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Serum Microrna222 And Nuclear Factor Erythroid 2–Related Factor 2 (Nrf2) As Biomarkers For The Diagnosis Of Papillary Thyroid Carcinoma

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Abstract
Papillary thyroid carcinoma (PTC) is the most common type of thyroid cancer, yet its diagnosis still faces challenges, particularly in distinguishing between benign and malignant cases using traditional methods such as fine-needle aspiration biopsy (FNAB). This study aimed to evaluate the potential diagnostic roles of microRNA-222 and Nrf2 as biomarkers in the detection of PTC. A total of 87 samples were analyzed, divided into three groups: PTC patients (n=16), patients with benign thyroid diseases (n=39), and healthy controls (n=32). Serum levels of Nrf2 were measured using ELISA, while microRNA-222 expression was analyzed using RT-PCR. The results revealed a significant decrease in serum Nrf2 and Folding Change microRNA-222 levels in PTC patients compared to healthy individuals, while Ct.microRNA-222 showed limited diagnostic value. ROC analysis showed that FC microRNA-222 had the highest sensitivity (75%) but lower specificity (48.72%). These findings suggest that both Nrf2 and FC microRNA-222 may serve as promising supplementary biomarkers to improve the diagnostic accuracy of PTC and help differentiate malignant from benign thyroid conditions.
Keywords: *Papillary Thyroid Carcinoma (PTC), microRNA-222, Nrf2, Biomarkers, Thyroid Nodules, ELISA, Gene Expression, ROC Curve*

INTRODUCTION:

The most common subtype of thyroid cancer is called papillary thyroid carcinoma (PTC) (1). Is a disease where the cells of the thyroid gland grow abnormally and can spread to other parts of the body if left untreated (2). This cancer of the epithelium has unique nuclear characteristics and follicular cell differentiation (3). From 4.9 per 100,000 in 1975 to 13.9 per 100,000 in 2023, the incidence of thyroid cancer has risen over the past 50 years (4). According to the Annual Report of the Iraqi Cancer Registry (2022), PTC is the predominant form of thyroid cancer, accounting for about 88.3% of all thyroid cancer cases which is the fourth most common cancer in Iraq (5). In 2020, thyroid cancer caused 43,646 new deaths worldwide and 586,202 (3%) new cases (6). Exposure to ionizing radiation during childhood has been the most thoroughly studied contributing risk factor in the development of thyroid cancer, female gender and a family history of thyroid cancer are factors that increase the risk for developing PTC after radiation exposure (7). Fine needle aspiration biopsy (FNAB) is considered to be the most dependable technique for assessing thyroid nodules (8). FNAB is highly sensitive thyroid morphological examination when used for differentiation between benign and malignant thyroid nodules (9). However, it is ,an invasive procedure and has a number of potential complications (10). In recent years, many researchers have increasingly focused their interest on the molecular biomarkers of PTC with the aim of providing improved diagnostic accuracy and avoiding unnecessary surgical treatments (11) . MicroRNAs (miRNA) are a class of endogenous noncoding RNA molecules. Mature miRNAs are small, single stranded RNA molecules that have a length 18 to 22 nucleotides in length (12) . Numerous miRNAs have now been shown to be paly a role in carcinogenesis, cancer progression, and prognosis either as tumor suppressor genes or as oncogenes (13). Recently several studies

have illustrated the association of miR-222 dysregulation with cancer initiation and progression (14)- (15) . NRF2 as a crucial cytoprotective factor, positively regulates a number cancer hallmarks, including the promotion of metastasis, evasion of cell apoptosis, and metabolic alteration. In normal cells, activation of NRF2 complies with the period of acute detoxification; whereas in cancer cells, a newly described phenomenon called "NRF2 addiction" occurs (16) . Activation of Nrf2 is generally known to play a dual role in cancer, on the one hand protecting normal cells against oxidative damage and thereby preventing carcinogenesis, while on the other hand conferring a survival advantage to established cancer cells (17). Nrf2 is commonly activated in PTC, where it regulates the antioxidant defense and promotes the survival of cancer cells (18) . Blood samples are simple to obtain and less invasive, making this source of miRNA attractive for exploring the role of potential circulating biomarkers (19). The identification and using of molecular biomarkers in cancer is to improve the diagnostic precision and help physicians in the preoperative management of patients (20). This study aimed to investigate the diagnostic potential of serum miR-222 and Nrf2, and their correlation with the clinical staging and grading of PTC.

MATERIALS AND METHODS:

This study includes a case-control study for a group of (87) samples: (16) samples for patients with papillary thyroid carcinoma, (39) samples for patients with benign thyroid diseases and (32) healthy control samples . Patients with other types of thyroid cancer or non-specified thyroid diseases were excluded . The study was conducted from October 2024 to September 2025, cases of thyroidectomy for different causes were analyzed ,regarding sex, age and type of thyroid pathology ,whether benign or malignant at Safeer Al-Imam Al-Hussain(A.S)Surgical Hospital and Al-Kafeel Superspeciality Hospital in Kerbala city. The sociodemographic aspects of the patients were collected through the self-reported technique (student questionnaire) including age, gender, history of family, smoking state, job, duration of disease also weights and heights . laboratory investigations was measured for patients such as thyroid function test(TFT) , random blood sugar (RBS), HbA1c , and complete blood count (CBC) .The surgical procedure was performed on all patients, and final diagnoses were based upon pathological examination whose formalin-fixed paraffin embeded confirmed PTC (based on examination of nuclear features and histopathological finding) or benign nodules was recorded. Regarding the ethical approval, the hospital ethics committee approved the study plan, and all patients or their relatives were informed. The Ethical Committee at Kerbala University- College of Medicine issued an ethics certificate(No. 24-64 dated October10, 2024), The kerbala health directorate approved our study ,No.3672 on October 21, 2024 and Al-Kafeel Superspeciality Hospital in Kerbala city gave their approval to study plan, No. 4562 on December 2 . Verbal approval was taken from all patients included in the study.

