A Comprehensive Diagnostic Assessment of Thyroid Nodules Utilizing Scintigraphy and Telomere Lengths (T/S ratios)

ABSTRACT

Objective: This study aims to evaluate the effectiveness and role of telomere length measurements in leukocytes, plasma free cell DNA (cfDNA), and biopsy cells, along with Tc-99m MIBI scintigraphy, as non-invasive methods for diagnosing malignant thyroid lesions.

Method: Data from 128 patients, who underwent ultrasound, Tc-99m MIBI scintigraphy, and fine-needle biopsy with a preliminary diagnosis of malignant thyroid nodules, were analyzed. In 98 patients, telomere lengths in leukocytes (from blood), cfDNA (from plasma), and biopsy cells were measured using the Q-PCR method, and the relative T/S ratio was calculated. Based on cytological examination results, patients were categorized into three groups: malignant, benign, and suspicious. Group differences were analyzed using the Kruskal-Wallis and Chi-square tests, and correlations between variables were examined with Spearman correlation analysis.

Results: The sensitivity, specificity, positive predictive value, negative predictive value, and accuracy of Tc-99m MIBI scintigraphy for diagnosing malignant thyroid nodules were 64.70%, 79.16%, 29.72%, 83.51%, and 67.96%, respectively. While these results align with the literature, the positive predictive value was notably lower. No significant differences were observed in telomere lengths (T/S ratios) in leukocytes, plasma, or tissue between the groups.

Conclusion: Tc-99m MIBI scintigraphy demonstrates reasonable diagnostic accuracy for identifying malignancy in thyroid nodules. Contrary to limited reports, telomere length measurements may not be a reliable method for predicting thyroid malignancy. Larger studies are needed to further explore these findings.

Keywords: Thyroid cancer, Tc-99m MIBI Scintigraphy, cfDNA, Liquid biopsy, ctDNA, Telomere

ÖZ

Amaç: Bu çalışma, lökositlerde, plazma serbest hücre DNA'sında (cfDNA) ve biyopsi hücrelerinde telomer uzunluğu ölçümleri ile Tc-99m MIBI sintigrafisinin, kötü huylu tiroid

lezyonlarının teşhisinde non-invaziv yöntemler olarak etkinliğini ve rolünü değerlendirmeyi amaçlamaktadır.

Yöntem: Malign tiroid nodülleri ön tanısı konulan ve ultrason, Tc-99m MIBI sintigrafisi ve ince iğne biyopsisi uygulanan 128 hastanın verileri analiz edilmiştir. 98 hastada, lökositlerde (kandan), cfDNA'da (plazmadan) ve biyopsi hücrelerinde telomer uzunlukları Q-PCR yöntemiyle ölçülmüş ve göreceli T/S oranı hesaplanmıştır. Sitolojik inceleme sonuçlarına göre hastalar; kötü huylu, iyi huylu ve şüpheli olmak üzere üç gruba ayrılmıştır. Gruplar arası farklar Kruskal-Wallis ve Ki-kare testleriyle analiz edilmiş, değişkenler arasındaki korelasyonlar Spearman korelasyon analizi ile değerlendirilmiştir.

Bulgular: Tc-99m MIBI sintigrafisinin kötü huylu tiroid nodüllerini teşhis etmedeki duyarlılık, özgüllük, pozitif öngörü değeri, negatif öngörü değeri ve doğruluk oranları sırasıyla %64,70, %79,16, %29,72, %83,51 ve %67,96 olarak bulunmuştur. Bu sonuçlar literatürle uyumlu olmakla birlikte, pozitif öngörü değerinin belirgin şekilde düşük olduğu gözlemlenmiştir. Lökosit, plazma veya doku örneklerindeki telomer uzunlukları (T/S oranları) açısından gruplar arasında anlamlı bir fark bulunmamıştır.

Sonuç: Tc-99m MIBI sintigrafisi, tiroid nodüllerinde malignitenin tespitinde makul bir tanısal doğruluk sunmaktadır. Ancak sınırlı raporlara rağmen, telomer uzunluğu ölçümlerinin tiroid malignitesini öngörmede güvenilir bir yöntem olmayabileceği anlaşılmaktadır. Bu bulguların daha kapsamlı çalışmalarla araştırılması gerekmektedir.

