






## Increased DNA Damage and Insufficient DNA Repair in Euthyroid Patients With Nodular Goiter Analyzed by $\gamma$ -H2AX and 53BP1 Foci Assay

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### ABSTRACT

**Objective:** Thyroid nodules are a common occurrence in adults. Although the majority of thyroid nodules are benign, a small percentage are cancerous. The combined phosphorylated histone H2AX ( $\gamma$ -H2AX) and p53-binding protein 1 (53BP1) assay was utilized to detect deoxyribonucleic acid (DNA) damage and DNA repair as biomarkers of the cellular stress response. Using the combined  $\gamma$ -H2AX and 53BP1 assay, we evaluated DNA damage, DNA repair capacity, and malignancy risk in peripheral blood mononuclear cells (PBMCs) of euthyroid individuals with nodular goiter.

**Materials and Methods:** Peripheral blood samples were collected from 33 euthyroid patients with newly diagnosed nodular goiter and 30 healthy control participants. A fully automatic image analysis system was used for analyzing DNA damage ( $\gamma$ -H2AX), including DNA double-strand breaks (DSBs), and DNA repair (53BP1).

**Results:** Euthyroid patients with nodular goiter exhibited a higher mean number of  $\gamma$ -H2AX foci per cell and a higher percentage of apoptotic cells compared to the control subjects ( $p=0.022$  and  $p=0.005$ , respectively).

**Conclusion:** This study found considerably higher DNA damage in euthyroid patients with nodular goiter than in control individuals. The increase in DNA damage occurs in the early stages of carcinogenesis. These patients were expected to exhibit compromised DNA repair along with enhanced DNA damage, increasing the risk of carcinogenesis. However, euthyroid patients with nodular goiter might be at a high risk of thyroid malignancy due to the high level of DNA damage. A long-term follow-up of these patients would provide better evidence of the relationship between DNA damage and the malignancy risk of thyroid.

**Keywords:** Cancer, DNA damage and repair,  $\gamma$ -H2AX, 53BP1, nodular goiter.

## INTRODUCTION

The prevalence of thyroid diseases is gradually increasing globally.<sup>1</sup> Thyroid nodules are the most common disease of the thyroid gland.<sup>2</sup> Most thyroid nodules are benign, but there is a 7–15% risk of cancer in all types of nodules, whether they are single nodules or multiple nodules within a thyroid gland.<sup>2–4</sup> The relationship between thyroid nodules and cancer development is still unclear and under investigation.

In human cells, various types of deoxyribonucleic acid (DNA) damage, such as base modifications, inter- or intra-strand cross-links, single-strand breaks, and double-strand breaks (DSBs), can be caused by both external and internal metabolic processes.<sup>5,6</sup> DSBs are one of the most dangerous types of DNA damage, although they are uncommon. If misrepaired or unrepaired, lesions/damage can lead to genomic instability or cell death, respectively, and subsequently to cancer. To maintain genome integrity, cells have evolved several DSB repair mechanisms, such as homologous recombination (HR) or non-homologous end joining (NHEJ).<sup>7,8</sup> H2AX, a member of the histone H2A subfamily from the octameric histone core proteins in nucleosomes, is phosphorylated on the serine 139 residue (to form  $\gamma$ -H2AX) in response to DNA DSBs.<sup>6,9</sup>  $\gamma$ -H2AX is one of the various DNA DSB markers and it indicates the presence of DSBs and promotes the recruitment of DNA damage response and repair factors to the sites of DSBs.<sup>7,10,11</sup> One of these factors, p53-binding protein 1 (53BP1), has important regulatory functions in the DNA repair pathway and it co-localizes with  $\gamma$ -H2AX at the DNA lesions.<sup>8</sup> The foci of  $\gamma$ -H2AX and 53BP1 on nuclei can be visualized by immunostaining using digital fluorescence microscopy. Automated analysis of DNA damage and repair has been performed in several previous studies.<sup>9,10,12,13</sup> The combined  $\gamma$ -H2AX and 53BP1 foci assay is a useful approach used for screening genotoxic substances, assessing radiotherapy and multidrug resistance in cancer therapy, and determining the effects of *in vitro* exposure to ionizing radiation.<sup>5,13–16</sup>

