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Study Of Serum Micro-RNA 221 Expression In Patients With Thyroid Nodules And Its Relation To Outcome

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***Abstract**: Background: Thyroid nodules are extremely common and are usually benign. 4%-6.5% of thyroid nodules are malignant. Among thyroid cancer investigations many diagnostic molecular biomarkers were found to have applications in thyroid nodules management and avoiding unnecessary thyroidectomy.(1)*

This study aims:To detect value of micro-RNA 221 expression in sera of patients with thyroid nodules and its relation to outcome after surgery.

Patients and Methods: Forty-five adult subjects aged between 18 to 70-years old who were diagnosed with suspicious thyroid nodules that required total thyroidectomy were offered participation in the study. Based on the patient's history, physical examination, US and FNAB findings patients who had thyroid nodules with U/S pattern suggestive of malignant potential (TIRADS score ≥ 3), also with indeterminate FNAB results (Bethesda III, IV) were selected. Samples of human serum were collected from the selected patients visiting the endocrine outpatient clinic and endocrine surgical department of Ain Shams University hospital. Results were correlated with postoperative pathology results.

Results: In our study there was no significant difference regarding outcome in patients (benign or malignant) with respect to serum microRNA 221. There was a significant difference between benign and malignant outcomes regarding size of dominant nodules by ultrasound with a mean value equals 4.6 cm in largest dimension in patients with malignant thyroid nodules and p value equals 0.027 indicating increased size of nodule may be associated with increased risk of malignancy.

Keywords: Thyroid nodules, FNA, thyroid malignancy, serum microRNA 221, thyroid ultrasound, thyroidectomy.

INTRODUCTION

The current first line of thyroid nodules' evaluation includes thyroid hormones, thyroid stimulation hormone, and ultrasonography of the gland ***(2)***, the last one is a simple non-invasive procedure which reveals several characters related to nodules pathology. Increasing malignancy risks were mostly associated with presence of microcalcifications, irregularities or speculated margins without halo, hypo-echogenicity, mostly solid composition, and taller than wider shape ***(3).*** Fine-needle aspiration biopsy (FNAB) was used to exclude malignancy in thyroid nodules**.** Despite FNA safety and availability as a commonly used procedure, many complications as swellings may occur. About 60%- 80% of FNAs outcomes are benign ***(4)***. In 20–30 % of cases, however, FNAB yields indeterminate cytological results and suspicious for malignancy. Surgical treatment was usually advised for

these nodules for its potential malignancy. Overall, approximately 25 % was confirmed histopathologically on thyroidectomy to be malignant. 75% of cytologically indeterminate thyroid nodules cases underwent unnecessary thyroid surgeries for nodules which prove to be benign only after surgery ***(5).*** Recent studies on thyroid cancer discovered many diagnostic molecular biomarkers and profiling panels for managing thyroid nodules ***(6).*** Among these markers are MicroRNAs. MicroRNAs (miRs) are 19–25 nucleotides that functionally regulate gene expression. MicroRNAs were shown to play many roles in gene expression regulation and there is evidence that they are involved in many physiological cellular functions including differentiation, proliferation, and apoptosis ***(7).***

Aim of the work

To detect value of Micro-RNA 221 expression in sera of patients with thyroid nodules and its relation to outcome after surgery.

METHODS

This is cross-sectional investgation that was conducted in period of June 2019 to December 2019 on forty five adult males and females (18 to 70-years old) who were diagnosed of having suspicious thyroid nodule that required total thyroidectomy. Approved informed consent was obtained from the study patients who underwent total thyroidectomy. The informed consent was approved by the Independent Ethical Committee, under institutional review board-approved protocols of our University, research ethics committee (FWA000017585).The need for total thyroidectomy was determined by an endocrinologist, a radiologist and a surgeon based on the patient's history, physical examination, US and FNAB findings. Samples of human sera were collected from patients visiting outpatient endocrine clinic and endocrine surgical department of our University hospital. Patients included in this study had thyroid nodules with U/S pattern suggestive of malignant potential (TIRADS score $\geq$ 3)**,** also with indeterminate FNAB results (Bethesda III, IV)**.** Patients with diseases affecting pattern of micro-RNA as chronic kidney disease and malignancies like liver, lung, breast and prostate cancer were excluded.

