

## Diagnostic Value of Circulating Cell-free DNA in Patients With Papillary Thyroid Cancer

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**Abstract.** *Aim: We investigated whether the occurrence and development of papillary thyroid cancer (PTC) might be predicted using levels of circulating cell-free DNA (cfDNA). Materials and Methods: The peripheral blood samples were collected from 68 patients with PTC, 31 patients with nodular goiter (NG), and 86 healthy controls (HC). The concentration of cfDNA was measured by qPCR using three primer sets:  $\beta$ -actin99,  $\beta$ -actin394 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in plasma samples. Results: It was demonstrated that plasma  $\beta$ -actin99 and  $\beta$ -actin394 in the PTC group were significantly higher compared to HC ( $p < 0.05$  and  $p < 0.001$ , respectively). The cfDNA integrity index was significantly higher in the PTC patients compared to HC and NG ( $p < 0.001$ ,  $p < 0.05$ , respectively). The cfDNA concentration in the NG group was significantly higher than in the PTC ( $p < 0.05$  and  $p < 0.001$ , respectively). Moreover, in most PTC patients with suppressed thyroglobulin, the  $\beta$ -actin394 and cfDNA integrity index was significantly decreased after surgery ( $p < 0.05$  and  $p < 0.001$ , respectively). ROC analysis revealed that cfDNA integrity index can be used as a potential marker in distinguishing PTC from HC (AUC 0.901,  $p < 0.001$ ) and NG (AUC 0.629,  $p < 0.05$ ). Conclusion: Increased concentration of cfDNA  $\beta$ -actin99 and  $\beta$ -actin394 may be a valuable biomarker that differentiates PTC patients from HC. Also, an increased cfDNA integrity index may be a suitable parameter which differentiates PTC patients from NG and HC.*

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**Key Words:** Papillary thyroid carcinoma, circulating cell-free DNA, cfDNA integrity index, GAPDH,  $\beta$ -actin.



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Papillary thyroid carcinoma (PTC) is the most common type of differentiated thyroid cancer, accounting for about 80-85% of all cases of thyroid cancer (1). Based on Global cancer statistics 2020, 586.202 new cases of thyroid cancer worldwide were diagnosed (2). For the majority of patients with PTC, the prognosis is good. However, PTC can be aggressive; up to 35% of patients have local tumor renewal or systemic spread and about 10% of cases may present with metastases at initial presentation. Around 60-70% of this subset of patients will become radioiodine refractory (1, 3-5). Non-invasive diagnostic methods such as ultrasound are primarily used to differentiate thyroid nodules. However, if thyroid cancer is suspected, invasive procedures such as fine-needle biopsy (FNB) must be performed. However, even the FNB is often unable to make a definitive diagnosis and we must undertake an even more aggressive invasion-surgery. FNB cytology by ultrasonography is still the main diagnostic method of PTC, but there is a technical limitation and 15-30% of cases are cytological indeterminate (6, 7).

A minimally invasive diagnostic test that can accurately diagnose the onset of the disease remains the subject of research. Plasma sampling is the easiest way to investigate the presence of malignancies. It has very recently been recognized as the sample of choice over serum and whole blood for investigating levels of cfDNA (8).

Circulating cell-free DNA (cfDNA) is different length nucleic acid fragments released to the bloodstream from body cells. The length of cfDNA fragments depends on the mechanism of cell death. Short cfDNA fragments, less than 180 bp, are usually produced to the bloodstream from normal cells after apoptosis, while longer cfDNA fragments occur due to necrosis in tumor cells (9, 10).

The cfDNA in blood plasma was first reported by Mandel and Metais in 1948, but only very recently has been recognized as the potential diagnostic and prognostic biomarker in a variety of tumors (11, 12). Current analysis shows potential for diagnostic use in colorectal, liver, kidney, breast, prostate and lung cancer, and hepatocellular carcinoma

(13-19). cfDNA can also be measured at higher levels in trauma, stroke, myocardial infarction and sepsis (20, 21). Unlike benign cells, tumor cells release longer DNA fragments in the blood because of necrosis or autophagy.

Recent studies demonstrate that longer DNA strands circulating in plasma can be considered a biomarker for thyroid cancer. However, the structural features of cfDNA for diagnosis and prognosis of differentiated thyroid cancer are poorly characterized. *GAPDH* as well as  $\beta$ -actin is the most commonly used endogenous housekeeping genes that have a stable expression pattern and other characteristics in cancer. Low amplification and deletion probability of *GAPDH* gene is detected in different types of cancer. Therefore, *GAPDH* is a useful reference gene in measuring cfDNA concentration released to the bloodstream from various types of cancer (21, 22).

Strong correlation between  $\beta$ -actin and a multitude of cancers was shown, but also controversial results were found. Some studies found that  $\beta$ -actin is deregulated in melanoma, liver, kidney, gastric, colorectal, oesophageal, lymphoma, prostate, pancreatic, lung, ovarian, breast, and leukaemia (15, 23). However,  $\beta$ -actin is upregulated in most cancer tissues. It was shown that  $\beta$ -actin polymerization and expression changes may be consistent with metastasis and invasive cancer. Thus, we chose *GAPDH* and  $\beta$ -actin genes to identify the role of cfDNA status for early non-invasive diagnosis and prognosis of papillary thyroid cancer. We investigated whether the occurrence and development of PTC might be predicted using levels of circulating cfDNA.

## Materials and Methods

**Sample collection.** PTC patients, patients with nodular goiter (NG) and healthy controls (HC) were involved in this study. Plasma samples were obtained from patients with PTC 24 h before and 4-6 weeks after surgery at the Hospital of Lithuanian University of Health Sciences Kaunas Clinics between 2020 and 2021. PTC patients underwent thyroidectomy, and the diagnosis of PTC was confirmed histopathologically after surgery. Classification of patients with PTC was performed according to the 8<sup>th</sup> edition of the AJCC/UICC staging system (24). Histological PTC subtypes were divided into two groups: aggressive (diffuse sclerosing variant and tall cell carcinoma) and non-aggressive subtypes (classical and follicular variant). The PTC group had no benign nodes of thyroid, severe or critical condition and no previous history of any other cancer.