Several studies have been conducted on the use of  $\gamma$ -H2AX and 53BP1 levels in detecting thyroid diseases, including nodular thyroid and some thyroid carcinomas.<sup>16–18</sup> It has been shown that the numbers of  $\gamma$ -H2AX and 53BP1 foci significantly increase initially soon after radioiodine therapy before progressively decreasing in the blood of patients.<sup>16,17</sup> Higher  $\gamma$ -H2AX expression levels have been found in malignant tissues than in benign (nodular goiter and normal adjacent) tissues, but no significant differences in  $\gamma$ -H2AX expression levels have been recorded between nodular goiter and normal adjacent tissues.<sup>18</sup> However, to date, there are no data on combined  $\gamma$ -H2AX and 53BP1 foci analysis in

peripheral blood samples collected from euthyroid patients with nodular goiter.

Therefore, the purpose of this study was to assess DNA damage (DSBs), DNA repair capacity, and malignancy risk using combined  $\gamma$ -H2AX and 53BP1 foci analysis in isolated peripheral blood mononuclear cells (PBMCs) of euthyroid patients with nodular goiter.

## MATERIALS AND METHODS

### Patient and Control Subjects

This study included 33 euthyroid patients with newly diagnosed nodular goiter (26 women and seven men), with a mean age of  $46.15 \pm 12.85$  years (mean  $\pm$  standard deviation, range 21–65 years), admitted to the Division of Endocrinology and Metabolism at Erciyes University's Faculty of Medicine between April 2016 and May 2017. The study also included 30 healthy controls (23 women and seven men), with a mean age of  $40.10 \pm 10.49$  years (range 22–58 years) and with matching socio-economic status (as socio-economic status may affect DNA damage). At the beginning of the study, the subjects (patient and control groups) were matched for age and gender. However, we had to exclude some participants who met exclusion criteria, especially patients who did not have enough cells for evaluation. Thyroid ultrasound examination was performed to detect nodules, but the ultrasound results for thyroid nodules were accessible for only 15 euthyroid patients with nodular goiter. All patients with nodular goiter were euthyroid and exhibited bilateral multiple nodules. Biopsy specimens, obtained via fine needle aspiration from these patients, were examined cytologically. The cervical lymph nodes were not enlarged.

A comprehensive history was taken from both the patients and control subjects, covering current health status, lifestyle, past medical history, drug use, and tobacco and alcohol consumption habits. Exclusion criteria for both patient and control subjects included: a history of environmental or occupational exposure to well-known genotoxic chemicals or radiation, medical exposure to X-ray/computed tomography within the last three months, addiction to tobacco or alcohol (or both), and the presence of concurrent conditions such as diabetes mellitus, obesity, hypertension, heart disease, and cancer. Levels of free triiodothyronine (FT3), free thyroxine (FT4), and thyroid-stimulating hormone (TSH) were measured in blood samples from euthyroid patients with nodular goiter using the electrochemiluminescence immunoassay (ECLIA) method (Cobas, Roche Diagnostics, Mannheim, Germany). The heights and weights of all participants were measured to calculate body mass index (BMI).

### Blood Sampling and $\gamma$ -H2AX and 53BP1 Immunofluorescence Staining

After obtaining informed consent, 4 mL of peripheral whole blood was collected from each euthyroid patient with nodular goiter and control subject between 9 and 10 a.m. Blood samples were drawn into ethylenediaminetetraacetic acid tripotassium salt dihydrate (K3 EDTA) tubes (Vacutest Kima, Arzergrande, PD, Italy) for  $\gamma$ -H2AX and 53BP1 foci analysis. All blood samples were transported to the laboratory and processed as soon as possible, typically within two hours after collection, and were kept at 4°C, protected from light during transport.