The vein samples were collected from all cases and control. 5 mL of blood was drowned from patients pre operation using 5 ml disposable syringes, blood samples were aliquot into EDTA tube then stored in deepfreeze at -80C◦for microRNA222 and gel tube for nuclear factor erythroid2-related factor2(Nrf2) biomarker measurement . Gel tube samples left for 15min at room temperature and serum was separated by centrifuging for 10 minutes at approximately 4000 x g . Serum samples were aliquot into two eppendroff and store at -80°C to avoiding multiple freezing-thawing cycles and used to check the level of(Nrf2) by ELISA test. MicroRNA-222 signatures in all samples and control was analysis using TransScript Green One-step RT/Rl Enzyme Mix (Korea) was used for total RNA mesearment according to the manufacturer's instructions. Total RNA was purified using an RNAClean XP Kit and RNase-Free DNase Set., and Nrf2 levels were measured by ELISA test using Elabscience® Human NFE2L2(Nuclear Factor ,ErythroidDerived2,like2) Elisa Kit (USA).

Statistical Analysis: Information from the questionnaire from all participants was entered into a data sheet and was assigned a serial identifier number. Multiple entries were used to avoid errors. The data analysis for this work was generated using graphical pad prism9. Descriptive statistics were performed on the participants' data of each group. Values were illustrated by n (%) for categorical. The distribution of the data was checked

using the Shapiro-Wilk test as numerical means of assessing normality. Significant differences in categorical variables among the parameters were confirmed through analytical statistical tests. Results of all hypothesis tests with p-values <0.05 (two-side) were considered to be statistically significant. The optimal threshold with high specificity and sensitivity for PTC was detected using receiver operating characteristic (ROC) analysis.

RESULTS:

The demographic characteristics of the study groups, including healthy controls, benign hyperplasia patients, and papillary thyroid carcinoma (PTC) patients, are presented in Table (1).

The Healthy Control Group consisted of individuals with a mean age of 39.09 ± 14.33 years and a mean Body Mass Index (BMI) of 27.42±4.39. The gender distribution in this group was predominantly female, comprising 26 individuals (81.3%), while males accounted for 6 individuals (18.7%). The Benign Hyperplasia Group comprised 39 participants, accounting for 44.8% of the total study population. For the Papillary Thyroid Carcinoma (PTC) Group, the table indicates that 16 individuals were classified as PTC Stage 1, representing 18.4% of the total study population.

Patients Groups consisted of individuals with a mean age of 44.81 ± 10.26 years. The gender distribution within this group showed a strong female predominance, with 48 individuals (86%) being female and 8 individuals (14%) being male. The mean Body Mass Index (BMI) for the patients' groups was 26.46 ± 4.40 Kg/m^2

Table (1) Demographic characteristics of study groups (benign hyperplasia, papillary thyroid carcinoma patients and healthy control)

		Patients Groups	Healthy control group
Age		44.81±10.26	39.09±14.33
Gender	**Male**	(8) , 14%	(6),18.7%
	Female	(48),86%	(26),81.3%
BMI		26.46±4.40	27.42±4.39
Study Groups	**Benign group**	(39) ,44.8%	(32) , 36 .8%
	PTC stage1	(16) , 18.4%	

Serum Levels of Nuclear Respiratory Factor 2 (Nrf2), Ct.microRNA222, and Folding Change microRNA222 Among Study Groups

Table (2) presents the mean serum levels and standard deviations (SD) for (Nrf2), Ct.microRNA222, Nuclear Factor Erythroid 2–Related Factor 2and Folding Change microRNA222 across the Benign, Malignant, and Healthy Control study groups.

A clear difference was observed in the serum levels of Nrf2 and Ct.microRNA222 between the healthy control group and the patient groups. The patient group exhibited significantly lower mean serum levels of Nrf2 (0.17 ± 0.13) compared to healthy controls (0.83 ± 0.61). Similarly, Folding Change microRNA222 levels were markedly reduced in patients (771 ± 677) compared to healthy individuals (7855 ± 2490). Conversely, the mean serum level of ct. microRNA222 was observed to be 20 ± 8.4 in healthy controls and remained comparable in the patient group at 26 ± 7.2.

Table (2) Mean of Serum Levels Nuclear Factor Erythroid 2–Related Factor 2 (Nrf2),Folding Change microRNA222, and CtmicroRNA222 among patients' groups (included benign thyroid hyperplasia & PTC) compared to control

Serum Biomarkers Level (Mean±SD)	Heathy control	Patients group
Nrf_2	0.83 ± 0.61	0.17 ± 0.13

Folding Change microRNA$_{222}$	7855 ± 2490	771 ± 677
Ct.microRNA$_{222}$	20± 8.4	26 ± 7.2