Plasma samples from patients with NG were obtained 24 h before surgery. NG patients underwent thyroidectomy, and the diagnosis of multiple hyperplastic nodes was confirmed histopathologically. The HC group had no thyroid disease, autoimmune illness or previous history of any cancer and their family history for thyroid diseases and cancer was negative.

Thyroid ultrasound, hormones (TSH and fT4) and anti-thyroid peroxidase (anti-TPO) antibodies were performed on all prospective subjects before inclusion to the study. Plasma thyroglobulin (Tg) was performed on all PTC patients three month after surgery, before I<sup>131</sup> therapy. Tg <0.1 ng/ml was considered suppressed.

Venous blood was drawn from PTC, NG and HC patients. All peripheral venous blood samples (10 ml) were collected in EDTA (BD Vacutainer PPT™ Plasma Preparation Tube; 13 x 100 mm/5 ml) tubes and separated by the centrifugation 1,900 × g for 10 min at 4°C. Supernatant then was transferred to a new 15 ml tube and centrifuged in conical tubes at 16,000 × g for 10 min at 4°C. Purified plasma was transferred to 1.5 ml aliquots and stored at -80°C until nucleic acid purification. Plasma cfDNA was extracted from 5 ml blood plasma using QIAamp Circulating Nucleic Acid Kit (Qiagen, Germany) according to the manufacturer's protocol. Eluted cfDNA was transferred into 0.2 ml Eppendorf tubes and stored at -80°C. Total cfDNA was quantified by the qPCR assay targeting the 67 bp amplicon on the APP gene.

The study was approved by the Kaunas Regional Committee of Biomedical Research (Lithuania, approval No. BE-2-64; 2020-02-07). Written informed consent was obtained from each participant of the study after a full explanation of the purpose and nature of all procedures used. This study was conducted in accordance with the Declaration of Helsinki.

**Measurement of cfDNA integrity and cfDNA concentration.** Plasma cfDNA concentration and cfDNA integrity index were analysed in our study. Plasma cfDNA was quantified by the qPCR assay targeting the 99 bp amplicon on the  $\beta$ -actin gene and 108 bp amplicon on the *GAPDH* gene.  $\beta$ -actin394 primers were used to amplify the long DNA fragments, released mainly from non-apoptotic cells. cfDNA integrity index was calculated as the ratio between the 394 and 99 bp amplicons of the  $\beta$ -actin gene as previously described. The concentration of cfDNA was measured by qPCR using three primer sets  $\beta$ -actin99 (F: 5'-CCACACTGTGCCCCATCTACG-3'; R: 5'-AGGATCTTCATGAGGAGTCAGTCAG-3'),  $\beta$ -actin394 (F: 5'-CCACACTGTGCCCCATCTACG-3'; R: 5'-TTAGCTTCCACAGCACAGCC-3') and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) (F: 5'-ATGTTTCGTCATGGGTGTGAA-3'; R: 5'-GGTGCTAAGCAGTTGGTGGT-3') as previously described.

qPCR was performed in 20 µl reaction volume with 0.4 µM of forward and reverse primers, 2 µl of cfDNA, 6.4 µl of nuclease free water and 10 µl PowerUp™ SYBR™ Green Master Mix (Applied Biosystems, Foster City, CA, USA). qPCR was performed in Applied Biosystems 7500 Fast Real-Time PCR System thermal cycler (Applied Biosystems, USA) using the following cycling profile: 50°C for 2 min, 95°C for 2 min, 40 cycles at 9°C for 15 s, 60°C for 15 s, and 72°C for 1 minute, and ended with a melt curve from 65 to 95°C for 5 s. All reactions were performed in triplicate. The amount of DNA fragments for each gene was determined using a standard curve created by performing qPCR with serial dilutions (from 2.5 to 25,000 ng/ml) of human genomic DNA.

**Statistical analysis.** The normality of data distribution was tested using Kolmogorov-Smirnov criteria. Student's *t* test and analysis of variance (ANOVA - for normal distribution) or Mann-Whitney *U*-test and Kruskal-Wallis H-test criterion - for abnormal distribution were used to determine the differences in quantitative traits between the comparison groups. The association between qualitative values in comparative groups was assessed by the Chi-square ( $\chi^2$ ) test. The relationships between the amount of cfDNA in the plasma and clinic - pathological parameters were determined by Pearson correlation. The predictive capability (diagnostic performance) of each biomarker was investigated by means of the area under the ROC (Receiver-Operating Characteristics) curve (AUC). Statistical analyses were performed

using SPSS 22.0 software (SPSS Inc., Chicago, IL, USA). The results were considered statistically significant at  $p < 0.05$ .

## Results

**Study population.** One hundred and eighty five patients were included in the study: 68 patients with a histologically confirmed diagnosis of PTC after surgical treatment, 31 patients with NG and 86 healthy controls. In the PTC group, there were 8 male patients (11.8%) and 60 female patients (88.2%), while in the NG group, 5 patients (16.1%) were male and 26 patients (83.9%) were female. In the HC group, there were 65 (75.6%) female and 21 (24.4%) male patients. The mean age [standard deviation (SD)] of PTC patients was 48.19 (14.9), 49.61 (17.16) of NG patients and 45.30 (12.07) of HC patients. Clinicopathological characteristics of the study population with PTC are shown in Table I.

**cfDNA concentration in PTC, NG and HC groups.** Plasma cfDNA concentration was quantified according to  $\beta$ -actin99 and GAPDH concentrations. The concentration of  $\beta$ -actin99 was significantly higher in the PTC patients compared to HC (1,005.97 vs. 623.12 ng/ml,  $p < 0.05$ ), while there was no significant difference of GAPDH between these groups (Figure 1 and Figure 2). The concentration of GAPDH was significantly higher in the NG group compared to the PTC and HC (1,167.50 vs. 879.76 and vs. 751.34 ng/ml,  $p < 0.05$  in both cases). The concentration of  $\beta$ -actin99 in the NG group was significantly higher compared to the PTC and HC (1,336.89 vs. 1,005.97 and vs. 623.12 ng/ml,  $p < 0.001$  and  $p < 0.05$ , respectively). The concentration of  $\beta$ -actin394 in the PTC group was significantly higher than in the HC (456.35 vs. 157.27 ng/ml,  $p < 0.001$ ). The NG group also had a higher concentration of long fragments than the control group (474.04 vs. 157.27 ng/ml,  $p < 0.001$ ) (Figure 3).

