

SERUM MICRORNA-222 EXPRESSION for MALIGNANCY PREDICTION in SAMPLE of EGYPTIAN EUTHYROID PATIENTS with INDETERMINATE THYROID NODULE

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Abstract:

Background: The Prevalence of thyroid nodules is rising nowadays, luckily most of them are benign. The risk of malignancy 5-15%, which necessitates the ultimate need to accurately distinguish benign from malignant nodule to avoid unnecessary thyroidectomy with risk of recurrent laryngeal nerve injury, postoperative hypothyroidism and lifetime thyroid replacement therapy, and other complications related to surgery and anaesthesia. Recent evidence suggests that circulating miRNA might have probable advantage as diagnostic or prognostic markers for numerous cancers. Given their reproducible and constant presence in sera, miRNA profiles have emerged as a non-invasive method to categorise a wide variety of human cancers. This study aimed to evaluate a possible relationship between the expression level of circulating miRNA-222 and the histological outcome of euthyroid patients undergoing thyroidectomy for thyroid nodules with indeterminate FNAC. This study included 45 euthyroid patients with indeterminate thyroid nodules diagnosed with ultrasound and FNAC which planned for thyroidectomy. Quantitative assay of serum micro RNA-222 expression by quantitative Real-Time polymerase chain reaction (qRT-PCR) performed preoperatively, and results compared with postoperative histopathology.

Results: The incidence of thyroid nodules was predominant in female gender in benign group and malignant group. Risk of malignancy increases as TI-RADS and Bethesda scores increases. Also, larger nodule in size has a more risk of malignancy ($p=0.027$). Expression level of circulating miRNA-222 in serum can't differentiate between benign and malignant patients where there was no significant difference between them statistically ($p=0.905$). Circulating miRNA-222 was a poor predictor for malignant nodules with sensitivity of 50%, specificity of 32.43%, with high negative predictive value (NPV=75%).

Conclusion: Although circulating miRNA-222 has been identified as novel minimally invasive biomarker for preoperative prediction of malignant nodules, but in our study, it did not show a value as a tool for discrimination of malignant nodules. Ultrasound remained important procedure in preoperative prediction and management of thyroid nodules especially when correlating to nodule size which had positive correlation with malignancy in our study.

Key words: Thyroid nodules, microRNA-222, FNAC, Bethesda, TI-RADS.

INTRODUCTION

The prevalence of thyroid nodules has increased over time predominantly due to advances in imaging techniques. Epidemiological studies have reviewed possible environmental factors influencing nodular formation [1]. 7%–15% of thyroid nodules may harbor malignant cells, depending on sex, age, family history, history of radiation exposure and other risk factors [2].

The current approaches to distinguish between benign and malignant nodules include history, physical examination, ultrasound, thyroid nuclear scanning, FNAB and surgical excision and histopathological examination. The gold standard is postoperative histopathological examination [1, 3]. FNAC is considered the most reliable method for preoperative diagnosis of thyroid nodules. However, the predictive value of FNA is still limited in patients with cytological features of atypia of undetermined significance, follicular neoplasm or suspicious malignancy which represent approximately 15%–40% of FNAC samples and carry a malignancy rate of 5%-15%, 15%-30%, and 60%-75% respectively, leading to unnecessary thyroid lobectomies or thyroidectomy [4, 5].

MiRNAs are an abundant class of gene regulatory molecules and modulate expression of multiple protein-coding genes. A single miRNA can potentially target hundreds of genes, and a single gene could be a potential target of many different miRNAs [6]. Studies have clearly demonstrated that miRNA plays a critical role in several cancer related biologic processes, including proliferation, differentiation, and cell death [7].

Previous studies have reported that certain miRNA have potential advantage as diagnostic or prognostic markers for numerous cancers [8]. The most comprehensive panel characterized in thyroid carcinomas, has included as many as seven markers (miR-187, miR-221, miR-222, miR-146b, miR-155, miR-224, and miR-197) to detect thyroid cancers in surgical and preoperative samples [9, 10, 11]. Most previous studies examined miRNA expression mainly in tumor cells and tissues from PTC patients. However, few studies have examined circulating miRNA expression in PTC patients, and have attempted to use serum miRNA to differentiate benign nodules from malignancies. Given their reproducible and stable existence in sera, miRNA profiles have emerged as a non-invasive way to classify a wide variety of human cancers [12, 13].