The blood samples were analyzed for combined  $\gamma$ -H2AX and 53BP1 foci using a  $\gamma$ -H2AX and 53BP1 immunofluorescence staining kit (The AKLIDES® Nuk Human Lymphocyte Complete Combi 4268, Medipan GMBH, Dahlewitz/Berlin, Germany). This kit is employed for performing an indirect immunofluorescence assay to quantify  $\gamma$ -H2AX and 53BP1 foci in lymphocytes derived from whole blood samples.<sup>9,12,13,16,19</sup> Samples were processed in accordance with the manufacturer's instructions, utilizing kit components. Briefly, blood samples were diluted 1:1 (v/v) with phosphate-buffered saline (PBS) and maintained at room temperature (20°C–25°C). Before centrifugation at 1,200 × g for 20 minutes at room temperature (20°C–25°C), separation medium was added to the diluted blood samples. The white band of the PBMCs fraction was collected and washed twice with PBS, then resuspended in PBS to achieve a concentration of 1.0 × 10<sup>6</sup> cells/ml. A 50 µL volume of the cell suspension was dispensed into each well of a 6-well microscopic slide. The slides were allowed to stand for 10 minutes at room temperature (20°C–25°C). Subsequently, 50 µL of a fixative (2% paraformaldehyde in PBS) was added to fix the cells onto the microscopic slides, followed by a 15-minute incubation at room temperature (20°C–25°C). After fixation, the slides were washed three times with PBS. The cells were then permeabilized with a permeabilization buffer containing 0.2% Triton™ X-100 and 1% bovine serum albumin (BSA). Following this, the slides were washed three times with 1% BSA/PBS. Cells affixed to the slides were incubated with the primary antibodies (anti-phospho-histone H2AX mouse monoclonal IgG and anti-53BP1 rabbit polyclonal IgG), diluted 1:20 in 1% BSA/PBS, for one hour at room temperature (20°C–25°C). After another washing step (three times with BSA/PBS), the slides were incubated with secondary antibodies (Alexa Fluor 488 goat anti-mouse IgG and Alexa Fluor 647 goat anti-rabbit IgG), diluted 1:50 in 1% BSA/PBS, for one hour at room temperature (20°C–25°C). Following a final wash with PBS, a small drop of mounting medium containing anti-fading reagent and 4',6-diamidino-2-phenylindole (DAPI) was applied to each well and covered with a coverslip. Each subject's sample was processed in duplicate across two microscopic wells.

### Automated Analysis of Combined $\gamma$ -H2AX and 53BP1 Foci

The analysis of combined  $\gamma$ -H2AX and 53BP1 foci was conducted using the fully automated AKLIDES® reading system (Medipan GmbH, Berlin, Germany), which facilitates an automated evaluation of immunofluorescence images and data processing.

The system's software module automatically focuses on and identifies cells within the blue DAPI channel, sensitively detects the  $\gamma$ -H2AX fluorescence signal in the green Fluorescein Isothiocyanate (FITC) channel, and the 53BP1 signal in the red Allophycocyanin (APC) channel, subsequently overlaying the signals from the DAPI, FITC, and APC channels. Image acquisition and analysis proceeded as described previously.<sup>9,12,19,20</sup> The precise focal plane of the nuclei in the DAPI channel was determined using autofocusing, based on Haralick's image texture analysis.<sup>9,20</sup> To ensure that we selected the correct cells between apoptotic cells and cells with a high quantity of induced DNA DSBs, only cells with convex nuclei and diameters ranging from 2 to 15 µm were analyzed. Cells with a  $\gamma$ -H2AX signal constituting ≥70% of the DAPI signal were excluded from the analysis and scored as apoptotic. Each focus was defined by criteria including size and intensity: a minimum diameter of 0.2 µm, a maximum diameter of 1.2 µm, and a minimum intensity of 70 gray values on an 8-bit grayscale.<sup>19,20</sup> For each well, a minimum of 100 cells per slide were evaluated using the following parameters: number of analyzed cells, number of cells with  $\gamma$ -H2AX foci, number of cells with 53BP1 foci, mean number of  $\gamma$ -H2AX foci per cell, mean number of 53BP1 foci per cell, percentage of  $\gamma$ -H2AX foci-positive cells, percentage of 53BP1 foci-positive cells, and percentage of apoptotic cells.