The cfDNA integrity index was significantly higher in the PTC patients compared to HC and NG (0.475 vs. 0.134 and vs. 0.359,  $p < 0.001$  and  $p < 0.05$ , respectively). While the index of NG patients was significantly higher compared to the HC group (0.359 vs. 0.134,  $p < 0.001$ ) (Figure 4).

**cfDNA concentrations in PTC patients before and after surgery.** The specificity of the assay was demonstrated considering the variation of plasma cfDNA concentrations from GAPDH and  $\beta$ -actin99 genes and cfDNA integrity index comparing before and after surgery. After surgery, samples showed a significantly lower concentration of  $\beta$ -actin394 and cfDNA integrity index as compared to before. Moreover, concentration of  $\beta$ -actin394 and cfDNA integrity index after the surgery significantly decreased in PTC patients with suppressed Tg concentration ( $p < 0.05$  and  $p < 0.001$ , respectively) (Figure 5 and Figure 6). However, after surgery concentrations of GAPDH and  $\beta$ -actin99 cfDNA decrease was not significantly lower (Table II).

Table I. Clinicopathological characteristics of the population with papillary thyroid cancer (PTC).

Characteristic	PTC patients (n=68)
Age at initial surgery (years)	
≤55 years, n (%)	44 (33.6)
>55 years n (%)	24 (18.3)
T (TNM), n (%)	
pT1a	27 (39.7)
pT1b	7 (10.3)
pT2	4 (5.9)
pT3a	19 (27.9)
pT3b	11 (16.2)
Lymph node metastases at initial surgery, n (%)	
Yes	16 (23.5)
No	52 (76.5)
Variant of PTC, n (%)	
The classical variant	29 (42.6)
The follicular variant	18 (26.5)
The diffuse sclerosing variant	17 (25.0)
The tall cell carcinoma	4 (5.9)
Multifocality, n (%)	
Yes	16 (23.5)
No	52 (76.5)
Extrathyroidal extension, n (%)	
Yes	30 (44.1)
No	38 (55.9)
Lymphovascular invasion, n (%)	
Yes	36 (52.9)
No	32 (47.1)
Autoimmune thyroiditis, n (%)	
Yes	23 (33.8)
No	45 (66.2)

**Association of cfDNA concentration with clinicopathological features of PTC.** A trend towards higher cfDNA concentrations has been observed between many clinicopathological features in the presence of more advanced PTC or in the presence of adverse disease features, although no significant differences were identified (Table III). We observed that GAPDH and  $\beta$ -actin99 concentrations were significantly higher in PTC with greater tumor size ( $>2$  cm) compared to lower ( $\leq 2$  cm) tumor size ( $p < 0.05$  in both cases). Similarly, PTC patients with a larger tumor size ( $>2$  cm) were more likely to have a higher cfDNA integrity index compared to lower ( $\leq 2$  cm) tumor size ( $p < 0.05$ ). The concentration of GAPDH,  $\beta$ -actin99,  $\beta$ -actin394 and cfDNA integrity index in aggressive histology variants subtypes of PTC to other, non-aggressive subtypes of PTC was compared, but no statistically significant relation was found (Table III).

The total tumor size was calculated as the sum of the diameters of all tumors in PTC multifocal cases. A weak positive correlation between the concentration of GAPDH and  $\beta$ -actin99 with the total size of PTC tumors was found ( $p < 0.05$ ,  $r = 0.245$  and  $p < 0.05$ ,  $r = 0.304$ , respectively) (Figure 7). The analysis showed a weak positive correlation between

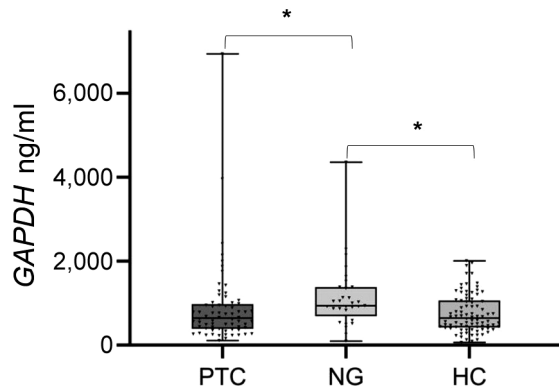


Figure 1. The comparison of *GAPDH* concentration in papillary thyroid cancer (PTC), nodular goiter (NG) and healthy controls (HC) patients. Analysis of Kruskal – Wallis H-test criterion was used to determine the differences in quantitative traits between the comparison groups. \* $p<0.05$ .

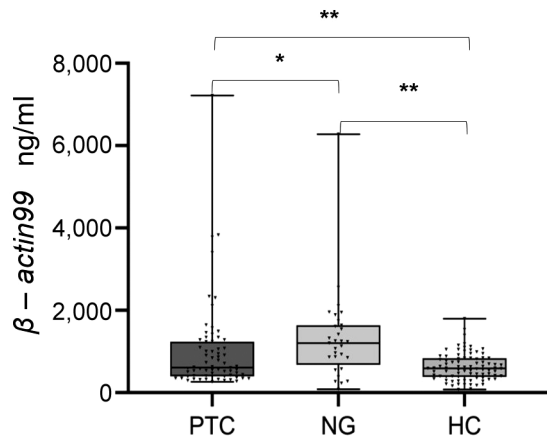


Figure 2. The comparison of  $\beta$ -actin99 concentration in papillary thyroid cancer (PTC), nodular goiter (NG) and healthy controls (HC) patients. Analysis of Kruskal – Wallis H-test criterion was used to determine the differences in quantitative traits between the comparison groups. \* $p<0.05$ , \*\* $p<0.001$ .

the concentration of  $\beta$ -actin394 and the total size of PTC tumors ( $p<0.05$ ,  $r=0.239$ ) (Figure 8).