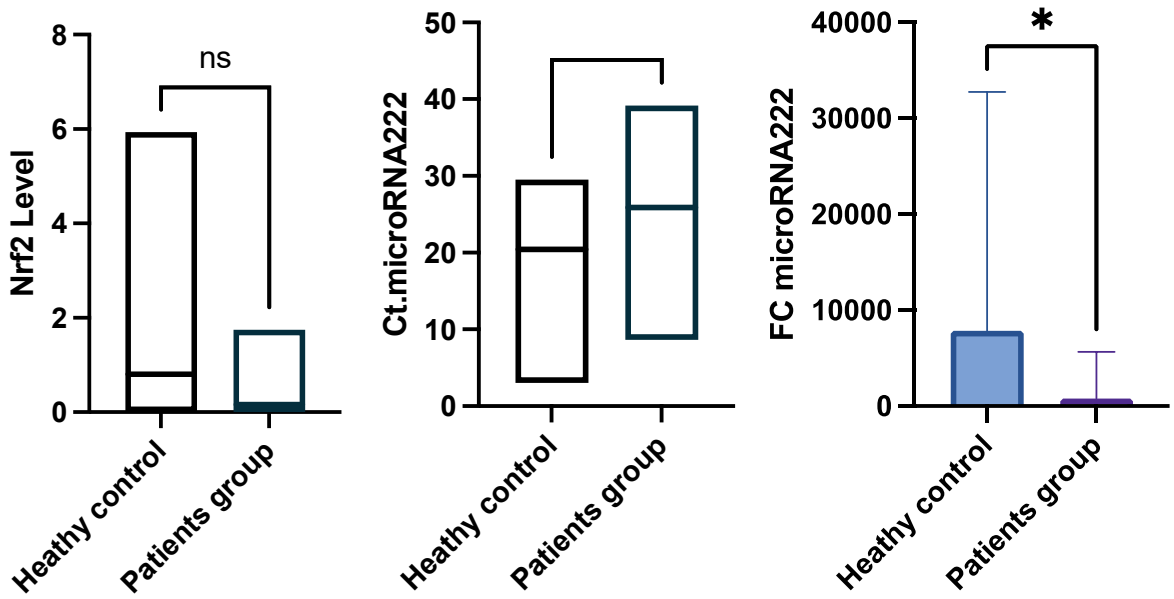

Figure (1) Distribution of Serum Levels of Nuclear Factor Erythroid 2–Related Factor 2 (Nrf2), and Folding Change microRNA222 among patients groups(included benign thyroid hyperplasia & PTC) compared to control (t- test was *: significant at p ≤ 0.05, **: significant at p ≤ 0.01, *: significant at p ≤ 0.001), ****: significant at p ≤ 0.0001**

On the other hand, a clear pattern emerged for Nrf2 levels, both the benign and malignant patient groups exhibited significantly lower mean serum Nrf2 levels compared to the healthy control group. The Nrf2 levels in the benign (0.17 ± 0.35) and malignant (0.16 ± 0.21) groups were very similar to each other, indicating a general reduction in Nrf2 in thyroid pathology compared to healthy group (0.80 ± 0.3), as presented in Table (3)

For Folding Change microRNA222 in healthy control group was (13.3 ± 8.8) and in benign group was (13 ± 11.4) both showed remarkably similar mean levels. However, the malignant group presented a drastic reduction in Folding Change microRNA222 (1.01 ± 0.9) when compared to both the healthy controls and the benign group. This might reflect that Folding Change microRNA222 might serve as a distinguishing biomarker for malignancy.

The consistently low serum Nrf2 levels observed in both benign and malignant thyroid patient groups, when compared to healthy controls, reinforce the notion that a compromised Nrf2 pathway is associated with thyroid disease.

Table (3) Serum Levels of Nuclear Factor Erythroid 2–Related Factor 2 (Nrf2), and Folding Change microRNA222 among study groups

Study Groups	**Serum Biomarkers Level** (Mean±SD)	
	Nrf_2	Folding Change microRNA$_{222}$
Benign	0.17 ± 0.35	13 ± 11.4
Malignant	0.16 ± 0.21	1.01 ± 0.9

Healthy control	0.80 ± 0.3	13.3 ± 8.8

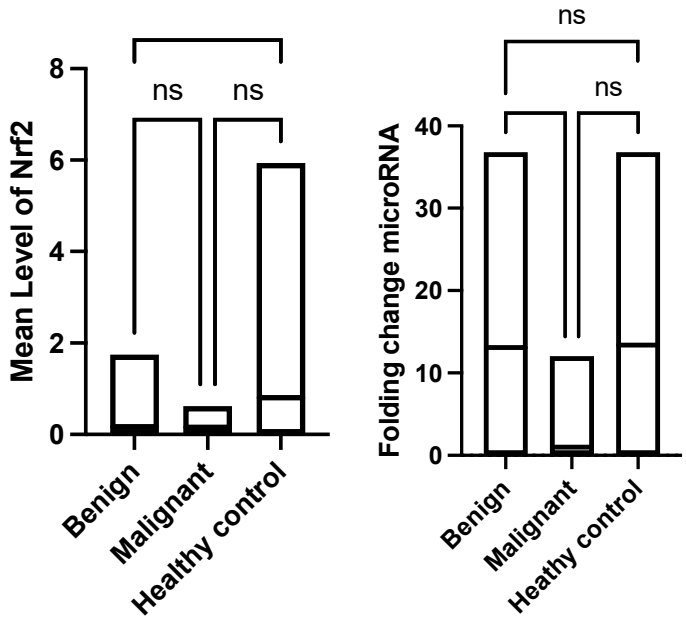

Figure (2) Distribution of Serum Levels of Nuclear Factor Erythroid 2–Related Factor 2 (Nrf2), and Folding Change microRNA222 among study groups (Post Hoc ANOVA test was *: significant at p ≤ 0.05, **: significant at p ≤ 0.01, *: significant at p ≤ 0.001), ****: significant at p ≤ 0.0001**

Diagnostic performance of studied biomarkers in discriminating between benign hyperplasia patients and healthy controls

For differentiating between patients with benign group and healthy controls, an evaluation of the biomarkers was performed.

Through comparing the three markers, Nrf2 and FC microRNA222 exhibited comparable, albeit modest, diagnostic utility, with AUC values of 0.617 and 0.600, respectively. Both markers showed fair discrimination between the benign group and healthy controls. In terms of sensitivity, FC microRNA222 had the highest sensitivity (75%), suggesting it is better at correctly identifying individuals in the benign group. However, its specificity was the lowest (48.72%), meaning it also had a higher rate of false positives. Nrf2 showed a better balance between sensitivity (53.12%) and specificity (66.67%).

In contrast, Ct.microRNA222 performed poorly as a diagnostic marker, with an AUC of 0.515, indicating minimal ability to distinguish between the two groups. Its sensitivity (50%) and specificity (61.54%) were also the lowest among the three markers, making it an unreliable indicator in this context.

Regarding predictive values, FC microRNA222 had the highest NPV (70.37%), suggesting it is relatively good at ruling out benign conditions when the test is negative. Nrf2 had a slightly lower NPV (63.41%) but a better PPV (56.67%) compared to FC microRNA222 (54.55%). Ct.microRNA222 had the lowest PPV and NPV, further supporting its limited diagnostic utility.