METHODS

This study was approved by the Research Ethics Committee, Faculty of medicine, Federal Wide Assurance No. FWA000017585. Informed consent was obtained from all patients included in our study. This cross-section study conducted on 45 patients, their ages ranging from 19 to 70-years old selected from endocrine outpatient clinic and endocrine surgical ward, in the period from June 2019 to December 2019. Patients with suspicious thyroid nodule that required total thyroidectomy (indeterminate FNAC and TIRADs 3 and 4 on US) were recruited and subsequently sub-grouped according to the final outcome of post-operative histopathology into 2 groups: Group (I): thirty-seven patients confirmed to had benign nodules (82%). Group (II): eight patients confirmed to had malignant nodules (18%).

All studied patients were subjected to full history taking and clinical physical examination. laboratory Investigations include thyroid function tests by ELISA kits manufactured by BioVendorR. Serum was prepared for quantitative assay of micro RNA-222 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 miR reverse transcription kit protocol (Applied Biosystems, Foster City, CA) and stored at -20 C until used. Candidate miRNA (miR-222 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) [14].

Ultrasonography (US) on thyroid gland performed by a radiology specialist to all patients to evaluate thyroid parenchyma. All data analysed and calculated according American College of Radiology (ACR-TIRADS) scoring system for malignant risk [15,16]. Suspicious lesions with TIRADs score 3, 4 and 5 were included in this study and referred for FNAC.

FNAC was performed by intervention radiologist for all patient according to the principles described by Werga et al., 2000 [17]. All slides were interpreted by qualified pathologist. Results are reported and calculated according BETHESDA classification of thyroid nodule for risk of malignancy [5]. Only suspicious lesions of BETHESDA 4 and 5 were included and then referred for surgery. According to the case we performed hemithyroidectomy, subtotal thyroidectomy, or total thyroidectomy at endocrine surgical department. The specimens were sent for histopathology examination to detect patients with thyroid cancer and results correlated to preoperative expression level of circulating microRNA-222, US criteria and FNAC results.

Statistical analysis

Statistical presentation and analysis was performed using mean, standard deviation, student t-test, Chi-square, and Mann-Whitney U test by SPSS (Statistical program for Social Science) version 20. ROC curves were used to analyze the diagnostic utility of differentially expressed miRNAs. The optimal cut-off point was selected as the value with the maximal sum of sensitivity and specificity. The levels of miRNA in each group were defined by the median and interquartile range (IQR). A P-value of $P < 0.05$ was assumed as a significant statistical difference.

RESULTS

Our patients were sub-grouped according to postoperative histology into 37 patients with benign nodules, 8 with malignant nodules according to post thyroidectomy pathology (four patients diagnosed papillary cell carcinoma, two invasive follicular variant of papillary carcinoma, one anaplastic on top of papillary carcinoma and one Hurthle cell neoplasm with encapsulated medullary micro-carcinoma). Mean age of benign and malignant were 41.405 ± 11.687 and 40.375 ± 12.397 years respectively, with female predominance. Median of expression level of circulating miRNA-222 of benign and malignant patients were 0.73 (0.49-1.25), 0.85 (0.43-1.63) respectively. with a non-significant difference between them statistically ($p=0.905$). And cutoff value > 0.62 with sensitivity of 50%, specificity of 32.43% and high negative predictive value (NPV=75%) (table 1,2, figure 1,2).

We compare size of the dominant nodule on US on its largest dimension and results revealed that benign group had nodules with mean size 3.180 ± 1.431 , while malignant group had nodules with mean size 4.600 ± 2.258 , with *significant* difference between them ($p=0.027$) (table 1). Patients had been regrouped into 2 groups according to diameter of dominant nodule on US, more or less than 4 cm, and we compared groups with postoperative pathology. we found that, 75.68% of benign nodules were less than 4 cm in diameter, while 62.5% of malignant nodules were more than 4 cm in diameter, with a statistically *significant* difference ($p=0.034$) (table 1).

Incidence of malignancy in nodules with TIRADS 3 was 16%, in nodules with TIRADS 4 was 18.75% and in nodules with TIRADS 5 was 25% (table 3). Incidence of malignancy in nodules with Bethesda 3 was 13.16%, and in nodules with Bethesda 4 was 42.86% (table 3).