Anahtar Kelimeler: Tiroid kanseri, Tc-99m MIBI Sintigrafi, cfDNA, Sıvı biyopsi, ctDNA, Telomer

Introduction

It is crucial to determine the functional status of the detected thyroid nodules and whether they are malignant. For this purpose, thyroid function tests, ultrasound (US) findings, and scintigraphic evaluations are conducted on patients (1-2).

Although thyroid function tests provide valuable information about the functional status of the thyroid gland and nodules, they do not indicate the presence of malignancy. Ultrasound imaging can assess the size, echogenicity, structure, presence of micro- and macro-calcifications, margin irregularity, peripheral halo, shape, and vascularity of nodules. Among

these features, hypoechogenicity, microcalcifications, and irregular margins are the characteristics most strongly associated with malignancy (3-4). Additionally, thyroid ultrasound can evaluate the presence of pathological lymph nodes in the neck or signs of invasion in surrounding tissues. However, no ultrasound finding alone is sufficiently sensitive and specific for detecting malignancy (5).

Another method used to assess the malignancy potential of thyroid nodules is thyroid scintigraphy. In thyroid scintigraphy with Tc-99m MIBI (Methoxyisobutylisonitrile), uptake rates and washout levels can provide insights into the presence of malignancy in nodules (6). Tc-99m MIBI is a tracer used to detect P-glycoprotein function and inhibition in vivo, which is associated with the multidrug resistance mechanism in tumors. The multidrug resistance P-glycoprotein (MDR1 Pgp) affects the uptake of MIBI in tumor cells, making it a preferred agent for differentiating benign from malignant thyroid nodules (6). Studies have shown that the likelihood of malignancy increases in hypofunctioning nodules with MIBI uptake, while nodules without uptake are generally benign. In another study, 35% of nodules showing uptake with Tc-99m MIBI were found to be malignant (7).

In recent years, liquid biopsy has emerged as an important innovation in distinguishing between benign and malignant thyroid nodules (8). Liquid biopsy enables the non-invasive detection of biological markers, such as cell-free DNA (cfDNA) released by tumors, in body fluids like blood. cfDNA can provide crucial insights into the presence of malignancies. These biomarkers reflect the genetic characteristics and mutations of the tumor, and can be used for cancer diagnosis, prognosis evaluation, monitoring treatment response, and determining those at high risk (9-10). Telomeric DNA lengths are a significant parameter in cancer biology, as changes in telomere length have been shown to be associated with tumor formation and progression. Telomeres are protective structures located at the ends of chromosomes that prevent cellular aging and apoptosis. The majority of cancer cells express the enzyme telomerase, which lengthens telomeres, granting the cells immortality. In particular, the telomerase enzyme is upregulated in many malignancies, including thyroid cancer. Therefore, telomere length and telomerase activity are being explored as potential biomarkers for cancer diagnosis. The release of cfDNA into the bloodstream is related to increased cell death and tumor microcirculation in cancer cells. However, it has also been shown that cfDNA can increase in other conditions, such as trauma, inflammation, and myocardial infarction, so the specificity of cfDNA measurements for cancer must be interpreted with caution (11-12).

Traditional methods used to detect malignancy in thyroid nodules, such as fine-needle aspiration biopsy (FNAB), are invasive techniques. However, since these methods take

samples from only specific parts of the tumor tissue, they may not fully reflect tumor heterogeneity (13-14). This can lead to ambiguous or uncertain results.

For these reasons, our study aims to investigate the diagnostic accuracy and role of non-invasive methods, including Tc-99m MIBI scintigraphy and telomere length measurements (a form of liquid biopsy), in detecting malignant thyroid lesions.

Methods

Study Population

A total of 128 patients were included in the study at Nuclear Medicine Clinic, who underwent Tc-99m MIBI thyroid-tumor scan scintigraphy due to suspected malignancy, fine-needle aspiration biopsy in the Endocrinology clinic, and cfDNA and telomere length measurements in blood, plasma, and biopsy samples in the Medical Genetics laboratory. While all patients underwent scintigraphy and biopsy, telomere lengths were measured in 98 of them. Patients with thyroiditis and insufficient biopsy material were excluded from the study.