The observed sensitivity of 53.12% and specificity of 66.67% for Nrf2 suggest that while Nrf2 is often modulated in benign conditions, its changes are not entirely unique or consistently pronounced enough to serve as a highly accurate standalone marker. The fair AUC indicates a detectable, but not strong, difference in Nrf2 levels between benign and healthy states, likely due to the complex interplay of stress, inflammation, and cellular responses across different benign conditions

The improved AUC (0.600) for FC microRNA222, despite still being modest, is significant. "Folding Change" implies a comparison of expression levels, likely relative to a reference gene or a control group. This suggests that while the absolute amount of miR-222 may not be a good indicator, the relative change in its expression,

perhaps reflecting a perturbation from an individual's baseline or a subtle, consistent shift within the benign group compared to controls, is more informative. This hints at a dynamic process where miR-222 expression is indeed altered, but the magnitude of this alteration, when normalized or compared, offers a better discriminatory signal. The higher sensitivity (75%) of FC microRNA222 suggests it might be better at detecting the presence of a benign condition, but its lower specificity (48.72%) means it also generates more false positives, indicating its expression is not exclusively tied to the benign state.

Table (4) AUC, optimal threshold, Sensitivity, and specificity of Serum Levels Nuclear Factor Erythroid 2–Related Factor 2 (Nrf2), Ct.microRNA222 and Folding Change microRNA222 benign Group and Healthy Control

	Cutpoint	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	AUC
Nrf2	0.063	53.12%	66.67%	56.67%	63.41%	0.617
microRNA222	24.12	50%	61.54%	51.61%	60%	0.515
FC	0.053043998	75%	48.72%	54.55%	70.37%	0.600

The classification results for Nrf2 show a relatively balanced performance in terms of true negatives (26) and true positives (17). The number of false positives (13) is slightly lower than false negatives (15). This indicates that while Nrf2 can correctly identify a fair proportion of both healthy individuals and those in the benign group, there's a tendency to miss slightly more individuals from the benign group than to misclassify healthy individuals as benign. This aligns with its moderate AUC value, suggesting a moderate ability to discriminate.

Ct.microRNA222 (Cutoff 24.12), Ct.microRNA222 demonstrates a nearly equal number of true negatives (24) and true positives (16), but with relatively high false positives (15) and false negatives (16). The close numbers for true classifications and false classifications (TN vs FN and FP vs TP) highlight its limited discriminatory power. The fact that the number of false negatives (16) is almost equal to the true positives (16) indicates a significant failure to correctly identify individuals with the benign condition. Similarly, the 15 false positives suggest a notable rate of misclassifying healthy individuals. This poor performance strongly supports the conclusion drawn from its very low AUC, indicating it's not an effective marker for distinguishing the benign group from healthy controls.

FC microRNA222 (Cutoff 0.053), FC microRNA222 shows the highest number of true positives (24) among the three markers, indicating its strong ability to correctly identify individuals in the benign group. This is consistent with its highest sensitivity (75%) reported previously. However, this comes at the cost of a higher number of false positives (20) compared to the other markers. This means that while it is good at "catching" true positives, it also frequently misclassifies healthy individuals as having the benign condition. The number of false negatives (8) is the lowest, reinforcing its high sensitivity. This pattern of high true positives but also high false positives explains its higher sensitivity but lower specificity, and its moderate AUC. It suggests that while there is a significant change in FC microRNA222 in the benign group, this change might also occur to a lesser extent or under different physiological conditions in some healthy individuals, leading to more false alarms.

Table (5) Distribution of benign Group according to Suggested Cutoff Value of Nuclear Factor Erythroid 2–Related Factor 2 (Nrf2), Ct.microRNA222 and Folding Change microRNA222

Cutoff value Nrf2			**Cutoff value: microRNA222**			**Cutoff value:FC**		
0.063			24.12			0.053		
	Negative	**Positive**		**Negative**	**Positive**		**Negative**	**Positive**
Negative	26 (TN)	13 (FP)	Negative	24 (TN)	15 (FP)	Negative	19 (TN)	20 (FP)
Positive	15 (FN)	17 (TP)	Positive	16 (FN)	16 (TP)	Positive	8 (FN)	24 (TP)

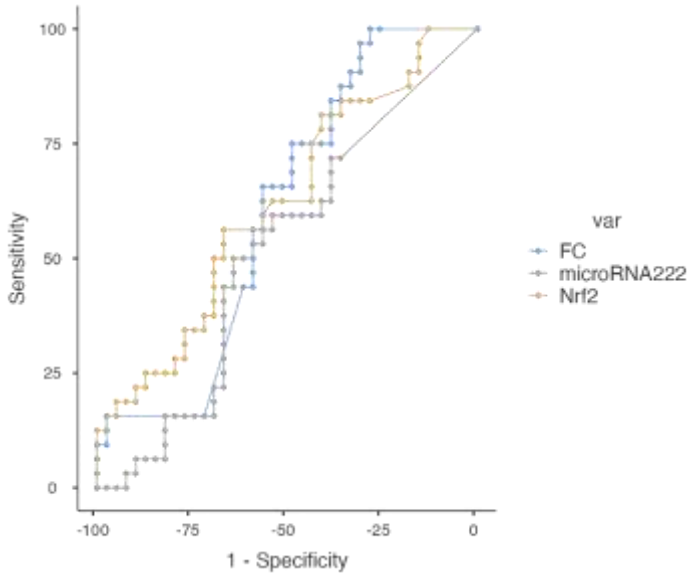

Figure (3) ROC curves of Nuclear Factor Erythroid 2–Related Factor 2 (Nrf2), Ct.microRNA222 and Folding Change microRNA222 serum levels among benign PTC Group to analyze the optimal diagnostic points for predicting such cases compared to control group

Diagnostic performance of studied biomarkers in discriminating between PTC patients and healthy controls

Table (6) presents the diagnostic performance of serum levels of Nuclear Factor Erythroid 2–Related Factor 2 (Nrf2) and Folding Change (FC) microRNA222 in differentiating between the Papillary Thyroid Carcinoma (PTC) group and healthy controls.

For Nrf2, with an optimal cutpoint of 0.03, the sensitivity was 83.33%, and specificity was 48.84%. The positive predictive value (PPV) was 31.25%, and the negative predictive value (NPV) was 91.3%. The area under the receiver operating characteristic curve (AUC) for Nrf2 was 0.672, indicating fair discriminatory ability.