Sampling:

Five milliliters (ml) of peripheral venous blood samples were aseptically withdrawn from each patient.
Serum was prepared for quantitative assay of micro RNA-221 by qRT-PCR through several steps which included briefly Total RNA extraction, including miRNA by miRNeasy Mini Kit provided by Qiagen as described by the manufacturer. Complementary deoxyribonucleic acid (cDNA) was synthesized from total RNA using gene-specific primers according to the TaqMan miRNA reverse transcription kit protocol (Applied Biosystems, Foster City, CA) and stored at -20 C until used. Candidate miRNA (miR-221 and miR-16) were reversibly-transcribed. Real-time polymerase chain reaction (RT PCR) was performed using 5 Plex Rotor Gene Real Time PCR Analyzer (Qiagen, Germany) (8).

Detection and calculation of results:

Results were reported in relative quantification. The relative expression level (fold change) for miRNA-***221*** in each sample was calculated by the comparative cycle threshold $2^{-(\Delta\Delta CT)}$ method.

The cycle threshold (C_t) was determined for (miRNA-*221* and miRNA-16) in each experimental sample using Step One Plus™ Software v2.1, which is the intersection between an amplification curve and a threshold line.

Statistical Analysis:

Data was analyzed by SPSS version 23. Comparing among studied groups as regards to qualitative data ***Chi-square test***. And among two independent groups (quantitative data and parametric distribution) by ***Independent t-test*** and independent groups regarded to (quantitative data with non-parametric distribution) by ***Mann-Whitney test.***

RESULTS

Forty-five adults (males and females) age ranged between 18 to 70-years old who were diagnosed with suspicious thyroid nodule that required total thyroidectomy were offered participation in the study.

- **Patients :** Included 45 euthyroid patients, 1 was male and 44 were females with mean age was (40.70 ± 11.37) years, their TIRADS scores on thyroid U/S were 25 patients had TIRADS score III, 16 patients had TIRADS score IV and 4 patients had TIRADS score V with mean thyroid nodule size (3.43±1.67), Their FNAC results reveals that 38 patients had Bethesda III and 7 patients had Bethesda IV, their post thyroidectomy pathology reveals 37 patients were benign, 8 patients were malignant

Distribution of Patients

Table (1): Descriptive for Nodule size, TIRADS score, Bethesda classification system, pathology in patients group (no. = 45).

		No.= 45
Nodule size	Mean±SD Range	**3.43**±1.67 (1.1-9)
TIRADS score	Median (IQR) Range	3(3–4) 3 – 5
	3 4 5	25 (55.6%) 16 (35.6%) 4 (8.9%)
Bethesda classification system	Median (IQR) Range	3(3–3) 3 - 4
	3 4	38 (84.4%) 7 (15.6%)
Pathology	Benign Malignant	37 (82.2%) 8 (17.8%)

Age in benign and malignant

Table (2): Showing range of age in both benign and malignant pathologies

Age	Pathology						T-Test	
	Benign			Malignant			t	P-value
Range	19	-	70	25	-	59	0.224	0.824
Mean ±SD	41.405	±	11.687	40.375	±	12.397		