The groups were formed based on the Bethesda classification (I: Non-diagnostic/unsatisfactory, II: Benign (colloid and follicular cells), III: Atypia of undetermined significance/follicular lesion of undetermined significance (follicular or lymphoid cells with atypical features), V: Suspicious for malignancy, VI: Malignant).

Patients with a Bethesda Class I diagnosis were excluded from the study. Those with a Bethesda Class II diagnosis were categorized as the benign group. Patients in Bethesda Classes III, IV and V were grouped as the suspicious group. Lastly, those with a Bethesda Class VI diagnosis were classified as the malignant group.

All participants were informed about the study prior to the collection of blood and biopsy samples, and they were included after obtaining signed consent forms. This study received ethical approval from the Clinical Research Ethics Committee of the Faculty of Medicine, XXX University, with the decision dated 12/03/2020 and numbered 04-01.

Tc-99m MIBI Scintigraphy

The Tc-99m MIBI scintigrahies were obtained by using a dual-head gamma camera system (Infinia, General Electric Medical Systems, Milwaukee, Wisconsin, USA) with a low-energy, parallel-hole, high-resolution collimator, a 20% window width, and a 140 KeV photopeak of Tc-99m. Early planar, 15. minutes SPECT and 2. hour planar imaging were performed after

the injection (0.31 mCi/kg sestamibi). The Tc-99m MIBI scintigraphy images were evaluated by two nuclear medicine experts. Lesions were categorized as "isoactive" if their uptake was equal to or less than the background activity and/or thyroid tissue uptake, and "hyperactive" if the lesion's uptake was greater than the background activity and/or thyroid tissue activity.

Measurement of Telomere Length by Q-PCR Method

Preparation of Standards

In all studies, DNA obtained from a venous blood sample taken from a healthy young individual was used as a calibrator. In studies normalized with the calibrator, a standard curve is not necessary for each experiment. Since the target/reference gene ratios of the samples were normalized with the calibrator target/reference gene ratio, the results are only affected by the different PCR efficiencies of the target and reference genes. To eliminate this effect, the PCR efficiency can be adjusted to 2 during analysis.

Inclusion of Samples in Telomere and Betaglobulin PCR Analysis

Primers synthesized by HPLC were diluted to 100 pmol/µl as main stocks, and intermediate stocks of 10 pmol/µl were prepared and stored at -20°C for PCR. The samples were subjected to PCR using telomere and beta-globin primers. The samples were analyzed using the Light Cycler 2.0 (Roche) device. The quantification process was performed on the FReal-time PCR (Roche Light Cycler 2.0) device. Samples were analyzed in duplicates.

Quantitative Assessment of Telomere Length

When PCR efficiency is assumed to be 100%, the telomere/single copy gene (T/S) ratio is calculated as follows:

$$\text{T/S ratio}=[2^{\text{Ct(telomer)}} / 2^{\text{Ct(beta globin)}}]^{-1} = [2^{[\text{Ct(telomer)-Ct(beta globin)}]}]^{-1} = 2^{-\Delta Ct}$$

$$\Delta Ct=^{[\text{Ct(telomer)-Ct(beta globin)}]}$$

$$\text{Relatif T/S ratio}=2^{-(\Delta Ct1 - \Delta Ct2)} = 2^{-\Delta\Delta Ct}$$

$$\Delta\Delta Ct= \Delta CtT-\Delta Ct \text{ calibrator}$$

Statistical Analysis

The data of the study were transferred to the SPSS 26.0 software for electronic analysis. Data control and analyses were conducted using this program. For the presentation of descriptive statistics, percentages, mean, standard deviation, median, minimum, and maximum values were used. Statistical evaluations included Chi-Square, Kruskal-Wallis, and Spearman correlation analyses. To determine the cutoff points for the relative T/S ratio values of whole

blood, plasma, and tissue in detecting malignancy, ROC Curve analysis was performed, and graphs were presented. A p-value of <0.05 was considered statistically significant.