**The diagnostic value of cfDNA.** To evaluate the possible diagnostic value of cfDNA, ROC analysis was performed. Concentration of  $\beta$ -actin99,  $\beta$ -actin394 and cfDNA integrity index had statistically significant satisfactory or good diagnostic values to differentiate PTC patients from HC (Figure 9C, 9E, 9G; Table IV). cfDNA integrity index had the highest AUC of 0.901 (95% CI=0.855-0.948), with 98.5% sensitivity and 64.0% specificity at the cutoff value of 0.065 (PTC vs. HC, Figure 9G). The concentration of *GAPDH* did

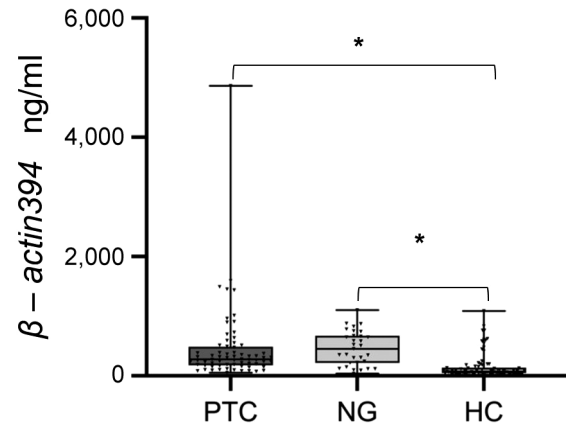


Figure 3. The comparison of  $\beta$ -actin394 concentration in papillary thyroid cancer (PTC), nodular goiter (NG) and healthy controls (HC) patients. Analysis of Kruskal – Wallis H-test criterion was used to determine the differences in quantitative traits between the comparison groups. \* $p<0.001$ .

not show statistically significant differences between PTC and HC (Figure 9A; Table IV). However, *GAPDH* and  $\beta$ -actin99 had statistically significant differences between PTC and NG. *GAPDH* had an AUC of 0.703 (95% CI=0.597-0.809),  $\beta$ -actin99 - 0.644 (95% CI=0.520-0.767) with a low sensitivity and specificity (Figure 9B, 9D; Table V). In addition, the comparison between PTC patients and those with benign nodules showed that the cfDNA integrity index had an AUC of 0.629 (95% CI=0.513-0.746), with 69.1% sensitivity and 67.6% specificity at the cutoff value of 0.279 ( $p<0.05$ ). This might be useful for differentiating PTC from NG (Figure 9H; Table V).

## Discussion

Currently, there are no non-invasive tests that can specifically identify the presence of early-stage PTC and give high-confidence results. cfDNA has been the subject of significant interest in the field of cancer researchers, but little has been published with regards to PTC. In order to expand our knowledge, we choose *GAPDH* gene,  $\beta$ -actin99 and  $\beta$ -actin394 genes to identify the role of cfDNA status for early non-invasive diagnosis and prognosis of PTC.

The annealing sites of  $\beta$ -actin99 are within the  $\beta$ -actin394 ones. The  $\beta$ -actin99 primers could amplify both shorter fragments, truncated by apoptosis ( $\beta$ -actin99), and longer DNA fragments ( $\beta$ -actin394), so the results of  $\beta$ -actin99 quantitation represent the total amount of cfDNA. However,  $\beta$ -actin394 primers amplify only longer DNA fragments. Therefore, the results of  $\beta$ -actin394 quantitation more closely represent the amounts of DNA released from necrotic cell death. The main origin of short cfDNA fragments in healthy individuals has been attributed to apoptotic cells,

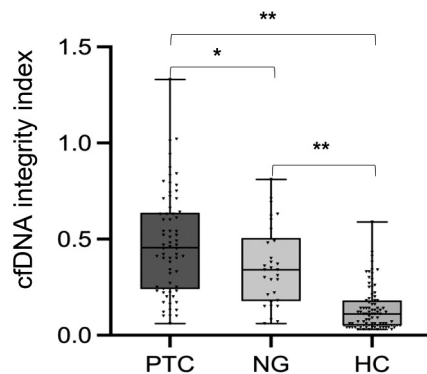


Figure 4. The comparison of cfDNA integrity index in papillary thyroid cancer (PTC), nodular goiter (NG) and healthy controls (HC) patients. Analysis of ANOVA - for normal distribution, was used to determine the differences in quantitative traits between the comparison groups. \* $p < 0.05$ , \*\* $p < 0.001$ .

while a majority of longer DNA fragments could represent a diagnostic biomarker for malignant tumors.

In this study, we compared cfDNA concentration determined by *GAPDH*,  $\beta$ -actin99 and  $\beta$ -actin394 and cfDNA integrity index in plasma samples of PTC, NG and HC. We observed that  $\beta$ -actin99 and  $\beta$ -actin394 concentrations and cfDNA integrity index in the PTC group were significantly higher compared to HC. Meanwhile, the concentration of *GAPDH* and  $\beta$ -actin99 in the NG group was significantly higher compared to the PTC and HC. Only the cfDNA integrity index was significantly higher between the PTC and NG groups. After surgery, PTC plasma samples showed a lower  $\beta$ -actin394 concentration and cfDNA integrity index, compared to the levels before. cfDNA (*GAPDH* and  $\beta$ -actin99) concentration,  $\beta$ -actin394 concentration and cfDNA integrity index was higher in tumors with greater tumor size ( $> 2$  cm) compared to lower ( $\leq 2$  cm) tumor size. The concentration of *GAPDH*,  $\beta$ -actin99 and  $\beta$ -actin394 correlated with total tumor size. ROC analysis revealed that cfDNA integrity index provides higher accuracy in distinguishing PTC from NG and may be used as a potential tumor marker.

A higher concentration of circulating  $\beta$ -actin has previously been observed in hepatocellular and renal cell carcinomas, when compared to healthy controls (15, 23). Zane *et al.* found a significantly higher concentration of cfDNA in thyroid cancer patients compared with healthy individuals (25). Similarly, Jianga *et al.* found a significantly higher concentration of plasma *GAPDH* cfDNA in the thyroid cancer patients, than that of healthy controls, while in nodular goiter, they were not significantly different from the other two groups (26). Our study showed that the concentration of plasma  $\beta$ -actin was higher in the PTC group compared to HC, while the concentration of *GAPDH* did not differ significantly.