Table 1: Comparison between benign, malignant and healthy controls as regard different parameters

		Groups				T-Test	
		Benign		Malignant		T	P-value
Age (year)	Range	19 – 70		25 – 59		0.224	0.824
	Mean ±SD	41.405±11.687		40.375±12.397			
TSH (uIU/ml)	Range	0.5-4		0.8 – 4		-1.065	0.293
	Mean ±SD	1.705±1.243		2.226±1.323			
FT3 (pg/ml)	Range	2 – 4		2 - 3.17		1.945	0.058
	Mean ±SD	2.909±0.481		2.551±0.420			
FT4 (ng/dl)	Range	0.8 - 1.6		0.8 - 1.5		1.216	0.231
	Mean ±SD	1.176±0.269		1.053±0.218			
						Mann-Whitney Test	P-value
miRNA-222 (fold change)	Range	0.12 - 6.45		0.23 - 4.44		0.119	0.905
	Median(IQR)	0.73(0.49-1.25)		0.85(0.43-1.63)			
						T-Test	P-value
Size of the dominant	Range	1.1 - 8.6		1.5 – 9		-2.284	0.027*
	Mean ±SD	3.180±1.431		4.600±2.258			
		N	%	N	%	Chi-Square	P-value
Diameter of dominant nodule	<4 cm	28	75.68	3	37.50	4.473	0.034*
	>4 cm	9	24.32	5	62.50		

Table 2: The estimated cutoff value, sensitivity, specificity and accuracy of miRNA-222

ROC curve between Pathology Benign and Malignant in Patients					
Cutoff	Sensitivity	Specificity	PPV	NPV	Accuracy
>0.62	50.00	32.43	13.8	75.0	51.4%

Table 3: Incidence of different TIRADS and BETHSEDA scores in benign and malignant groups

TIRADS	Pathology			
	Benign (n=37)		Malignant (n=8)	
	No.	%	No.	%
TIRADS 3	21	84	4	16
TIRADS 4	13	81.25	3	18.75
TIRADS 5	3	75	1	25
BETHSEDA				
BETHSEDA 3	33	86.84	5	13.16
BETHSEDA 4	4	57.14	3	42.86

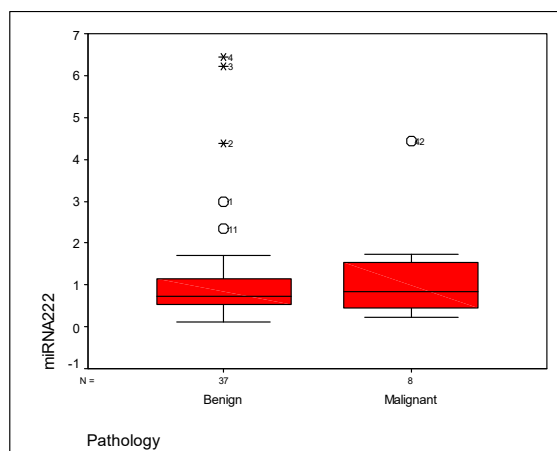


Figure 1: Comparison between benign and malignant groups as regard expression level of circulating miRNA-222

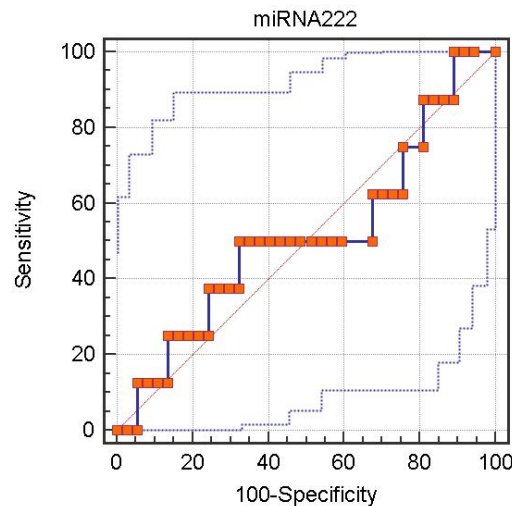


Figure 2: Receiver operating characteristic curve (ROC) for plasma level of circulating miRNA-222 to differentiate between benign and malignant groups.

DISCUSSION

Incidence of thyroid nodules have increased nowadays. The gold standard is postoperative histopathological examination [1, 3]. But still indeterminate nodules stay in gray zone between benign and malignant categories. The ability to improve the diagnostic accuracy of indeterminate nodules preoperatively could potentially have an impact on patient management, clinical outcomes, and health care costs by reducing unnecessary thyroid resections [18].

Since discovering miRNA in *C.elegans*, it have become one of the most characterized molecules. Most previous studies examined miRNA expression mainly in tumor cells and tissues from patients with malignant nodules. However, few studies have examined circulating miRNA expression in patients with malignant nodules, and have attempted to use serum miRNA to differentiate benign nodules from malignancies. Given their reproducible and stable existence in sera, miRNA profiles have emerged as a non-invasive way to classify a wide variety of human cancers [12, 13].