### Statistical Analysis

Statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS) software (version 22, SPSS Inc., Armonk, NY, USA). Before the study started, the G\*Power program was used to calculate the sample size needed for each group to achieve 80% test power. Data distributions for normality were assessed with the Shapiro-Wilk test, histograms, and Q-Q plots. The parameters for  $\gamma$ -H2AX and 53BP1 foci (number of  $\gamma$ -H2AX and 53BP1 foci per cell, percentages of  $\gamma$ -H2AX and 53BP1 foci-positive cells, and percentage of apoptotic cells) were compared between euthyroid patients with nodular goiter and control individuals using the Mann-Whitney U test to determine whether there were any significant differences. For comparing the age and BMI values between euthyroid patients with nodular goiter and controls, an independent t-test was used. The strength of the correlation between values of  $\gamma$ -H2AX and 53BP1, age, BMI, thyroid hormones (FT3, FT4, and TSH), and thyroid nodule

**Table 1.** Basic characteristics of euthyroid patients with nodular goiter (mean±SD)

Variables	Euthyroid patients with nodular goiter (n=33)	Control subjects (n=30)
Females (n)	26	23
Males (n)	7	7
Age (years)	46.15±12.85*	40.10±10.49
BMI (kg/m <sup>2</sup> )	26.76±3.98*	25.99±4.78
FT3 (normal range 2.0–4.4 pg/mL)	3.46±0.86	–
FT4 (normal range 0.93–1.97 ng/dL)	1.26±0.21	–
TSH (normal range 0.27–4.20 µIU/mL)	1.49±1.09	–
Thyroid nodule size (mm)	18.63±9.37	–

\*: P>0.05 between the control subjects and euthyroid patients with nodular goiter. BMI: Body mass index; FT3: Free triiodothyronine; FT4: Free thyroxine; SD: Standard deviation; TSH: Thyroid-stimulating hormone.

**Table 2.** Median (minimum–maximum) data for γ-H2AX and 53BP1 foci per cell, percentages of γ-H2AX and 53BP1 foci-positive cells, and percentage of apoptotic cells in euthyroid patients with nodular goiter and control subjects

	Euthyroid patients with nodular goiter (n=33)	Control subjects (n=30)	p-value (Mann-Whitney U test)
Mean number of γ-H2AX foci per cell	0.20 (0.00–3.30)	0.08 (0.00–1.53)	<b>0.022</b>
Percentage of γ-H2AX foci-positive cells (%)	7.80 (0.00–70.00)	3.49 (0.00–36.84)	0.052
Mean number of 53BP1 foci per cell	9.22 (1.01–53.51)	5.98 (0.00–58.90)	0.989
Percentage of 53BP1 foci-positive cells (%)	18.49 (0.00–61.06)	11.14 (0.00–57.90)	0.165
Percentage of apoptotic cells (%)	10.26 (0.00–426.67)	2.17 (0.00–38.64)	<b>0.005</b>

53BP1: p53-binding protein 1.

size was assessed using Spearman’s rho correlation analysis in euthyroid patients with nodular goiter and controls. A p-value of <0.05 was considered statistically significant.

### Ethics Committee Approval

The study protocol received approval from the Institutional Ethics Committee of Erciyes University (approval No. 2015/214), and the study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). All subjects provided signed informed consent before the commencement of the study.

### RESULTS

The basic characteristics of euthyroid patients with nodular goiter and control subjects are presented in Table 1. The sizes of the thyroid nodules in euthyroid patients with nodular goiter ranged from 5 to 40 mm, with a mean size of 18.63±9.37 mm (mean±standard deviation). The fine-needle aspiration biopsy specimens from the patients were cytologically benign. No statistically significant difference (p>0.05) was observed in the BMI (kg/m<sup>2</sup>) values between euthyroid patients with nodular

goiter (26.76±3.98, mean±standard deviation) and control subjects (25.99±4.78). The FT3, FT4, and TSH values of the patients fell within the reference intervals (Table 1).

Data on the γ-H2AX and 53BP1 foci in nuclei of PBMCs from euthyroid patients with nodular goiter and control subjects were analyzed using the fully automated AKLIDES® reading system. Table 2 displays the statistical comparisons of the mean number of γ-H2AX and 53BP1 foci per cell, percentages of γ-H2AX and 53BP1 foci-positive cells, and the percentage of apoptotic cells in euthyroid patients with nodular goiter and control subjects. Euthyroid patients with nodular goiter exhibited a significantly higher mean number of γ-H2AX foci per cell and a higher percentage of apoptotic cells compared to control subjects (p=0.022 and p=0.005, respectively) (Table 2). Although the percentage of γ-H2AX foci-positive cells was also higher in euthyroid patients with nodular goiter than in control subjects, this difference was not statistically significant (p>0.05). However, a large inter-individual variability was observed in the percentage of γ-H2AX foci-positive cells, the number of p53BP1 foci per cell, and the percentage of p53BP1 foci-positive cells.