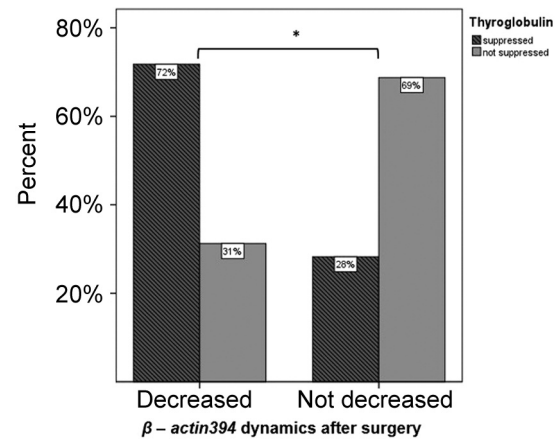


Figure 5. The comparison of  $\beta$ -actin394 dynamic after surgery in papillary thyroid cancer (PTC) patients with suppressed and not suppressed thyroglobulin. \* $p < 0.05$ .

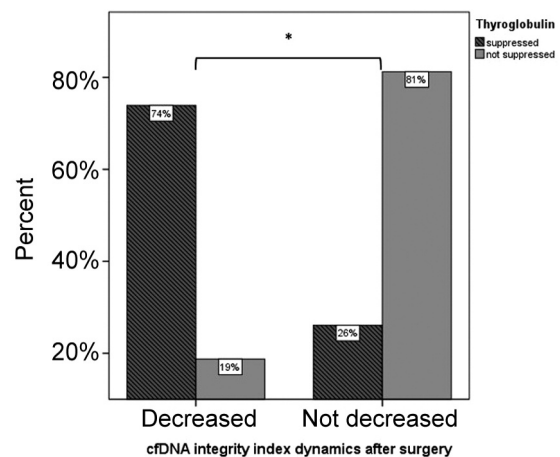


Figure 6. The comparison of cfDNA integrity index dynamic after surgery in papillary thyroid cancer (PTC) patients with suppressed and not suppressed thyroglobulin. \* $p < 0.001$ .

The concentration of cfDNA (*GAPDH* and  $\beta$ -actin99) and  $\beta$ -actin394 in the NG group was significantly higher compared to the PTC and HC. Given the fact that the large-sized nodes ( $> 2$  cm - 61.5% in the NG group) were diagnosed for the majority of NG group patients, concentration differences could be related to the size of the lesions. Thakur *et al.* observed that concentration of cfDNA was significantly higher in the plasma samples of patients with benign nodules compared to thyroid cancer patients (27). This is consistent with a few studies focused on other cancers that documented the cfDNA concentration correspondence to tumor size (28, 29). Tissue necrosis may develop at the center of large benign thyroid nodules due to circulatory and homeostatic disorders.

Table II. Plasma GAPDH,  $\beta$ -actin99,  $\beta$ -actin394 concentration and cfDNA integrity index in papillary thyroid cancer (PTC) patients before and after thyroidectomy.

Marker	Concentration: MEAN (SD)		p-Value
	Pre-operative PTC (n=68)	Post-operative PTC (n=62)	
GAPDH	879.76 (972.96)	752.82 (348.11)	0.253
$\beta$ -actin99	1,005.97 (1086.18)	805.96 (652.75)	0.062
$\beta$ -actin394	456.358 (647.17)	255.023 (166.555)	<b>&lt;0.05</b>
cfDNA integrity index	0.475 (0.266)	0.345 (0.134)	<b>&lt;0.05</b>

Bold values indicate statistical significance.

Table III. Correlation between clinicopathological features of papillary thyroid cancer (PTC) and relative concentration of GAPDH,  $\beta$ -actin99,  $\beta$ -actin394 and cfDNA integrity index in plasma samples.

Characteristic	GAPDH	$\beta$ -actin99	$\beta$ -actin394	cfDNA integrity index
	879.76 (108.37-6,943.71)	1,005.97 (266.61-7,213.34)	456.35 (54.15-4,863.18)	0.475 (0.06-1.33)
	C(ng/ml) p-Value	C(ng/ml) p-Value	C(ng/ml) p-Value	p-Value
Sex	0.732	0.332	0.954	0.945
Male	785.30 (353.10-1,751.93)	1,206.63 (295.73-3,398.12)	372.81 (152.86-897.36)	0.469 (0.12-0.78)
Female	892.36 (108.36-6,943.70)	979.22 (266.61-7,213.34)	467.49 (54.15-4,863.18)	0.476 (0.06-1.33)
Age at initial surgery (years)	0.817	0.663	0.878	0.877
≤55 years	449.88 (89.21-937.34)	1081.07 (266.61-7,213.34)	505.50 (54.15-4,863.18)	0.479 (0.06-1.33)
>55 years	609.76 (237.82-1,214.32)	868.30 (294.51-3,829.23)	366.25 (89.87-1,437.05)	0.469 (0.12-1.01)
pT (TNM)	0.264	0.524	0.234	0.474
pT1	718.25 (108.37-2,412.85)	847.31 (266.61-3,829.23)	366.10 (54.15-1,490.30)	0.459 (0.06-1.01)
pT2-3	1041.28 (237.18-6,943.71)	1,161.63 (278.21-7,213.34)	546.61 (89.82-4,813.18)	0.492 (0.12-1.33)
Tumor size (cm)	<b>&lt;0.05</b>	<b>&lt;0.05</b>	<b>&lt;0.05</b>	<b>&lt;0.05</b>
≤2	678.35 (108.37-2,141.14)	742.75 (266.61-2,302.36)	324.84 (54.15-1,453.77)	0.428 (0.06-1.01)
>2	1591.44 (237.18-6,943.70)	1,936.02 (294.51-7,213.34)	921.03 (187.18-4,863.18)	0.644 (0.13-1.33)
Lymph node metastases at initial surgery	0.553	0.686	0.497	0.745
Yes	769.08 (237.17-2,412.84)	1,429.84 (278.21-7,213.34)	741.65 (82.95-4,863.18)	0.456 (0.10-0.94)
No	1,239.47 (108.36-6,943.70)	875.55 (266.61-3,829.23)	368.57 (54.15-1,453.77)	0.481 (0.06-1.33)
Variant of PTC	0.974	0.695	0.051	0.648
Aggressive histology of PTC	768.99 (237.18-2,414.85)	949.82 (294.51-3,829.23)	310.04 (54.15-1,437.05)	0.382 (0.06-1.02)
Non-aggressive subtypes of PTC	929.26 (108.37-6,943.70)	1,031.06 (266.61-7,213.34)	521.73 (82.95-4,863.18)	0.5175 (0.10-1.33)
Extrathyroidal extension	0.415	0.843	0.537	0.723
Yes	1,074.84 (237.17-6,943.70)	1,194.82 (278.21-7,213.34)	562.84 (89.87-4,863.18)	0.462 (0.12-1.02)
No	725.76 (108.36-2,412.84)	856.89 (266.61-3,829.23)	372.28 (54.15-1,490.30)	0.486 (0.06-1.33)
Lymphovascular invasion	0.941	0.185	0.410	0.101
Yes	1,022.53 (161.39-6,943.70)	555.40 (144.83-1,344.81)	548.08 (54.15-4,863.18)	0.497 (0.10-1.02)
No	719.15 (108.36-2,141.13)	528.32 (189.30-1,101.94)	353.17 (71.41-1,453.77)	0.451 (0.06-1.33)