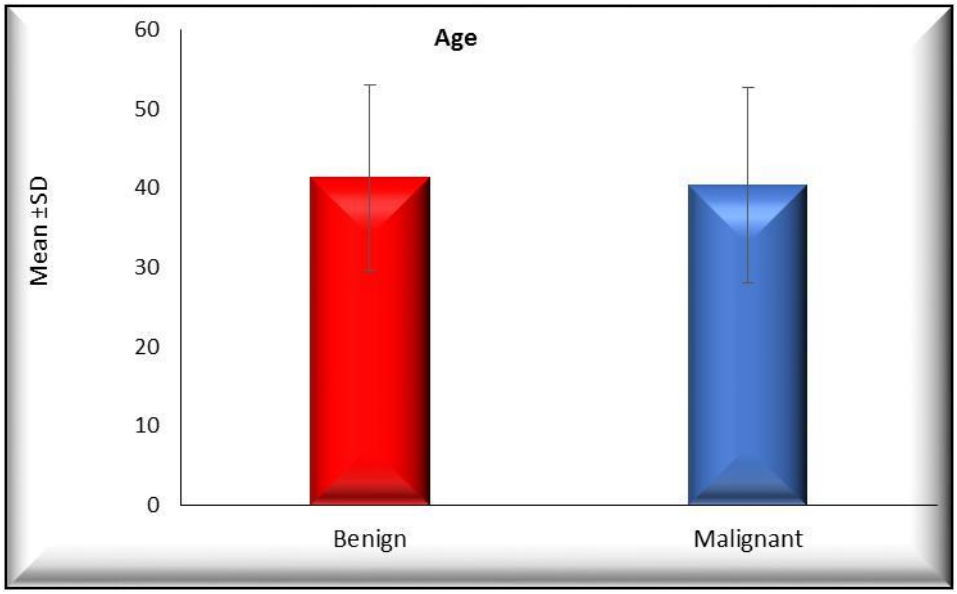

Figure (1): Showing range of age in both benign and malignant pathologies

From the above table and figures apparently , in our study there was no difference regarding outcome in patients (benign or malignant) with respect to age in both benign and malignant patients showing almost similar range of age, mean age was 41 years in benign and 40 years in malignant.

Predictability of different modalities in detection of malignancy

1. Ultrasound in detecting benign and malignant:

A. TIRADS scoring:

Table (3): Showing distribution of malignant and benign pathologies with different TIRADS values

TIRADS	Pathology						Chi-Square	
	Benign		Malignant		Total			
	N	%	N	%	N	%	X^2	P-value
3 TIRADS	21	56.76	4	50.00	25	55.56	0.122	0.727
4 &5 TIRADS	16	43.24	4	50.00	20	44.44		
Total	37	100.00	8	100.00	45	100.00		

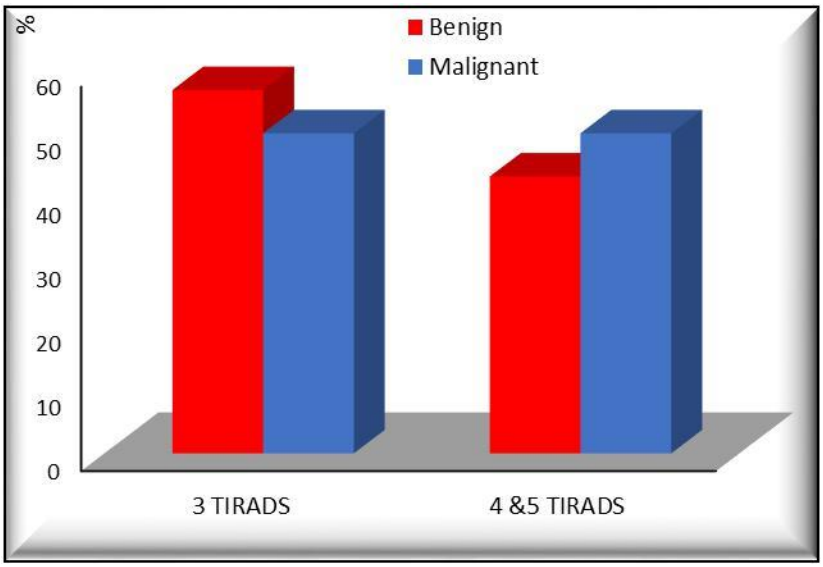

Figure (2): Showing distribution of malignant and benign pathologies with different TIRADS values

Apparently, in our study there was no difference regarding outcome in patients (benign or malignant) with respect to TIRADS scoring from 3 and above in malignant patients 4 were TIRADS III and 4 were TIRADS IV and above.

B. Size of the dominant nodule

Table (4): Showing distribution of malignant and benign pathologies with different sizes of dominant nodules.

