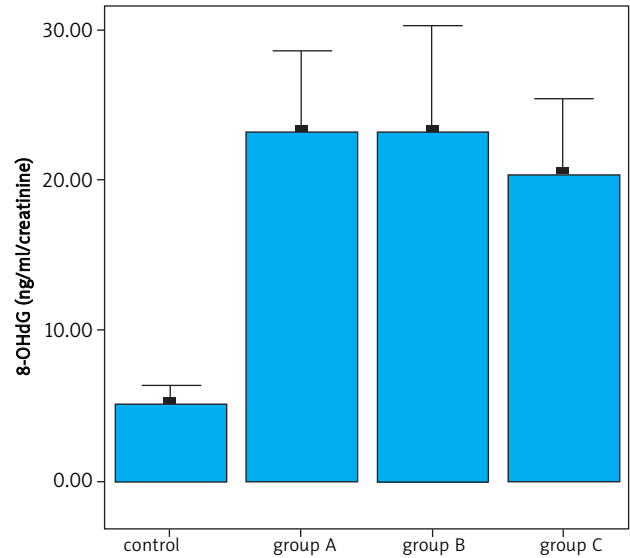

Fig. 2. Examination of DNA damage indicator between the patient and the control groups. A difference is determined in terms of 8-OHdG levels between the patients and the control group ($p < 0.05$). No difference is determined in terms of 8-OHdG levels between the patient groups ($p > 0.05$)

Table 3a. Distribution ratios of the cytological data obtained according to fine needle aspiration biopsy carried out from hypoactive thyroid nodule/nodules between the groups

Biopsy	Group A		Group B		Group C	
	%	n	%	n	%	n
benign	95	19	70	14	65	13
malign	5	1	10	2	10	2
non-diagnostic	–		20	4	25	5

Table 3b. Cytological distribution of FNAs after repetition of non-diagnostics

Biopsy	Group A		Group B		Group C	
	%	n	%	n	%	n
benign	95	19	90	18	80	16
malign	5	1	10	2	20	4

Table 4. Pre-operative cytology and post-operative histology results

	Pre-operative cytology	Post-operative malign	Post-operative benign
indeterminate	$n = 11$	2/11	9/11
malign	$n = 5$	5/5	–

patients in the GD group ($n = 1$), 10% of the patients in the TG group ($n = 2$), and 20% of the patients in the HT group ($n = 4$); the difference between the 3 patient groups was not significant. The Graves' disease and Hashimoto's thyroiditis cases were thought to have autoimmune origin, as compared to the toxic multinodular goiter cases in terms of cancer frequency. The combined carcinoma frequency rate in the GD and HT groups was 12.5%, versus 10% in the TG group, and the difference was not significant. Mean age of the patients diagnosed with carcinoma was 44.28 ±15.05 years, versus 51.81 ±13.40 years in those without carcinoma; the difference was not significant.

We did not detect a significant relation among nodule dimension, nodule number, HIF-1α, and 8-OHdG. Additionally, there was no correlation between cytological findings in FNAB specimens (malignant/benign/non-diagnostic) and the number of nodules or nodule dimension. There was no relationship between patient age and the number or dimension of nodules, or between patient age and HIF-1α or 8-OHdG levels.

A statistically significant difference was not observed in HIF-1α or 8-OHdG levels between the patients with and without papillary carcinoma, nor was there a difference in HIF-1α or 8-OHdG levels between the patient groups with biopsy results that were benign, malignant, or non-diagnostic.

Discussion

Most thyroid nodules are histologically diagnosed as thyroid adenoma and differentiated from thyroid cancer. These lesions differ from autonomous functional thyroid nodules – including TSHR mutations – because they are associated with many disorders of genetic origin [18]. Functional disorders associated with cold thyroid nodules are caused by failed iodine transport or organification of iodine. Reduced expression of the Na/I symporter (NIS) and consequent insufficient iodine transport are observed in thyroid cancers, as well as in benign cold thyroid nodules [19]. The present study aimed to investigate the relationship between the indicators of inflammation, DNA damage, and cancer pathogenesis, and

the disease and the nodule in patients with thyroid diseases of autoimmune origin having hypoactive thyroid nodule and with toxic multinodular goiter.

In the present study the toxic multinodular goiter group had the highest mean age, whereas the Graves' disease group had the lowest, as previously reported. There were more females than males in each of the 3 patient groups. Nodular disease was observed 5–15 times more in the female patients; therefore, genetic predisposition and the effect of steroid hormones are considered causal factors. The growth stimulation effect of estrogen was reported in thyroid cancer cell lines in rats *in vitro* [20, 21].

In previous studies, it was found that the prevalence of thyroid autoimmunity was correlated with the increase of age and thyroid nodularity [22, 23]. However, a correlation was not found among thyroid autoimmunity, age and thyroid nodularity in our study.