Results

Descriptive Findings

A total of 128 patients, including 101 women (%79) and 27 men (%21) aged between 19 and 85 years (mean age 52.66±13.14), were included in our study. All patients had results from Tc-99m MIBI thyroid scintigraphy and thyroid biopsies, while the telomere lengths in blood, plasma, and tissue could be measured genetically in 98 patients (Table1).

According to the pathology results, the average age (min-max) and gender distribution of the cases divided into 3 groups. No statistically significant difference was found between the groups regarding age (p=0.544) and gender (p=0.083) (Table 1).

Comparison of Ultrasonographic Features of Thyroid Nodules

When comparing the nodule diameters among these three groups, no significant difference was found (p=0.456) (Table 1). Our study population consisted of patients categorized as TIRADS 3-4-5 according to current guidelines indicating the need for biopsy. When the distribution of TIRADS categories in the benign, suspicious, and malignant groups was examined, it was found that the benign group had the highest proportion of TIRADS 3 (54.16%), the suspicious group had the highest proportion of TIRADS 4 (40%), and the malignant group had the highest proportion of TIRADS 5 (70,58%), which is a clinically expected result. As the risk of malignancy increases within the TIRADS categories, the likelihood of obtaining a malignant pathological result also increases. Statistical evaluation revealed that this difference is significant. (p=0.001) (Table 1).

Among the patients evaluated as TIRADS 3-4-5 on ultrasound and underwent biopsy, the cytological results obtained according to the Bethesda classification showed that 96 patients had benign results, 11 patients had atypia of undetermined significance/follicular lesion of undetermined significance (AUS/FLUS), 3 patients had follicular neoplasia or suspicion (including Hurtle cell type), 1 patient had suspicion of malignancy, and malignancy was reported in 17 patients (Table 2). Of the 17 cases with thyroid malignancy identified, 14 were papillary carcinoma, 2 were follicular variant papillary carcinoma, and 1 was primary thyroid lymphoma based on histopathological type.

Comparison of Tc-99m MIBI Scintigraphy Results

The power and adequacy of Tc-99m MIBI thyroid scintigraphy, performed to investigate the presence of malignancy in thyroid nodules, were statistically evaluated. The specificity of Tc-99m MIBI thyroid scintigraphy in detecting benign nodules was calculated as 79.16%, and the negative predictive value was 83.51%. The sensitivity of Tc-99m MIBI thyroid scintigraphy in detecting malignant nodules was calculated as 64.70%, and the positive predictive value was 29.72%. Additionally, the diagnostic accuracy of Tc-99m MIBI thyroid scintigraphy was found to be 67.96% (Table 3).

In the suspicious group (15 individuals), among the biopsied nodules, 6 (%40) were classified as isofunctional, and 9 (%60) were classified as hyperfunctional. Due to the inability to access subsequent biopsy results or postoperative biopsy results for the patients in this group, the validity and reliability of scintigraphy in this group could not be calculated.

Figure 1 shows examples of true positive, false positive, true negative, and false negative patients in Tc-99m MIBI thyroid scintigraphy. The image of a primary thyroid lymphoma case, identified apart from differentiated thyroid malignancies, is shown in Figure 2, where early and late images revealed radioactivity retention in a firm, fixed nodule covering the entire thyroid. Additionally, a metastatic right supraclavicular lymph node, confirmed histopathologically and showing Tc-99m MIBI retention in both early and late images, was identified.

Comparison of Telomere Lengths in Blood, Plasma, and Tissue

There was no statistically significant difference in the relative T/S ratio values of whole blood, plasma, and tissue among the groups (Table 4). Although no statistical difference was found, it was observed that the relative T/S ratio values in whole blood for patients in the malignant group were larger than those in the other groups. Conversely, for the plasma relative T/S ratio values, higher average values were obtained in the benign group compared to the suspicious and malignant groups. Additionally, the close similarity of the average tissue relative T/S ratios in the malignant and benign groups is noteworthy. When examining telomere lengths in all three groups, the order of telomere lengths was found to be Whole Blood > Plasma > Tissue in each group (Table 5).