**Table 3.** Spearman’s rho correlation coefficient and significance values for age, BMI, FT3, FT4, TSH, and thyroid nodule size with mean numbers of  $\gamma$ -H2AX and 53BP1 foci per cell, percentage of  $\gamma$ -H2AX and 53BP1 foci-positive cells, and percentage of apoptotic cells in euthyroid patients with nodular goiter and control subjects

	Mean Number of $\gamma$ -H2AX foci per cell	Percentage of $\gamma$ -H2AX foci-positive cells (%)	Mean number of 53BP1 foci per cell	Percentage of 53BP1 foci-positive cells (%)	Percentage of apoptotic cells (%)
<b>Euthyroid patients with nodular goiter (n=33)</b>					
Age					
r	-0.012	-0.106	-0.107	0.058	-0.143
p	0.946	0.557	0.552	0.750	0.426
BMI (kg/m <sup>2</sup> )					
r	0.278	<b>0.380</b>	0.018	0.117	0.087
p	0.117	<b>0.029</b>	0.922	0.515	0.631
FT3 (pg/mL)					
r	<b>-0.488</b>	-0.327	-0.332	-0.225	-0.266
p	<b>0.007</b>	0.084	0.079	0.241	0.162
FT4 (ng/dL)					
r	<b>-0.362</b>	-0.327	-0.150	-0.268	<b>-0.349</b>
p	<b>0.038</b>	0.063	0.405	0.132	<b>0.046</b>
TSH ( $\mu$ U/mL)					
r	0.305	<b>0.366</b>	<b>0.396</b>	0.084	0.336
p	0.085	<b>0.036</b>	<b>0.022</b>	0.643	0.056
Thyroid nodule size (mm)					
r	0.047	0.126	<b>-0.627</b>	-0.073	-0.392
p	0.869	0.653	<b>0.012</b>	0.795	0.148
<b>Control subjects (n=30)</b>					
Age					
r	-0.057	-0.037	-0.006	-0.048	<b>0.369</b>
p	0.766	0.846	0.975	0.800	<b>0.045</b>
BMI (kg/m <sup>2</sup> )					
r	0.079	0.087	-0.197	-0.220	-0.144
p	0.677	0.647	0.298	0.242	0.449

BMI: Body mass index; FT3: Free triiodothyronine; FT4: Free thyroxine; TSH: Thyroid-stimulating hormone; r: Spearman’s rho correlation coefficient value; p: Spearman’s rho significance (2-tailed) value.

Table 3 presents Spearman’s rho correlation coefficients and significance values for age, BMI, FT3, FT4, TSH, and thyroid nodule size with the mean numbers of  $\gamma$ -H2AX and 53BP1 foci per cell, percentages of  $\gamma$ -H2AX and 53BP1 foci-positive cells, and the percentage of apoptotic cells in euthyroid patients with nodular goiter and control subjects. Weak but significant negative correlations were found between FT3 and the mean number of  $\gamma$ -H2AX foci per cell ( $p=0.007$ ,  $r=-0.488$ ), between

FT4 and the mean number of  $\gamma$ -H2AX foci per cell ( $p=0.038$ ,  $r=-0.362$ ), between FT4 and the percentage of apoptotic cells ( $p=0.046$ ,  $r=-0.349$ ), and between thyroid nodule size and the mean number of 53BP1 foci per cell ( $p=0.012$ ,  $r=-0.627$ ) in euthyroid patients with nodular goiter. Similarly, weak but significant positive correlations were observed between BMI and the percentage of  $\gamma$ -H2AX foci-positive cells ( $p=0.029$ ,  $r=0.380$ ), between TSH and the percentage of  $\gamma$ -H2AX foci-



positive cells ( $p=0.036$ ,  $r=0.366$ ), and between TSH and the mean number of 53BP1 foci per cell ( $p=0.022$ ,  $r=0.396$ ) in euthyroid patients with nodular goiter, along with a significant positive correlation between age and the percentage of apoptotic cells ( $p=0.045$ ,  $r=0.369$ ) in control subjects. However, there was no statistically significant correlation between age, BMI, FT3, FT4, TSH, thyroid nodule size, and the percentage of 53BP1 foci-positive cells in euthyroid patients with nodular goiter, not between BMI and the  $\gamma$ -H2AX and 53BP1 foci parameters in control subjects ( $p>0.05$ ) (Table 3).