Size of the dominant	Pathology		T-Test	
	Benign	Malignant	t	P-value
Range	1.1 - 8.6	1.5 - 9	-2.284	0.027*
Mean ±SD	3.180 ± 1.431	4.600 ± 2.258		

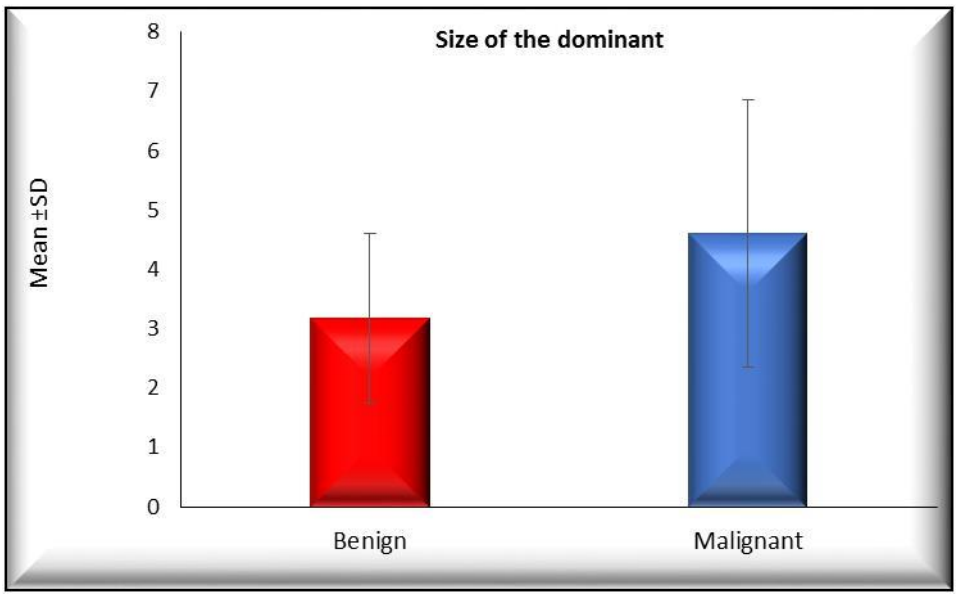

Figure (3): Showing distribution of malignant and benign pathologies with different sizes of dominant nodules.

There was a significant difference between benign and malignant outcomes regarding size of dominant nodules with a mean value 4.6 cm in largest dimension in patients with malignant thyroid nodules and p value 0.027 indicating increased size of nodule may be associated with increased risk of malignancy.

2. Bethesda in detecting benign and malignant

Table (5): Showing distribution of malignant and benign pathologies with different Bethesda values.

BETHSEDA	Pathology						Chi-Square	
	Benign		Malignant		Total			
	N	%	N	%	N	%	X^2	P-value
3 BETHSEDA	33	89.19	5	62.50	38	84.44	3.567	0.059
4 BETHSEDA	4	10.81	3	37.50	7	15.56		
Total	37	100.00	8	100.00	45	100.00		

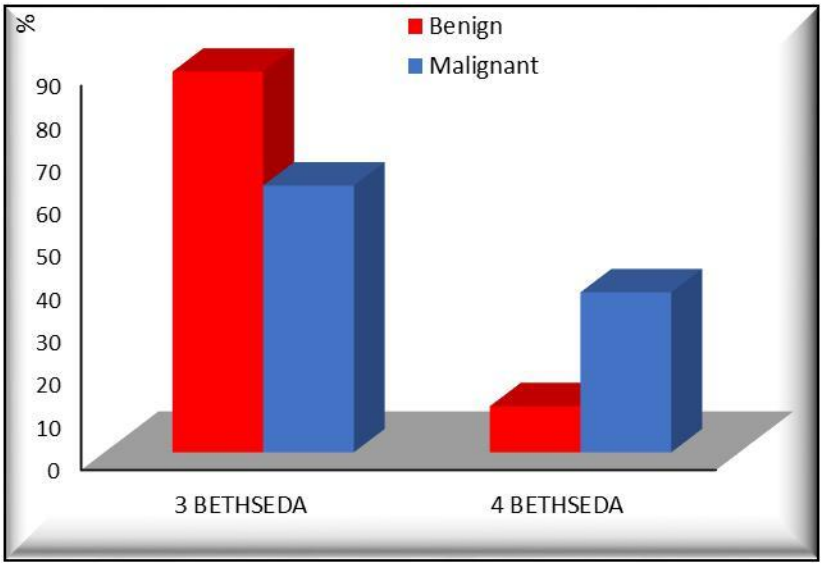

Figure (4): Showing distribution of malignant and benign pathologies with different Bethesda values.