Bold values indicate statistical significance.

Therefore, we hypothesize that the increase in cfDNA concentration in the NG group could be related to necrosis of the thyroid nodule cells.

Previous studies suggested that cfDNA integrity index was significantly higher in patients with gastrointestinal and hepatocellular tumors, melanoma, urologic tumor and breast cancer, as compared to healthy controls. Most studies have

used GAPDH, ALU and  $\beta$ -actin qPCR to calculate the cfDNA integrity index (23, 30-33). It was observed, that the integrity index was most specific parameter indicating tumor status (9). Our results also confirm that cfDNA integrity index is a more specific parameter in differentiating PTC from NG and HC than measurement of  $\beta$ -actin99 and  $\beta$ -actin394 concentrations separately. Salvianti *et al.* found a

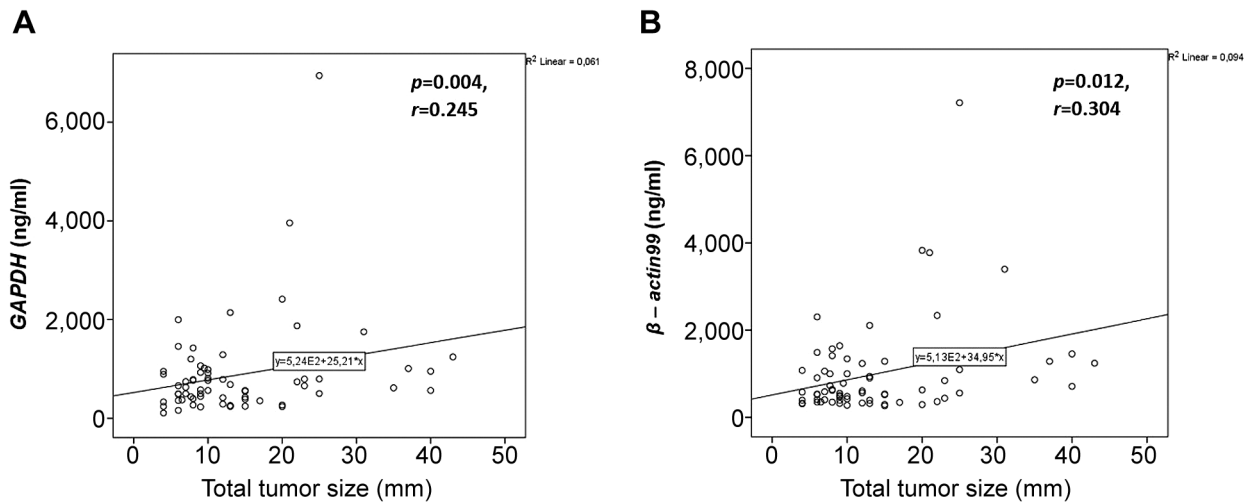


Figure 7. The correlation between *GAPDH* (A) and  $\beta$ -actin99 (B) and total tumor size.

higher cfDNA integrity index in thyroid cancer patients compared to healthy individuals and patients with NG (21). In accordance, our findings also suggest that cfDNA integrity index is significantly higher in PTC compared with NG and HC groups. This finding may support the cfDNA integrity index as a promising biomarker in differentiating PTC patients from thyroid nodules and healthy individuals.

Moreover, in most PTC patients with suppressed Tg, the  $\beta$ -actin394 and cfDNA integrity index was significantly decreased after surgery. The decrease in Tg levels may take up to six or twelve months. In our study, we obtained significant differences only three months after surgery. Our findings suggest that the plasma concentration of  $\beta$ -actin394 and cfDNA integrity index changed significantly after surgery and it therefore might be an indication of successful removal of the tumor and a useful prognostic marker after thyroidectomy. However, it would be useful to assess the changes after six or twelve months.

The findings by Salvianti *et al.* also suggest that cfDNA integrity index was lower after surgery than that taken before surgery in patients with thyroid cancer (21). Gang *et al.* also found the relation between cfDNA integrity index and the presence of thyroid tumor (32). Further studies of plasma cfDNA concentration measurements before and after surgery with more participants and a longer follow-up period would disclose more information about cfDNA as a prognostic biomarker of PTC patients.