On the other hand, in the ROC curve analyses, the area under the curve (AUC) for the whole blood relative T/S ratio was found to be AUC=0.507; for the plasma relative T/S ratio, AUC=0.558; and for the tissue relative T/S ratio, AUC=0.507. These values indicate insufficient diagnostic accuracy (Table 6,7,8).

Discussion

Due to the limitations of existing methods for detecting thyroid cancer, research on new non-invasive approaches is ongoing. In this study, we aimed to evaluate the role of scintigraphic imaging with Tc-99m MIBI and DNA telomere length measurement in distinguishing malignant from benign thyroid nodules.

Tc-99m MIBI scintigraphy, used in the evaluation of malignancies such as lung, breast, brain, lymphoma, bone, and soft tissue cancers, has also been investigated for predicting thyroid cancers. The studies reported show significant differences in diagnostic performance values, in addition to heterogeneity in inclusion criteria and methods used (15-17). Overall, Tc-99m MIBI scintigraphy appears to be a cost-effective method that contributes to the evaluation of thyroid nodules. However, current guidelines do not recommend the use of Tc-99m MIBI scintigraphy to predict the cancer risk of thyroid nodules (6,18,19).

In a meta-analysis by Treglia G et al., which included 21 studies, the pooled sensitivity and specificity of Tc-99m MIBI scintigraphy in detecting malignant thyroid nodules were 85.1% and 45.7%, respectively, per lesion, regardless of previous technetium pertechnetate or iodine-123 scan results. When a limited sub-analysis was performed with data on hypofunctioning nodules from Tc-99m pertechnetate or iodine-123 scans, the pooled sensitivity and specificity were 82.1% and 62.8%, respectively. The researchers concluded that this imaging method could be helpful in patients with thyroid nodules suspected of malignancy based on conventional diagnostic techniques, and that higher specificity could be achieved in hypofunctioning thyroid nodules (7,20). Based on a prospective study results, the researchers stated that Tc-99m MIBI scintigraphy was significantly more accurate than mutation analysis and that a negative Tc-99m MIBI scan could reliably rule out malignancy.[18] Another study reported that a strong negative relationship was found between Tc-99m MIBI uptake in thyroid lesions and multidrug resistance-associated protein-1 (MRP1) (21).

Although many studies have reported a high sensitivity, Tc-99m MIBI scintigraphy has low specificity and is not recommended as a first-line investigation. Instead, it is suggested as a second-line test to help reduce unnecessary surgeries (22,23).

In our study, we found the sensitivity, specificity, positive predictive value, negative predictive value, and accuracy of Tc-99m MIBI scintigraphy in distinguishing between malignant and benign thyroid nodules to be 64.7%, 79.16%, 29.72%, 83.51%, and 67.96%, respectively. Our results are generally consistent with those reported in the literature; however, the heterogeneity in patient selection and methods used in the studies makes direct comparison difficult.

One liquid biopsy method measures the telomere lengths of cells, focusing on two primary telomere repair mechanisms: telomerase activation and the alternative lengthening of telomeres (ALT) pathway, which functions independently of telomerase. While 85-95% of cancer cells express telomerase, 5-15% activate the ALT pathway (24-25). Telomerase is active in embryonic and stem cells and is upregulated in over 90% of cancers, including thyroid cancer, allowing indefinite replication of cancer cells. Telomerase comprises two subunits: telomerase RNA and telomerase reverse transcriptase (TERT) (26). TERT mutations occur in 11.3% of well-differentiated papillary thyroid cancers, 17.1% of follicular thyroid cancers, 32% of widely invasive Hurthle cell carcinomas, 43.2% of poorly differentiated thyroid cancers, and 40.1% of anaplastic thyroid cancers (27).