In our study the postoperative histology confirm thirty seven patients were had benign follicular adenoma and eight patients with malignant nodules with different subtypes which included four patients diagnosed papillary cell carcinoma (PTC), two patients diagnosed invasive follicular variant of papillary carcinoma (IFVPC), one patient diagnosed anaplastic on top of papillary carcinoma (ATC) and one patient diagnosed Hurthle cell neoplasm with encapsulated medullary micro-carcinoma (MTC).

As previously reported that, the most comprehensive miRNA panel known to be upregulated in thyroid malignancy included seven markers (miR-187, miR-221, miR-222, miR-146b, miR-155, miR-224, and miR-197) to detect thyroid cancers in surgical and preoperative samples [9,10]. We chose miRNA-222 from this panel to evaluate its ability in preoperative malignancy prediction, as in previous studies, miRNA-222 was up-regulated several folds among the PTCs, FTCs, IFVPC, MTC and ATCs. while their expression was relatively low among the follicular adenomas [9, 10, 11].

We performed quantitative assay of serum micro RNA-222 expression by quantitative Real-Time polymerase chain reaction (qRT-PCR) for all patients preoperatively in our study. Our

results revealed that expression levels of circulating miRNA-222 couldn't discriminate malignant nodules from benign one ($p=0.905$). With sensitivity of 50% and specificity of 32.43%, and accuracy 51.4%.

Yu et al., 2012; Yoruker et al., 2016; Lee et al., 2013; Li et al., 2013; were reported that circulating miRNA-222 was up-regulated in papillary thyroid carcinoma. And it has a diagnostic value and may serve as novel minimally invasive biomarkers for the preoperative diagnosis of malignant nodules. with sensitivity of 81.1% and specificity of 89.5% in Yu et al., 2012, study [3, 19, 20, 21].

Pai et al., 2012, who studied four miRNA markers (miR-187, miR-221, miR-222, and miR-224) to differentiate the benign from the malignant thyroid tumors using miRNA derived from thirty-eight formalin fixed paraffin embedded thyroidectomy samples. they reported that miR-222 were able to significantly ($P < 0.001$) discriminate the malignant samples (PTC, follicular variants of PTC, FTC, ATC, and PDC) from the non-malignant neoplasms (FA), both with a two-fold and a more stringent three-fold higher cut-off. with sensitivity and specificity, 81% and 91% respectively, and accuracy 84.3% [10].

Nikiforova et al., 2008, reported that various histopathological types of thyroid tumors have distinct miRNA profiles, which further differ within the same tumor type, reflecting specific oncogenic mutations [9].

To the best of our knowledge, our study is the first one performed to study the predictive value of circulating miRNA-222 for malignant thyroid nodules in Egyptian population. As Yoruker et al., 2016, performed their study on Turkish race, Yu et al., 2012, and Li et al., 2013, conducted their studies on Chinese race, Lee et al., 2013, conducted their study on Australians [19, 3, 21, 20]. Also, our study, limited by number of nodules confirmed to be malignant, with small number of samples pertaining to each subtype of thyroid malignancy.

We had studied the ACR TI-RADS score of thyroid nodules with postoperative histopathology and we found that, risk of malignancy was 16% in nodules with TIRADS 3. And risk of malignancy was 18.75% in nodules with TIRADS 4. While incidence of malignancy was 25% in nodules with TIRADS 5. Barbosa et al., 2019, reported that the risk of malignancy was TIRADS 3 (23.3%), TIRADS 4 (49.6%), and TIRADS 5 (92.9%). Due to the variability of cancer risk observed in their study for TIRADS 4 score (4a – 28.1%; 4b – 55.6% and 4c – 65.2%), they subdivided this category as they observed that TIRADS 4a had a behavior more similar to that of low risk, while TIRADS 4b and 4c had a behavior similar to that of high risk [22].

We compare size of the dominant nodule on US on its largest dimension of our studied patients, we found a significant difference between benign and malignant groups ($p=0.027$). Also, we divided patients according to diameter of dominant nodule into more or less than 4 cm and comparing between them as regard postoperative pathology, and revealed that, 75.68% of benign nodules were less than 4 cm in diameter, while 62.5% of malignant nodules were more than 4 cm in diameter with significant statistical difference ($p=0.034$). These results in agreement with Shin et al., 2015; they reported that greater nodule size influences cancer risk. Also, Kuru et al., 2010, reported that nodule size greater than or equal to 4 cm was an independent factor associated with malignancy. Kamran et al., 2013, reported that nodules greater than or equal to 4 cm had a higher prevalence of malignancy than

nodules less than 4 cm., reported that large nodules have a higher probability of malignancy [23, 24, 25].