FC microRNA222, at a cutpoint of 2.42, showed a sensitivity of 50% and a specificity of 65.12%. The PPV was 28.57%, and the NPV was 82.35%. Its AUC was 0.467, suggesting very poor discriminatory power, approaching that of random chance and even slightly worse than a random classifier (AUC of 0.5).

Table (6) AUC, optimal threshold, Sensitivity, and specificity of Serum Levels Nuclear Factor Erythroid 2–Related Factor 2 (Nrf2) and Folding Change microRNA222 in PTC Group compared to Healthy Control

	Cutpoint	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	AUC
Nrf2	0.03	83.33%	48.84%	31.25%	91.3%	0.672
FC	2.42	50%	65.12%	28.57%	82.35%	0.467

Table (6) demonstrated a comparison performance of Nrf2 and FC microRNA222 in distinguishing Papillary Thyroid Carcinoma (PTC) from healthy controls, distinct patterns emerge for each marker at their respective optimal cutoffs.

Results were shown that Nrf2 was characterized by high sensitivity but low specificity. It excels at identifying individuals with the disease but struggles to accurately rule out the disease in healthy individuals.

Furthermore, FC microRNA222 exhibits lower sensitivity but higher specificity compared to Nrf2. It is better at correctly identifying healthy individuals but misses a significant proportion of actual PTC cases. Its overall

performance is notably poorer, as indicated by its lower number of true positives and higher number of false negatives compared to Nrf2.

In essence, Nrf2 appears to be a better screening marker for PTC, designed to cast a wide net and ensure most cases are caught, even at the cost of more false positives. FC microRNA222, on the other hand, shows very limited diagnostic utility, performing poorly in both sensitivity and overall discrimination, making it an unreliable marker for PTC based on these findings.

Table (7) Distribution of PTC Group according to Suggested Cutoff Value of Nuclear Factor Erythroid 2–Related Factor 2 (Nrf2), and Folding Change microRNA222

Cutoff value Nrf2			**Cutoff value: FC**		
0.03			2.42		
	Negative	**Positive**		**Negative**	**Positive**
Negative	21 (TN)	22 (FP)	Negative	28 (TN)	15 (FP)
Positive	2 (FN)	10 (TP)	Positive	6 (FN)	6 (TP)

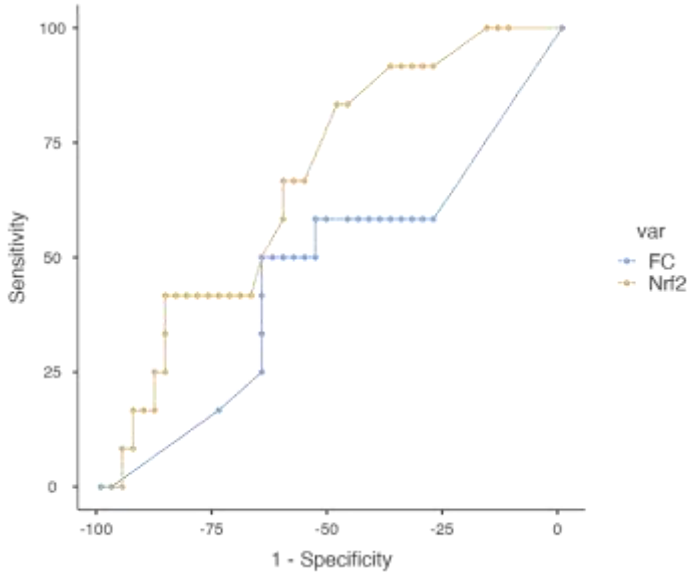

Figure (4) ROC curves of Nuclear Factor Erythroid 2–Related Factor 2 (Nrf2), and Folding Change microRNA222 serum levels among malignant PTC Group to analyze the optimal diagnostic points for predicting such cases compared to control group

DISCUSSION:

The demographic data presented show a generally acceptable balance among the study groups in terms of age and Body Mass Index (BMI), with slight variations that may reflect the pathological nature of the participants. The mean age in the healthy control group was 39.09 ± 14.33 years, while it was higher in the patient groups, reaching 44.81 ± 10.26 years. This age difference may reflect the increasing likelihood of developing thyroid disorders, such as benign hyperplasia and papillary thyroid carcinoma (PTC), with advancing age, which is consistent with findings in previous medical literature.

Regarding BMI, the groups showed relatively similar values: 27.42 ± 4.39 kg/m² in the control group compared to 26.46 ± 4.40 kg/m² in the patient groups. Despite this similarity, the potential association between overweight/obesity and certain thyroid disorders cannot be overlooked and may warrant further investigation in future studies.

As for gender distribution, the study showed a clear female predominance across all groups, especially in the patient groups, where females constituted 86% of the cases. This pattern aligns with the well-documented observation that thyroid disorders, particularly PTC and benign hyperplasia, are more common in females than in males. This is likely due to hormonal and immunological factors that increase susceptibility among women.

Serum Levels of Nuclear Factor Erythroid 2–Related Factor 2 (Nrf2), Ct.microRNA222, and Folding Change microRNA222 Among Study Groups

A clear difference was observed in the serum levels of Nrf2 and Ct.microRNA222 between the healthy control group and the patient groups. The patient group exhibited significantly lower mean serum levels of Nrf2 (0.17 ± 0.13) compared to healthy controls (0.83 ± 0.61). Similarly, Folding Change microRNA222 levels were markedly reduced in patients (771 ± 677) compared to healthy individuals (7855 ± 2490). Conversely, the mean serum level of ct. microRNA222 was observed to be 20 ± 8.4 in healthy controls and remained comparable in the patient group at 26 ± 7.2.