From the above table and figures apparently, in our study there was no difference regarding outcome in patients (benign or malignant) with respect to Bethesda scoring III and IV. In malignant patients 5 subjects were Bethesda III and 3 subjects were Bethesda IV.

3. MicroRNA 221 in benign and malignant:

Table (6): Showing range of microRNA 221 in both benign and malignant pathologies

miRNA221	Pathology		Mann-Whitney Test	
	Benign	Malignant	Z	P-value
Range	0.11 - 37.53	0.4 - 8.28	0.148	0.882
Median(IQR)	3.25(0.90-5.18)	3.37(0.49-7.70)		

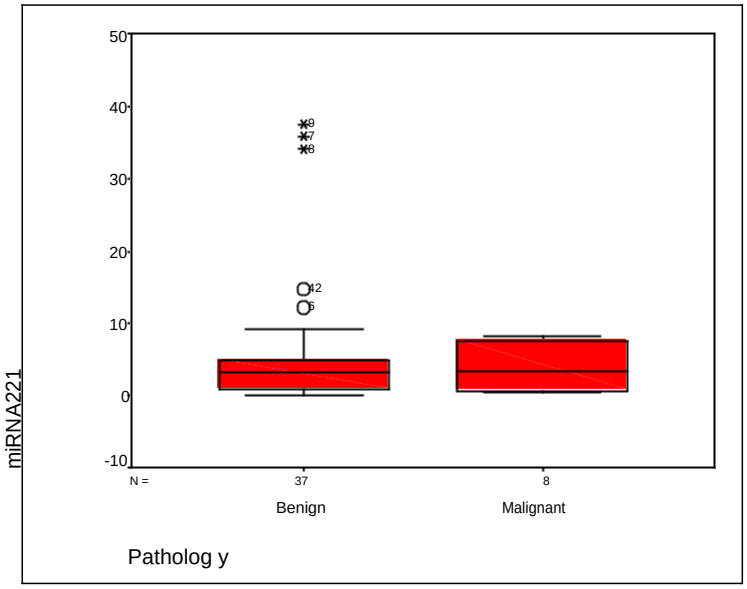

Figure (5): Showing range of microRNA221 in both benign and malignant pathologies

From the above table and figures apparently, in our study there was no significant difference regarding outcome in patients (benign or malignant) with respect to microRNA 221 values, both benign and malignant patients showing almost similar range of microRNA 221, with a median 3.25 in benign and 3.37 in malignant, and p value 0.882.

DISCUSSION

The current American thyroid association recommends follow up for non-diagnostic thyroid nodules surgery in category III and surgical excision in category IV ***(9).***

Our work aims was to detect value of Micro-RNA 221 expression in sera of patients with thyroid nodules and its relation to outcome after surgery either benign or malignant.

Previous researches have detected the ability of RNA-based risk classifiers (using tissue and serum) to help in management of indeterminate thyroid nodules, ***Woody et al.*** performed molecular tests for indeterminate thyroid nodules with Bethesda III or IV category by cytology with no history of thyroid cancer. Also examined all cases that underwent surgical treatments for thyroid nodules depending on positive or negative microRNA results, negative outcomes had higher survival rates without 81% (Lobectomy) or 85% (thyroidectomy) surgery post 2 years later. Positive outcome had higher risks for lobectomy and even more for total thyroidectomy **(10).**

Woody et al Identified thyroid tumor-associate microRNAs (miRNAs) in serum for development of a unique PTC recurrence markers where 754 miRNAs in serum collected from 11 PTC cases pre and post 30 days. Samples were processed using quantitative polymerase chain reactions in independent cohort with PTC or benign nodules and 20 healthy subjects analyzed. **(11)**

Eight miRNAs (miR-221, miR-222, miR-146a, miR-24-3p, miR-146b, miR-191, miR-103a, and miR-28) displayed high levels in pre-thyroidectomy in serum of PTC cases and exceeded levels of post-thyroidectomy. Most significantly marker were: miR-146a and miR-221 in 20 PTC cases.