Some studies have reported a relationship between oxidative DNA damage, and cancer and inflammatory diseases. An increase in the formation of oxygen radicals and a reduction in antioxidant enzyme levels, and/or the existence of a defect in DNA repair mechanisms lead to an increase in oxidative DNA damage [24, 25]. Human studies showed that DNA damage is an important mutagenic and carcinogenic factor [26]. It is thought that autoimmune thyroid diseases are associated with oxidative stress and 8-OHdG levels in mononuclear cell cultures obtained from patients with Graves' disease and Hashimoto's thyroiditis. In the present study a correlation between the 8-OHdG level and thyroglobulin antibodies was observed, which supports the existence of autoimmunity in patients with Hashimoto's thyroiditis ($p = 0.03$).

The level of 8-OHdG in patients with Graves' disease and Hashimoto's thyroiditis, which were untreated, was significantly higher than in healthy controls [11]. Thyroid hyperplasia increased the proliferation together with possible DNA damage due to H_2O_2 action. Similarly, in the present study 8-OHdG, an indicator of DNA damage, was significantly higher in the patients with Graves' disease and Hashimoto's thyroiditis than in the controls ($p < 0.05$).

Recent studies underline the importance of pH in the occurrence of cell death under hypoxic conditions [1]. The drop in oxygen pressure in the environment activates heterodimeric transcriptional regulators such as HIF-1. HIF-1 contributes to angiogenesis by directly increasing VEGF (vascular endothelial growth factor) formation [32]. It is argued with the data obtained from the study carried out by Pennacchietti and colleagues that *Met* plays a key role in the pathogenesis of thyroid papillary carcinoma. Two sections to which *HIF-1* can be bound were determined on the *Met* promoter region, and this reminded that it is responsible for increased gene transcription in some cancer types [33]. In the study carried out by Stefania Scarpino and colleagues, it is underlined that *Met* protein phosphorylation and synthesis increase in tumor tissue, and there is a relation between *HIF-1* and *Met*, and also the amount of both of them is high in tumor tissue, and this relation may be responsible for the invasion [6]. In our study, *HIF-1α* is an indicator that is thought to play a role in cancer pathogenesis in all patients receiving the diagnosis of Graves' disease, toxic multinodular goiter, and Hashimoto

thyroid. It was found to be significantly higher compared to the control group ($p < 0.05$).

In our study, there was no significant relation among age, nodule dimension, the number of nodules, and *HIF-1α* and *8-OHdG* parameters. Also, there was no significant difference in terms of *HIF-1α* and *8-OHdG* between the cases with and without papillary carcinoma. Similarly, no significant difference was detected among the patients whose fine-needle aspiration biopsy result is benign/malign/non-diagnostic/indeterminate. In the literature, no study was found comparing fine needle aspiration cytology, histology, and markers (*HIF-1α*, *8-OHdG*).

In a study carried out by Fiore and colleagues, a correlation between thyroid cancer and its autoimmunity was determined. In another study, it was suggested that thyroid autoimmunity may provide resistance to thyroid carcinoma [34, 35].

In some studies, it was found that the cancer development in hypoactive nodules of patients with toxic multinodular goiter was 7%, 21%, 9%, respectively [31–33]. In other studies, it was reported that the cancer frequency in patients with non-toxic multinodular goiter was 6.2%, 9.7%, 10.58%, respectively [33–35].

There are publications in the literature about hypoactive nodules – autoimmune thyroid diseases–cancer relations [36, 37]. In our study, there was no difference in terms of cancer formation between diseases of autoimmune origin (Graves and Hashimoto) and other patient group (toxic multinodular goiter). Similarly, there were reported conflicting results in the literature associated with the frequency of thyroid cancer in patients with toxic and nontoxic multinodular goiter [33, 38–40].

In the study carried out by Baier and colleagues, it was observed that the cases with malign cytology were younger (≤ 45 years). And the patients with non-diagnostic cytology were over 75 years old [39]. However, we did not detect a correlation among age, benign and malign cytological diagnosis in our study.

Kirtee Reparia and colleagues reported that the cancer rate was 19% in cases with nodule dimension smaller than 2 cm. And this rate in those with nodules bigger than 2 cm was 47% [40]. In our study, we did not find a relation between cancer and nodule size, because there was a limited number of cancer cases.

The results of thyroid fine-needle aspiration biopsy can change depending on many factors (ultrasound device, endocrinologist, pathologist etc.). In our study, a high rate of malignancy (11.6%) was encountered. There are conflicting publications related to cytological diagnosis (benign/malign/non-diagnostic/indeterminate) in the literature [38, 39, 41, 42].

Ultimately, serum HIF-1α and 8-OHdG levels in the determination of carcinogenic potential in hypoactive nodules could be used as an indicator for early detection of cancer. Our results provide insights into the potential role of 8-OHdG and HIF-1α levels in patients with thyroid cancer. Also analyzing 8-OHdG from urine is an advantage due to the fact that it is a non-invasive method to detect oxidative stress. While the elevation of serum HIF-1α and 8-OHdG levels in thyroid disorders was found to be at a significant level, its relation with cancer was not significant. This was connect-

ed with the limited number of cases of cancer. It is difficult to signify as "malignant" nodules by evaluating these markers in the light of current literature and our data in clinical practice. However, considering the role of solid tumors other than thyroid, these markers are thought to play a role in thyroid cancer. In the next step, we have planned a study involving further testing with larger study groups.

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