The observed significant decrease in serum Nrf2 levels in the patient groups (comprising benign thyroid hyperplasia and PTC) compared to healthy controls is a notable finding. Nrf2 is a master regulator of the antioxidant response, playing a crucial role in cellular protection against oxidative stress. Reduced Nrf2 levels could indicate a compromised ability of thyroid cells in these patients to counteract oxidative damage, which is often implicated in the initiation and progression of various thyroid disorders, including hyperplasia and carcinogenesis. A suppressed Nrf2 pathway might contribute to an environment conducive to cellular proliferation and malignant transformation. (21)

The drastic reduction in "Folding Change microRNA222" levels in the patient group was also significant. Folding Change microRNA222 was substantially decreased and indicated a significant dysregulation of this microRNA in thyroid disease. MicroRNAs are critical regulators of gene expression, and miR-222 has been implicated in cell proliferation, apoptosis, and differentiation in various cancers, including thyroid cancer. Its downregulation could lead to the dysregulation of its target genes, thereby influencing pathways involved in cell growth, survival, and differentiation, potentially contributing to the development or progression of thyroid hyperplasia and PTC. (22)

Conversely, the observed increased in "Ct.microRNA222" levels in the patient group (26 ± 7.2) compared to healthy controls (20 ± 8.4) was important when interpreting Ct values in the context of quantitative PCR. In qPCR, a higher Ct value indicates a lower initial concentration of the target molecule. Therefore, an increase in Ct.microRNA222 in the patient group suggests that the absolute quantity of microRNA222 is lower in these patients. This aligns with the reduction observed in "Folding Change microRNA222" both metrics were reflective of the abundance of miR-222, and indicated that miR-222 was downregulated in the patient group, suggesting its potential role as a tumor suppressor or its involvement in maintaining thyroid homeostasis, since microRNA222 biogenesis machinery plays a critical role in maintaining a differentiated thyroid state and its impairment induces an aggressive tumoral state (23)

Results were shown a significant alteration in the Nrf2 pathway and microRNA222 expression in patients with benign thyroid hyperplasia and PTC. The diminished Nrf2 levels imply compromised antioxidant defense, while the reduced microRNA222 levels (indicated by both "Folding Change" and higher "Ct" values) point to a substantial deregulation of this key regulatory molecule. These changes could play a crucial role in the pathogenesis and progression of thyroid disorders. (24)

For Folding Change microRNA222 in healthy control group was (13.3 ± 8.8) and in benign group was (13 ± 11.4) both showed remarkably similar mean levels. However, the malignant group presented a drastic reduction in Folding Change microRNA222 (1.01 ± 0.9) when compared to both the healthy controls and the benign group. This might reflect that Folding Change microRNA222 might serve as a distinguishing biomarker for malignancy.

The consistently low serum Nrf2 levels observed in both benign and malignant thyroid patient groups, when compared to healthy controls, reinforce the notion that a compromised Nrf2 pathway is associated with

thyroid disease. Nrf2 is a critical regulator of cellular defense against oxidative stress. Its downregulation in both benign and malignant conditions suggests a general susceptibility to oxidative damage in diseased thyroid tissues. This impaired antioxidant response could contribute to the initiation and progression of thyroid abnormalities, irrespective of their benign or malignant nature (25) . The lack of a significant difference in Nrf2 levels between benign and malignant groups might indicate that Nrf2 dysregulation is an early event in thyroid pathology, or that its levels alone may not be sufficient to distinguish between benign and malignant thyroid lesions. (26)

Diagnostic performance of studied biomarkers in discriminating between benign hyperplasia patients and healthy controls

For differentiating between patients with benign group and healthy controls, an evaluation of the biomarkers was performed.

Through comparing the three markers, Nrf2 and FC microRNA222 exhibited comparable, albeit modest, diagnostic utility, with AUC values of 0.617 and 0.600, respectively. Both markers showed fair discrimination between the benign group and healthy controls. In terms of sensitivity, FC microRNA222 had the highest sensitivity (75%), suggesting it is better at correctly identifying individuals in the benign group. However, its specificity was the lowest (48.72%), meaning it also had a higher rate of false positives. Nrf2 showed a better balance between sensitivity (53.12%) and specificity (66.67%).

In contrast, Ct.microRNA222 performed poorly as a diagnostic marker, with an AUC of 0.515, indicating minimal ability to distinguish between the two groups. Its sensitivity (50%) and specificity (61.54%) were also the lowest among the three markers, making it an unreliable indicator in this context.

Regarding predictive values, FC microRNA222 had the highest NPV (70.37%), suggesting it is relatively good at ruling out benign conditions when the test is negative. Nrf2 had a slightly lower NPV (63.41%) but a better PPV (56.67%) compared to FC microRNA222 (54.55%). Ct.microRNA222 had the lowest PPV and NPV, further supporting its limited diagnostic utility.

The results were demonstrated that both Nrf2 and FC microRNA222 hold some potential as diagnostic markers for differentiating between benign conditions and healthy controls, although their discriminatory power is only fair. An AUC value above 0.7 is generally considered good, and values below 0.6 are often considered poor. In this study, none of the markers reached the "good" category.

Nrf2, a key regulator of cellular defense against oxidative stress, has been implicated in various disease processes, including those with benign presentations. Its moderate AUC and balanced sensitivity and specificity suggest its involvement in the underlying physiological changes in the benign group, making it a potentially useful, albeit not definitive, diagnostic aid.

MicroRNAs play crucial roles in gene regulation and have emerged as promising biomarkers. The differential performance of Ct.microRNA222 and FC microRNA222 is noteworthy. The very low AUC for Ct.microRNA222 indicates that the absolute quantification (Ct value) of microRNA222 itself may not be a strong indicator in this context. However, the slightly better performance of FC microRNA222 (Folding Change) suggests that the relative change in microRNA222 expression, perhaps reflecting a dynamic shift in its levels, might be more diagnostically relevant than its absolute concentration. This highlights the importance of how microRNA data is analyzed and presented, as relative changes often capture biological significance more accurately than raw abundance.

The limitations of these markers, as indicated by their modest AUC values, suggest that none of them individually can serve as a definitive diagnostic tool. Their utility might be enhanced when combined with other clinical markers or integrated into a panel of biomarkers.