On the other hand, Cavallo et al., 2017, reported that the risk of malignancy is inversely related to the nodule size in her study. Also, she postulated that demonstrating size of the nodule alone should not be considered as an independent risk factor for malignancy [26]. Shrestha et al., 2012, reported no suggested difference in malignancy prevalence according to size [27].

We also, had studied the Bethesda Criteria for cytology with postoperative histopathology in our study we found that, incidence of malignancy was 13.15% in nodules with Bethesda 3 (Atypia of undetermined significance or follicular lesion of undetermined significance). While incidence of malignancy was 42.85% in nodules with Bethesda 4 (Follicular Lesion/Suspicious for Follicular Lesion). These results in agreement with Cibas and Ali, 2017, Faquin et al., 2016; reported that Bethesda III had risk of malignancy 10–30% if noninvasive follicular thyroid neoplasm with papillary-like features (NIFTP) is considered a cancer and 6% to 18% risk of malignancy if NIFTP is not a cancer. And for Bethesda IV risk of malignancy (10%–40%), Malignancy rates are similarly decreased when NIFTP is reclassified as non-malignant (10%–40% vs 25%–40%) [5, 28]. Iskandar et al., 2015, The malignancy rate in resected thyroid nodules was 13% (6/48) for Bethesda 3. While rate of malignancy was 28% (20/71) for Bethesda 4 which was against our results [29].

On the other hand, Strickland et al., 2015, reported in his study that rates of malignancy for, AUS/FLUS, SFN, 39.2%, 45.5%, respectively. If noninvasive follicular variant of papillary thyroid carcinoma (NFVPTC) were no longer termed carcinoma, these rates would drop to, 21.6% (45% decrease), 37.5% (18% decrease), respectively. These differences between studies may be due to these studies conducted on different number of patients with different races [30].

CONCLUSION

Although circulating miRNA-222 has been identified as novel minimally invasive biomarker for preoperative prediction of malignant nodules, but in our study, it did not show a value as a tool for discrimination of malignant nodules. Ultrasound remained important procedure in preoperative prediction and management of thyroid nodules especially when correlating to nodule size which had positive correlation with malignancy in our study.

RECOMMENDATIONS

We recommend further studies to analyse miRNA profile through Solexa sequencing procedure and qRT-PCR for initial biomarker screening, in different thyroid malignancy subtypes, in tissue and sera, pre and post nodule resection, and correlate results in a larger study to further validate the usefulness of circulating mi-RNA in prediction and diagnosis of thyroid malignancy preoperatively.

Abbreviations

ACR	American College of Radiology
ATC	anaplastic thyroid carcinoma
AUS/FLUS	Atypia of undetermined significance or follicular lesion of undetermined significance
FA	Follicular Adenoma
FNAC	Fine-needle aspiration cytology

IFVPC	invasive follicular variant of papillary carcinoma
IQR	interquartile range
MTC	medullary micro-carcinoma
PDC	poorly differentiated carcinoma
PTC	papillary cell carcinoma
qRT-PCR	quantitative Real-Time polymerase chain reaction
ROC	Receiver operating characteristic curve
TI-RADS	Thyroid Imaging, Reporting, and Data System
US	Ultrasound

DECLARATIONS

- **Ethical approval and consent to participate:** This study was approved by the Research Ethics Committee, Faculty of medicine, Ain Shams University. Federal Wide Assurance No. FWA000017585. Written and oral informed consent was obtained from all patients included in the study.
- **Consent for publication:** Not applicable.
- **Availability of data and material:** Data and material are available upon request.
- **Competing Interest:** The authors declare that they have no competing interests.
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- **Authors' Contributions:**
 - SA participated in formatting the design of the research, collected patients included in the study, data collection, statistical analysis of data, have drafted the work, wrote the manuscript and final approval of the version to be published
 - MH has designed the research, substantively revised it, and approved the submitted version
 - YE participated in preparing the design of the research, revised the manuscript, and approved the submitted version
 - MM, were following the process of the study, interpretation of data, revised the manuscript, and approved the submitted version
 - LS conducting the laboratory work, participated in drafting the work, revised the manuscript and approved the submitted version
 - SE, data collection, interpretation of data, revised the manuscript and approved the submitted versionall authors had read and approved the manuscript

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