It has been shown that cfDNA levels correlate with disease stage and primary tumor size (28). Umetani *et al.* found that cfDNA integrity index positively correlated with the size of invasive cancers and was significantly higher in the presence of lymphovascular invasion and lymph node metastasis. Our findings suggest that plasma

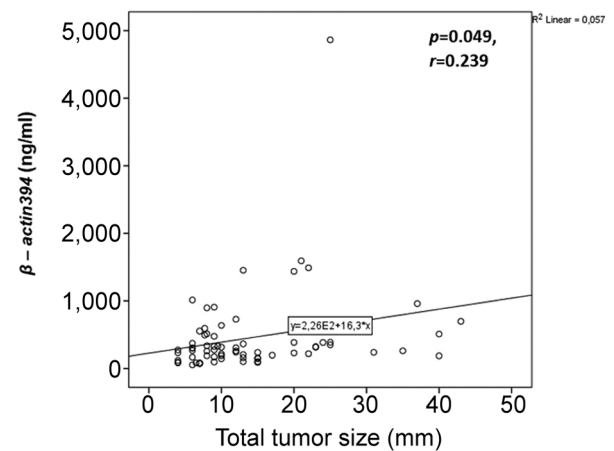


Figure 8. The correlation between  $\beta$ -actin394 concentration and the total tumor size.

concentration of cfDNA and  $\beta$ -actin394 and cfDNA integrity index were significantly higher in patients with greater tumor size. The concentration of *GAPDH*,  $\beta$ -actin99 and  $\beta$ -actin394 positively correlated with total tumor size. However, there was no correlation between lymphovascular invasion, lymph node metastasis and other clinico - pathological features. It is known that thyroid cancer releases lower amounts of cfDNA to the bloodstream compared with other more aggressive cancer types. Moreover, about half of the patients in our study were diagnosed with non-aggressive PTC subtype and pT1 stage. Therefore, we can assume that we do not have cancer invasion or metastasis for these reasons.