Nrf2 is a master transcription factor that plays a crucial role in cellular defense against oxidative stress and inflammation. Under normal conditions, Nrf2 is kept at low levels through continuous degradation mediated by the Keap1-Cul3-Rbx1 ubiquitin ligase complex. However, in response to oxidative stress or electrophilic insults, Nrf2 is released from Keap1, translocates to the nucleus, and binds to antioxidant response elements (AREs) in the promoter regions of target genes. This activation leads to the upregulation of numerous

cytoprotective genes, including those involved in antioxidant enzymes, detoxification enzymes, and proteins that maintain redox homeostasis. (27)

Benign conditions, often involve some degree of cellular stress, inflammation, or altered cellular proliferation. Many certain inflammatory benign tumors, are characterized by increased oxidative stress and chronic low-grade inflammation. Nrf2's activation is a natural cellular response to counteract these detrimental processes. An elevated Nrf2 level in the benign group could reflect the body's attempt to mitigate this stress . The AUC of 0.617 for Nrf2 suggests that while Nrf2 pathways are likely involved, the degree or consistency of their activation might vary, leading to only a fair discriminatory ability.

also influences cell proliferation and survival. In some contexts, Nrf2 activation can promote cell survival. In benign hyperplasia, there's an increase in cell number. While not uncontrolled like cancer, this proliferation might still engage Nrf2-mediated protective mechanisms to maintain cellular integrity in the face of growth signals or metabolic demands. (28)

The observed sensitivity of 53.12% and specificity of 66.67% for Nrf2 suggest that while Nrf2 is often modulated in benign conditions, its changes are not entirely unique or consistently pronounced enough to serve as a highly accurate standalone marker. The fair AUC indicates a detectable, but not strong, difference in Nrf2 levels between benign and healthy states, likely due to the complex interplay of stress, inflammation, and cellular responses across different benign conditions. miR-222 has a complex and often context-dependent role, acting as both an oncogene (promoting cancer) and a tumor suppressor in different types of tissues and conditions. (29) miR-222 has also been implicated in modulating inflammatory responses and angiogenesis (formation of new blood vessels), processes that can be relevant in the development or progression of certain benign conditions. (30)

The very low AUC (0.515) for Ct.microRNA222 is striking. This suggests that the absolute abundance of miR-222 in serum is largely indistinguishable between the benign group and healthy controls. This could be due to several reasons:

High inter-individual variability in baseline miR-222 levels even among healthy individuals.

The changes in absolute miR-222 levels in benign conditions might be too subtle or inconsistent to be reliably detected as a diagnostic marker.

The role of miR-222 might be highly localized within specific tissues, and serum levels may not accurately reflect these localized changes. The improved AUC (0.600) for FC microRNA222, despite still being modest, is significant. "Folding Change" implies a comparison of expression levels, likely relative to a reference gene or a control group. This suggests that while the absolute amount of miR-222 may not be a good indicator, the relative change in its expression, perhaps reflecting a perturbation from an individual's baseline or a subtle, consistent shift within the benign group compared to controls, is more informative. This hints at a dynamic process where miR-222 expression is indeed altered, but the magnitude of this alteration, when normalized or compared, offers a better discriminatory signal. The higher sensitivity (75%) of FC microRNA222 suggests it might be better at detecting the presence of a benign condition, but its lower specificity (48.72%) means it also generates more false positives, indicating its expression is not exclusively tied to the benign state.

Diagnostic performance of studied biomarkers in discriminating between PTC patients and healthy controls

When comparing the two markers in the context of differentiating PTC from healthy controls, Nrf2 demonstrates a notably better diagnostic performance than FC microRNA222.

Nrf2 has a higher AUC of 0.672, indicating a more reasonable ability to discriminate between the PTC group and healthy controls compared to FC microRNA222, which has an AUC of 0.467. The latter's AUC value suggests that it performs worse than random chance in this specific distinction, implying that higher levels of FC microRNA222 might be associated with healthy controls rather than PTC, or its expression pattern is too erratic to be useful.

In terms of sensitivity, Nrf2 is significantly superior (83.33%) to FC microRNA222 (50%). This means Nrf2 is much better at correctly identifying individuals with PTC. However, Nrf2's high sensitivity comes at the cost of lower specificity (48.84%) compared to FC microRNA222 (65.12%). This implies that while Nrf2 is good at detecting PTC, it also generates a substantial number of false positives.

Regarding predictive values, Nrf2 shows a high Negative Predictive Value (NPV) of 91.3%, indicating that a negative Nrf2 test result is highly reliable in ruling out PTC. Its Positive Predictive Value (PPV) is low at 31.25%, meaning a positive Nrf2 test is not very reliable for confirming PTC. FC microRNA222 also has a relatively high NPV (82.35%) but a very low PPV (28.57%).

Overall, Nrf2 appears to be a more promising marker for screening or ruling out PTC due to its high sensitivity and NPV, despite its modest specificity and PPV. FC microRNA222, on the other hand, shows very limited utility as a diagnostic marker for PTC based on these findings.

The results of this study suggest differential roles and utility for Nrf2 and FC microRNA222 as serum biomarkers for Papillary Thyroid Carcinoma (PTC) compared to healthy controls.

The relatively high AUC (0.672) and excellent sensitivity (83.33%) of Nrf2 in distinguishing PTC from healthy controls are noteworthy. Nrf2 is a critical transcription factor involved in the cellular response to oxidative stress, inflammation, and carcinogenesis. Cancer cells, including those in PTC, often exhibit increased oxidative stress and metabolic reprogramming. Upregulation of Nrf2 is a common feature in many cancers, acting as a survival mechanism that allows cancer cells to counteract oxidative damage, resist chemotherapy, and promote proliferation. Therefore, elevated serum Nrf2 levels in PTC patients could reflect the activated Nrf2 pathway within the tumor, spilling over into the circulation.

The high sensitivity means Nrf2 could be valuable as a screening tool or a "rule-out" test for PTC. A negative Nrf2 result (below the cutoff) would strongly suggest the absence of PTC due to its high NPV (91.3%). However, the low specificity (48.84%) and PPV (31.25%) mean that a positive Nrf2 result does not definitively confirm PTC. This lack of specificity could be attributed to several factors: Nrf2 activation is a general response to various stressors and pathological conditions (e.g., inflammation, other benign thyroid conditions, or even systemic conditions) that might also be present in healthy individuals, leading to false positives. Thus, while Nrf2 shows promise for initial screening, it would need to be complemented by other diagnostic methods for confirmation.