## DISCUSSION

Thyroid nodules are common worldwide and may be cancerous, although the majority are benign.<sup>2–4</sup> In this study, we assessed DSBs, DNA repair capacity, and cancer risk using combined  $\gamma$ -H2AX and 53BP1 foci analysis in PBMCs of euthyroid patients with nodular goiter, as cancer is characterized by increased genomic alterations.

The  $\gamma$ -H2AX assay involves the immunocytochemical detection of  $\gamma$ -H2AX molecules in the chromatin surrounding the DSB sites. Unlike other genotoxicity assays, such as chromosome aberration (CA) and cytokinesis-block micronucleus (CBMN), the formation of  $\gamma$ -H2AX foci can occur in both proliferating and non-proliferating cells. CA and CBMN assays are performed on metaphase spreads and binucleated cells after the first mitosis, respectively, making the CBMN assay an indirect technique for detecting chromosomal DNA damage.<sup>6,21</sup> Additionally, the  $\gamma$ -H2AX assay is a specific and sensitive test that can also be co-analyzed with 53BP1 foci, which contribute to the repair of DSBs.<sup>9,13–17,19,20,22</sup>

To date, the combined numbers of  $\gamma$ -H2AX and 53BP1 foci had not been studied in patients with nodular goiter. However, some early studies reported that  $\gamma$ -H2AX and 53BP1 foci per cell were induced in peripheral blood lymphocytes of patients with thyroid cancer following radioiodine therapy.<sup>16,17</sup> In this study, for the first time, we found that the mean number of  $\gamma$ -H2AX foci per cell was significantly higher in PBMCs of euthyroid patients with nodular goiter than in control subjects. The higher mean number of  $\gamma$ -H2AX foci per cell in euthyroid patients with nodular goiter indicates that DNA damage (DSBs) was more prevalent in these patients than in the control subjects. Similarly, in our previous study, patients with multinodular goiter exhibited higher levels of oxidative (8-OHdG levels) and chromosomal (MN frequency) DNA damage, along with increased numbers of apoptotic and necrotic cells, compared to healthy subjects in the control group.<sup>21</sup> This elevated DNA damage suggests a deficiency in DNA repair mechanisms in these patients. However, a previous study on hyperthyroid patients revealed that high baseline markers of oxidative DNA and ribonucleic

acid (RNA) damage significantly decreased after treatment.<sup>23</sup> Furthermore, in the present study, there was an increase in the percentage of apoptotic cells in euthyroid patients with nodular goiter. These findings suggest that excessive DNA damage and/or inadequate DNA repair lead to cell death and the oncogenic transformation of cells in these patients. Moreover, apoptotic cells can exhibit a high number of  $\gamma$ -H2AX foci, indicating that the observed findings could be a result of increased apoptosis.

The tumor suppressor 53BP1 plays essential regulatory and mediator roles in response to DSBs, acting in conjunction with the DSB repair pathway to maintain genomic integrity.<sup>7,11</sup> It triggers the repair of DSBs by NHEJ, which is active in the G0 and G1 phases of the cell cycle and prevents HR functioning in the late S/G2 phase of the cell cycle when homologous sister chromatids are undamaged.<sup>8,11,20</sup> Additionally, 53BP1 accumulates at  $\gamma$ -H2AX foci in nuclei.<sup>11,20</sup> In this study, despite the high level of DNA damage in euthyroid patients with nodular goiter, there was no difference in the mean number of 53BP1 foci per cell or the percentage of 53BP1 foci-positive cells between patients and control subjects. Thus, our results suggest that DSBs are either incorrectly repaired or that DNA repair mechanisms are impaired, leading to more severe DNA damage. Furthermore, ongoing replication stress may cause an overall increase in  $\gamma$ -H2AX foci numbers due to the complexity of DNA damage.<sup>10,11</sup>