In a meta-analysis by Ma H et al., which included 21 studies, shorter telomeres were found to be significantly associated with cancer risk, particularly in cancers related to smoking, such as bladder and lung cancers, as well as cancers of the digestive and urogenital systems (28). Telomerase activity was reported to increase and telomere length to decrease in thyroid cancer tissues and follicular adenomas compared to normal peritumoral tissues. In a study by Capezzone M et al., the average relative telomere length of familial papillary thyroid carcinoma (PTC) patients was found to be short in neoplastic thyroid tissues (0.87 ± 0.2), but no significant difference was observed compared to normal contralateral thyroid tissues (0.85 ± 0.11) and extrathyroidal tissues (0.85 ± 0.31). Conversely, in patients with sporadic PTC, the average relative telomere length was significantly shorter in neoplastic tissues (1.73 ± 0.63) than in normal contralateral tissues (2.58 ± 0.89) and extrathyroidal tissues (2.5 ± 0.86) (29). Caria P et al. found that the relative telomere length was significantly shorter in familial PTC samples than in sporadic PTC samples (mean 0.93 vs. 1.9). In a study comparing telomere lengths among thyroid cancer subgroups, very short telomeres were detected in 12 out of 15 PTCs (80%), 1 out of 4 follicular carcinomas (25%), all 3 Hurthle cell carcinomas (100%), and 4 out of 12 FVPTCs (33%) when compared to adjacent normal thyroid tissue. In the same study, the average telomere lengths of thyroid tumors were statistically significantly shorter in the following order: PTC, FC/HC, FVPTC, FA/HA, and hyperplastic nodules (30). Another study found that the TINF2 gene was associated with melanoma and PTC, and, in contrast to other studies, increased telomere length was observed in patients with these mutations (31).

In a prospective study measuring telomere length in leukocytes, a reduced average relative telomere length was found to be significantly associated with a higher risk of PTC (32). Another regression analysis investigating telomere length in leukocytes found that shorter

relative telomere length was significantly associated with an increased risk of renal cell carcinoma (RCC), with an RTL of 3.18 ± 1.50 in the RCC group compared to 4.39 ± 1.99 in the control group (33). In a study measuring telomere length in hematological malignancies, patients with hematological malignancies had shorter average relative telomere lengths. This was observed in both familial and non-familial cases of hematological malignancies (34).

A study investigating the relationship between cfDNA levels and various biological and lifestyle factors found that acute exercise, pesticide exposure, and stress increased cfDNA. Smoking, body mass index, hypertension, circadian rhythm, gender, age, and chronic exercise caused inconsistent changes, ionizing radiation decreased cfDNA, and alcohol consumption and menstruation did not cause any changes (35).

In studies on cfDNA telomere lengths during cancer development, shortened telomeres in serum samples were linked to increased gastric cancer risk and early breast cancer detection. Shorter telomeres were also observed in patients with ductal carcinoma in situ (34-35). In HBV patients, longer telomeres in baseline serum were associated with HCC risk. While shortened telomeres are known to promote cancer by inducing chromosomal instability, recent studies suggest that both shortened and abnormally elongated telomeres are linked to various cancers (36).

Unlike the reported studies, our research compared telomere lengths in leukocytes, plasma cfDNA, and tumor cells, finding no statistically significant difference between relative T/S values across these groups. Although not statistically significant, the relative leukocyte T/S value was found to be higher in the malignant group compared to other groups. The relative cfDNA T/S value was longer in the benign group than in the suspicious and malignant groups. Notably, the average relative T/S ratios in tissues were very close between the malignant and benign groups.

Study Limitations

In our study, the small total number of patients and subgroup sizes limited the ability to conduct reliable statistical analyses. Therefore, further prospective, case-controlled studies with larger sample sizes and standardization of methods are needed.

In conclusion

In this study, we investigated the validity and reliability of non-invasive methods, such as Tc-99m MIBI scintigraphy and DNA telomere length measurements, in predicting the presence of malignancy in thyroid nodules.

The results indicate that Tc-99m MIBI scintigraphy has a reasonable diagnostic performance in predicting the risk of malignancy in thyroid nodules, in line with the literature. With its high negative predictive value, a negative Tc-99m MIBI scan can reliably rule out malignancy.

According to our findings, the relative telomere lengths in blood (leukocytes), plasma (cfDNA), and tissue samples did not provide statistically significant results in distinguishing between malignant and benign groups. This finding is inconsistent with the limited literature suggesting that relative telomeric length may be a valid method for predicting thyroid malignancy.

In the evaluation of malignancy in thyroid nodules, Tc-99m MIBI scintigraphy appears to be a more accurate and cost-effective method compared to telomere length analysis.

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