In our study ultrasound remained a corner stone in diagnosis and management of thyroid nodules especially when correlating to nodule size which had positive correlation with malignancy in addition to TIRADS classification.

Bethesda classification among the selected patients was either III or IV with no significant difference among patient outcome whether benign or malignant.

No significant differences regarding age in determination whether the nodules were benign or malignant, in contrast to ***Kwong et al*** who stated that patients with benign nodules are older than those with malignant nodules, cases aged < 55 years have higher Thyroid malignancy risks than older subjects. Moreover, after further analysis, age < 55 years was confirmed to be an independent risk factor for thyroid malignancy. **(12)**

Statistical relation shows thyroid function had no effect on nodule malignancy in contrast to ***Carles et al.*** who stated that TSH was higher in thyroid carcinoma. DTC cases had larger malignant nodule size than non-malignant nodule, Correlation was detected between malignant nodule size and TSH values, but not between TSH levels and size of the largest benign nodule.**(13)**

SUMMARY

Thyroid nodules are extremely common, especially in young adults and children. By age 60, about 50% of whole population considered to have a thyroid nodule that can be found either through examination or with imaging.

The aim of our study was to assess a new diagnostic tool to decrease un-necessary diagnostic thyroidectomies in patients with fine needle cytology results showing intermediate results.

Our study showed no significant correlation between serum microRNA 221 and malignancy, there was many limitations among which was sample size, variability of pathologies, and variability of microRNA panels.

Holistic approach of patients with thyroid nodules remains the logic approach including age, family history of thyroid malignancy, Ultrasound with TIRADS scoring and size, fine needle aspiration cytology.

CONCLUSION

Non-significant correlation between serum microRNA 221 and malignancy, which may indicate variability of microRNA panel between different ethinicities, other molecular biomarkers including micro-RNA-222, microRNA-146b, larger sample size and larger panel may be needed to confirm among Egyptian patients with thyroid nodules.

LIMITATIONS

Our investigation had some limitations as small sample size, and we were unable to use a wide panel of markers due to financial issues.

Declarations
-Ethics approval and consent to participate
-Approved informed consent was obtained from the study patients who underwent total thyroidectomy. The informed consent was approved by the Independent Ethical Committee,

under institutional review board-approved protocols of Ain Shams University, Faculty of Medicine, research ethics committee (FWA000017585).
-Consent for publication: 'Not applicable'
-Availability of data and material 'Not applicable'
-Competing interests: We have no conflicts of interest to disclose.
-Funding No funding available to mention
-Authors' contributions:
S.M. as the primary author, data collection, analysis interpretation
L.S. for conducting the laboratory work, sample analysis and interpretation of the results
R.B. for the design of work and drafting
A.E. for critical revision of the paper
H.A for critical revision of the paper

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"All authors have read and approved the manuscript"

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List of abbreviations

- FNAB…fine needle aspiration biopsy.
- MiR…micro-RNA.
- MiRNA…microRNA
- qRT-PCR… real time quantitative reverse transcription polymerase chain reaction
- DTC….differentiated thyroid cancer.
- ATA…American thyroid association.
- RT...Reverse Transcription.
- IQR…inter-quartile range.

Figure legend

- Figure (1): Showing range of age in both benign and malignant pathologies
- Figure (2): Showing distribution of malignant and benign pathologies with different TIRADS values
- Figure (3): Showing distribution of malignant and benign pathologies with different sizes of dominant nodules.
- Figure (4): Showing distribution of malignant and benign pathologies with different Bethesda values.
- Figure (5): Showing range of microRNA- 221 in both benign and malignant pathologies