In this study, the FT3, FT4, and TSH levels of euthyroid patients with nodular goiter were within the hospital laboratory reference intervals. However, we observed significant positive correlations between TSH levels and  $\gamma$ -H2AX and 53BP1 foci values in euthyroid patients with nodular goiter. This finding suggests that higher DNA DSBs and insufficient DNA repair are related to increased TSH levels. We also found that higher DNA DSBs are associated with a high BMI in euthyroid patients with nodular goiter, whereas there was no association between BMI and  $\gamma$ -H2AX and 53BP1 foci parameters in the control subjects. Previous studies have reported that increases in micronucleus (MN) frequency (chromosomal DNA damage) and  $\gamma$ -H2AX levels are related to BMI in overweight/obese adults and children.<sup>24–26</sup> Additionally, positive relationships between the proportion of  $\gamma$ -H2AX foci-positive cells and TSH and BMI values suggest that  $\gamma$ -H2AX foci are more closely related to metabolism rather than to toxicity. We also found significant negative correlations between FT3 and FT4 levels and the number of  $\gamma$ -H2AX foci in euthyroid patients with nodular goiter, indicating that high DNA DSBs are associated with low FT3 and FT4 levels. In contrast, in our previous study, we found that MN frequency was not related to TSH and FT4 levels in patients with multinodular goiter, but we observed a negative

association between plasma 8-hydroxy-2'-deoxyguanosine (8-OHdG) levels and FT4.<sup>21</sup> Although the associations between thyroid hormone levels and  $\gamma$ -H2AX and 53BP1 foci were considered speculative, Hu et al.<sup>18</sup> demonstrated that  $\gamma$ -H2AX was associated with FT3, FT4, and TSH in thyroid cancer. Thus, our results support the use of  $\gamma$ -H2AX foci expressions as a biomarker of thyroid cancer development. Additionally, we found that thyroid nodule size was negatively correlated with the mean number of 53BP1 foci per cell in euthyroid patients with nodular goiter. In this study, the healthy control group, with a mean age of 40.10±10.49 years, was younger than the group of patients with nodular goiter, who had a mean age of 46.15±12.85 years. Furthermore, it was unsurprisingly exceedingly challenging to recruit healthy individuals as their age increased (particularly beyond 50, and even after 40). However, we found no correlation between the frequencies of  $\gamma$ -H2AX and 53BP1 foci and age in euthyroid patients with nodular goiter or control subjects, except for a positive association between age and the percentage of apoptotic cells in control subjects. These findings contradict those of previous studies suggesting that  $\gamma$ -H2AX expression is age-dependent.<sup>27,28</sup> Conversely, Firsanov et al.<sup>29</sup> reported that the induction of  $\gamma$ -H2AX foci was age-independent, which aligns with our findings. However, the data on age-dependent  $\gamma$ -H2AX foci numbers seem to be highly debatable.<sup>15,27–29</sup>

Lastly, a high number of  $\gamma$ -H2AX foci can serve as a marker of DSBs or insufficient DNA repair, thus identifying genetically predisposed patients and determining the increased risk of cancer in normal subjects.<sup>6,9,11,15,16,18,30</sup>

One of the limitations of the study was the small sample size of euthyroid patients with nodular goiter, due to limited financial support for the project. It is probable that the parameters of  $\gamma$ -H2AX and 53BP1 foci could have reached statistical significance with a larger number of patients. Furthermore, information on the sizes of thyroid nodules in all euthyroid patients with nodular goiter, as well as the FT3, FT4, and TSH values in the control subjects, was not available.

## CONCLUSION

In this study, DNA damage was observed to be higher in euthyroid patients with nodular goiter compared to control individuals. The increase in DNA damage occurs in the early stages of carcinogenesis. These patients were expected to exhibit compromised DNA repair along with enhanced DNA damage, increasing the risk of carcinogenesis. Therefore, euthyroid patients with nodular goiter might have a heightened risk of thyroid malignancy due to a high level of DNA damage. A long-term follow-up of these patients could illuminate the relationship between DNA damage and the risk of thyroid nodule malignancy.

**Ethics Committee Approval:** The Erciyes University Clinical Research Ethics Committee granted approval for this study (date: 17.04.2015, number: #2015/214).

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