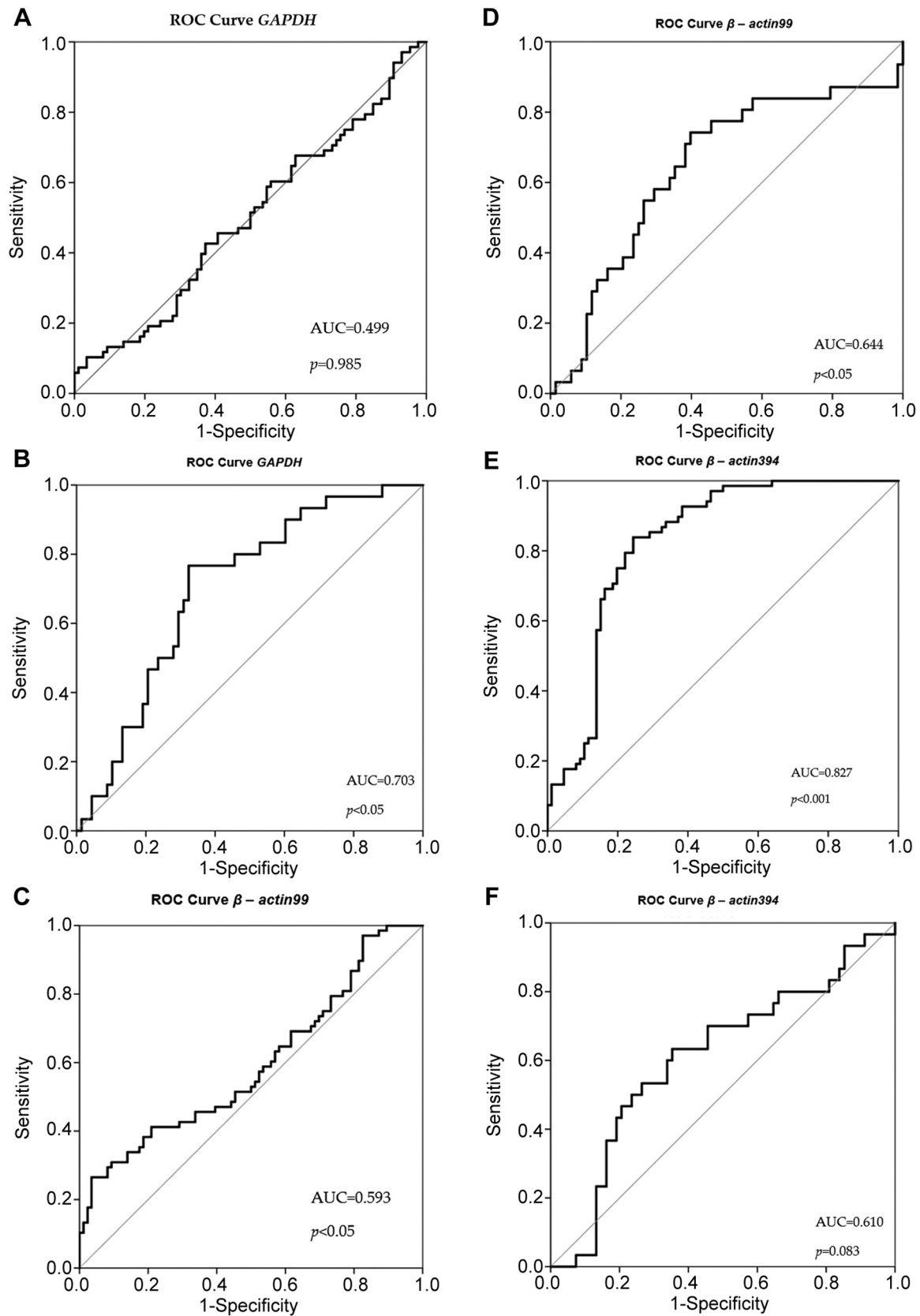


Figure 9. Continued

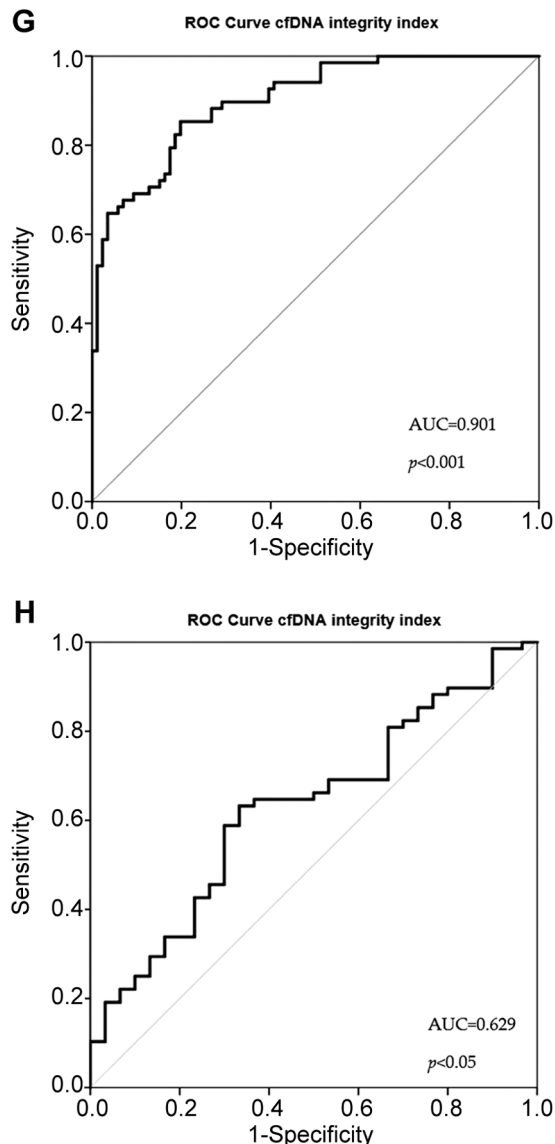


Figure 9. Diagnostic value of circulating cfDNA (*GAPDH*,  $\beta$ -actin99,  $\beta$ -actin394) and cfDNA integrity index in discriminating papillary thyroid cancer (PTC) from nodular goiter (NG) and healthy controls (HC). Receiver operating characteristic (ROC) curves were used to distinguish the groups. (A) Plasma *GAPDH* in PTC vs. HC and (B) NG. (C) Plasma  $\beta$ -actin99 in PTC vs. HC and (D) NG. (E) Plasma  $\beta$ -actin394 in PTC vs. HC and (F) NG. (G) Plasma cfDNA integrity index in PTC vs. HC and (H) NG. AUC, Area under the curve.

Previous studies suggest the significant diagnostic value of cfDNA concentration and integrity index in breast cancer patients. In our study, ROC curves were used to evaluate the diagnostic value of *GAPDH*,  $\beta$ -actin99,  $\beta$ -actin394 concentration and cfDNA integrity index in PTC, HC, and NG groups. Our findings indicated that  $\beta$ -actin99,  $\beta$ -

actin394 concentrations and cfDNA integrity index changes may be used as parameters in differentiating PTC patients from HC, but the diagnostic value of *GAPDH*,  $\beta$ -actin99 and  $\beta$ -actin394 concentrations does not allow to differentiate NG from PTC. Meanwhile, ROC curve analysis confirmed that only plasma cfDNA integrity index might be a reliable marker in discriminating PTC from NG [AUC of 0.629 (95% CI=0.513-0.746)].

cfDNA has several limitations that restrict its use as a marker for PTC detection. Firstly, cfDNA is not specific and increase in its level could be observed in other malignancies and conditions such as in infections, severe diseases or inflammations; therefore, it must be evaluated with other findings. Secondly, due to a short patient follow-up period after the surgery it is difficult to determine prognostic value of cfDNA in PTC. Another limitation of this study is the relatively small sample size and lack of advanced disease cases. Finally, further large-scale and longer follow-up prospective investigations are needed to confirm our results in the diagnosis and prognostic prediction of PTC. It would also be useful to include more genes in the study that may be relevant to the pathogenesis of PTC.

In conclusion, the changes of cfDNA integrity index are expected to be a sensitive and specific parameter for the diagnosis of PTC and differentiating PTC from NG. The concentration of  $\beta$ -actin99 and  $\beta$ -actin394 are superior to *GAPDH* in differentiating PTC from HC, but further studies with larger sample size and longer follow-up period are necessary to confirm circulating cfDNA relevance as a biomarker for non-invasive prognostic tool of PTC.

## Conflicts of Interest

The Authors declare that there are no conflicts of interest that could be perceived as prejudicing the impartiality of the research reported.

## Authors' Contributions

Conceptualization, A.D. and B.Z.; methodology, M.K., D.D., A.D.; validation, R.K., A.D., R.V.; formal analysis, R.K., A.K., M.K.; investigation, A.K., R.K., M.K., A.D.; resources, B.Z., A.D., R.V.; data curation, A.K., M.K., R.K., A.D., D.D.; writing – original draft preparation, R.K., M.K.; supervision, D.D., A.D., B.Z.; writing – review and editing, A.D., D.D., B.Z., R.V.; visualization, R.K., M.K., D.D., A.D.; project administration, A.D., B.Z.; funding acquisition, B.Z., A.D. All authors have read and agreed to the published version of the manuscript.

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Table IV. ROC curves analysis for plasma concentration of GAPDH,  $\beta$ -actin99,  $\beta$ -actin394 and cfDNA integrity index to discriminate papillary thyroid cancer patients from a healthy control.

Marker	AUC	Asymptotic 95% Confidence interval		p-Value	Sensitivity	Specificity
		Lower bound	Upper bound			
GAPDH	0.499	0.407	0.592	0.985	64.7%	62.8%
$\beta$ -actin99	0.593	0.501	0.685	<b>&lt;0.05</b>	75.0%	73.3%
$\beta$ -actin394	0.827	0.761	0.894	<b>&lt;0.05</b>	98.5%	64.0%
cfDNA integrity index	0.901	0.855	0.948	<b>&lt;0.05</b>	98.5%	64.0%

Bold values indicate statistical significance.

Table V. ROC curves analysis for plasma concentration of GAPDH,  $\beta$ -actin99,  $\beta$ -actin394 and cfDNA integrity index to discriminate papillary thyroid cancer patients from nodular goiter.

Marker	AUC	Asymptotic 95% Confidence interval		p-Value	Sensitivity	Specificity
		Lower bound	Upper bound			
GAPDH	0.703	0.597	0.809	<b>&lt;0.05</b>	46.7%	23.5%
$\beta$ -actin99	0.644	0.520	0.767	<b>&lt;0.05</b>	38.7%	23.5%
$\beta$ -actin394	0.610	0.486	0.735	0.083	46.7%	23.5%
cfDNA integrity index	0.629	0.513	0.746	<b>&lt;0.05</b>	69.1%	66.7%

Bold values indicate statistical significance.

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