In stark contrast, FC microRNA222 performed very poorly, with an AUC of 0.467. An AUC below 0.5 indicates that the marker ability to discriminate is worse than random chance, suggesting that its levels in the PTC group might be lower than in healthy controls, or its expression pattern is highly inconsistent and not indicative of the disease. MicroRNA-222 (miR-222) has been reported to be dysregulated in various cancers, including thyroid cancer, with some studies showing its upregulation and others reporting downregulation depending on the specific context and cancer type. In the context of PTC, previous research has indicated that miR-222 can act as an oncogene by promoting cell proliferation, migration, and invasion, often by targeting tumor suppressor genes.

The current finding of an AUC less than 0.5 for FC microRNA222 in serum suggests that, in this specific study population and measurement context, the "folding change" in miR-222 levels does not serve as a useful diagnostic marker for PTC. This could be due to many reported thoughts

In term of complex regulation, the circulating levels of miRNAs are influenced by various factors, including release from tumor cells, normal cells, exosomes, and degradation. The "folding change" might not accurately capture the biologically relevant changes, or perhaps only very specific subtypes of PTC show this alteration. (31)

While in context-dependency, the role of miR-222 might be highly context-dependent, that it might be upregulated intracellularly in PTC cells, its release into the serum or its stability in circulation might differ, leading to inconsistent or undetectable "folding changes" as a biomarker. Also, **heterogeneity of PTC might be involved,** PTC is heterogeneous, and different molecular subtypes might have varying miR-222 expression profiles. (32)

Nrf2 is a critical transcription factor that orchestrates the cellular antioxidant response. Under normal conditions, Nrf2 is bound by Keap1 in the cytoplasm, leading to its ubiquitination and degradation. However, in cancerous states, Nrf2 can become constitutively activated through various mechanisms, including: Cancer cells often experience higher levels of reactive oxygen species (ROS) due to altered metabolism and rapid proliferation. This oxidative stress can modify critical cysteine residues on Keap1, disrupting its interaction with Nrf2 and leading to Nrf2 stabilization and nuclear translocation. Also, **genetic Alterations** in NFE2L2 (the gene encoding Nrf2) or KEAP1 (the gene encoding Keap1) can directly lead to Nrf2 overactivation. For instance, loss-of-function mutations in KEAP1 or gain-of-function mutations in NFE2L2 can render Nrf2 insensitive to degradation. (27)

Activation of certain oncogenic pathways (e.g., MAPK/ERK, PI3K/Akt) frequently seen in cancers, including PTC, can indirectly stabilize Nrf2.While Nrf2 is generally protective in normal cells by mitigating oxidative damage and preventing transformation, in established cancer, its constitutive activation can become a pro-tumorigenic force. This "dark side" of Nrf2 activation allows cancer cells to **Evade Apoptosis. Through** upregulating antioxidant and detoxification enzymes, Nrf2 helps cancer cells survive the inherent oxidative stress of rapid growth and evade programmed cell death. (33).

Studies have consistently shown that Nrf2 is commonly activated in PTC. Immunohistochemical analyses reveal higher Nrf2 protein levels in PTC tissues compared to normal or benign thyroid tissue, often associated with increased oxidative stress markers within the tumors (34) .

The observed high sensitivity (83.33%) for serum Nrf2 in identifying PTC patients aligns with this mechanistic understanding: the activated Nrf2 pathway in PTC cells likely leads to an increase in circulating Nrf2, making it a detectable indicator of the disease. The relatively good AUC (0.672) further supports its utility as a marker reflecting the activated Nrf2 pathway in PTC. However, its low specificity (48.84%) suggests that Nrf2 activation, or elevated circulating Nrf2, is not exclusive to PTC. Other thyroid pathologies (e.g., goiter with oxidative stress) or systemic inflammatory conditions could also induce Nrf2, leading to false positives. This highlights that while Nrf2 is involved in PTC, its presence in serum reflects a broader cellular stress response, limiting its specificity as a standalone diagnostic tool.

Numerous studies have shown that miR-222 (and miR-221) are significantly upregulated in PTC tissues compared to normal thyroid tissue. This upregulation is often associated with aggressive tumor phenotypes, lymph node metastasis, and extrathyroidal extension. Given its established oncogenic role and consistent upregulation in PTC tissues, one would generally expect elevated levels of circulating miR-222 (or a positive folding change) to be a strong biomarker for PTC. (35)

The finding that FC microRNA222 has an AUC of 0.467 (worse than random chance) for distinguishing PTC from healthy controls is highly paradoxical and warrants careful consideration of mechanistic interpretation.

Serum miRNAs can originate from various sources (tumor cells, normal cells, immune cells, exosomes). The "folding change" in serum might not accurately reflect the changes happening within the tumor. There could be complex shedding patterns or rapid degradation in circulation. While miR-222 might be highly expressed within PTC cells, its release into the bloodstream and its stability there might be inconsistent or altered in a way that doesn't correlate directly with disease presence. (36)

The heterogeneity of Disease might also effect, not all PTCs could exhibit the same extent or pattern of miR-222 dysregulation, especially in serum. Other systemic conditions or individual variations in healthy controls could lead to higher baseline levels of miR-222, masking any subtle disease-specific increase from PTC. (37)

while Nrf2's performance aligns with its established role in cancer-associated oxidative stress and survival, the surprising inverse or random association of FC microRNA222 in serum for PTC highlights the complexities of translating tissue-level mechanistic insights into reliable circulating biomarkers. It underscores that while a molecule may play a critical role in tumor biology, its utility as a serum biomarker is contingent on

consistent and distinct changes in its circulating levels, which can be influenced by numerous factors beyond its direct cellular function.

CONCLUSION

The study findings demonstrated a significant decrease in serum levels of Nrf2 and Folding Change microRNA-222 among PTC patients compared to healthy controls, supporting their possible involvement in tumor development. While Ct.microRNA-222 showed poor diagnostic reliability, both Nrf2 and FC microRNA-222 presented moderate diagnostic value, particularly in distinguishing benign conditions from healthy states. Although FC microRNA-222 had high sensitivity, its low specificity limits its use as a standalone diagnostic tool. These results suggest that these biomarkers may serve as useful adjuncts rather than replacements for conventional methods such as FNAB.

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