



Diagnostic Value of High Resolution Neck ultrasonography, Fine Needle Aspiration Cytology and BRAFV600E Mutation in Diagnosis of Malignant Thyroid Nodules

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Abstract

Background: Thyroid malignancy is rapidly raising, nearly 9-15% of the thyroid nodules are malignant nodules. Finding an optimal mean to diagnose malignant thyroid nodule preoperative is a challenge. Combination of molecular analysis, imaging and cytopathology may be helpful. This study aimed to evaluate the value of adding BRAFV600E analysis to nodules with suspicious ultra-sonographic criteria.

Patients and methodology: 50 patients from Kasr alainy endocrine outpatient clinic with solitary or multiple thyroid nodules are included, All patients subjected to full history and examination, thyroid profile, high resolution neck High resolution neck ultrasound (U/S), Fine needle Aspiration Cytology (FNAC), U/S guided FNAC and BRAFV600E analysis on FNAC using DNA sequencing then high resolution melt curve analysis (HRMA) for confirmation.

Results: The incidence of BRAFV600E mutation among papillary thyroid cancer (PTC) patients was 55.6%. it's sensitivity 42.9%, specificity was 100% in diagnosing PTC. Sensitivity of high resolution neck ultrasonography in detecting malignancy was 88.2%, specificity 72.7%. Addition of ultrasonography to BRAFV600E analysis increased ultrasound sensitivity in detecting PTC preoperative to 92%. There was a positive correlation between most suspicious ultrasonography findings and presence of BRAFV600E mutation (increased AP/T diameter, Increase intra-nodular blood flow, cervical lymphadenopathy, absent or incomplete halo (all p value<0.001), irregular border p value 0.004, micro calcifications p value 0.007.

Conclusion: Adding BRAF-V600E analysis to work up of suspicious thyroid nodule would increase the sensitivity of preoperative diagnosis of PTC especially in cases of indefinite pathological findings. HRMA is simple, low cost tool for BRAF-V600E analysis in comparison to DNA sequencing method.

Keywords: Thyroid nodule; Thyroid cancer; High resolution neck ultrasound (U/S); Fine needle aspiration cytology (FNAC); BRAFV600E mutation

Abbreviation: (U/S): High Resolution Neck Ultrasound; FNAC: Fine Needle Aspiration Cytology, PTC: Papillary Thyroid Cancer; HRMA: High Resolution Melt Curve Analysis

Introduction

Thyroid nodules is a common problem their prevalence reaching 35% by ultrasound examination in some studies [1] and 8-65% in autopsy data [2] There is an increasing rate of

thyroid cancer incidence worldwide, 9~15 % of all thyroid nodules are malignant [3,4]. Accordingly the initial evaluation of thyroid nodule should focus on exclusion of malignancy [3].

High resolution U/S is the most sensitive diagnostic imaging technique for the detection of the thyroid nodules [4], it can characterize suspicious nodules by some sonographic [5], but it depends on the skills and accuracy of the operator [6].

FNAC is the most important step in the work up of the thyroid nodule; Diagnostic accuracy has improved using US needle localization due to a decreased number of inadequate specimens and false negative results [7]. The pathology report from FNAB according to Bethesda may be read as non-diagnostic, benign, indeterminate lesion (atypia or follicular lesion of undetermined significance), Follicular neoplasm or suspicious for follicular neoplasm, Suspicious for malignancy and malignant [8,9]. Benign lesions on FNAB have an approximate 3% risk of malignancy (vary with patient population), and may be followed clinically with ultrasound or with a repeat FNAB [9], which, if also benign, decreases the risk of a false negative to 1.3% [10], the only malignant pathology reliably diagnosed through FNAB is papillary thyroid carcinoma, as features such as 'Orphan Annie' nuclei, nuclear grooves, intra-nuclear inclusions, and psammoma bodies can be sufficient for a diagnosis [11]. Benign and malignant follicular neoplasms and oncocytic (Hurthle cell) adenomas and carcinomas cannot be distinguished on the basis of cytology alone, as tissue architecture is required to make the diagnosis of malignancy through observation of capsular or angio-lymphatic invasion [12].

Indeterminate cytology findings vary from 15% to 25% [13]. Indeterminate FNAC results and cyto-diagnostic errors are unavoidable due to overlapping cytological features particularly among hyperplastic adenomatous nodules, follicular neoplasms and follicular variants of papillary carcinoma [14]. The correlation of U/S features with FNAB results helps to overcome

the limitations of FNAB alone [15] even when cytological results were the same, the malignancy rate was higher when nodules had suspicious U/S findings [16]. So, U/S features and FNAB results are complementary to each other [17].

From that comes the importance of a new diagnostic tool to solve the problem, to overcome the limitations of FNA and to avoid unnecessary surgery. Several molecular factors have been added to improve the diagnostic accuracy of U/S-guided FNAB [2,18] one of the promising genetic factors is the analysis of the V600E mutation [19,20].

Aim of the Work

To detect an accurate and useful tool for early preoperative determination of thyroid malignancy in the patients with thyroid nodule and assess its value in addition to the previous modalities (ultrasonography, and FNAC) that may increase the diagnostic accuracy and prevent unnecessary surgery

Patients and Methods

This single group comparative descriptive study included 50 patients from Kasr Alainy endocrine outpatient clinic, Cairo University. The thyroid examined contained either solitary or multiple nodules. Four patients with four nodules were hypothyroid (8%), and two nodules were in hyperthyroid patients (4%), and the remaining part 44 nodules were in euthyroid patients (88%). 18 examined nodules were solitary nodule and 32 were a nodule in MNG. Nodules' size ranges from 0.6-28mm³, Mean 6.6mm³±5.8mm³ SD, Median 6.0mm³. Patients' ages ranged from 22-65 years old median 43.0±9.6 SD, Median 43.5. five males (10%) and 45 females (90%). Demographic data are included in Table 1.

Table 1: Descriptive Data.

	Frequency (n=50)	%
Male	5	10
Female	45	90
Nodule site		
Right lobe	26	52
Left lobe	22	44
Ismuth	1	2
Whole gland	1	2
Count		
Single	32	64
MNG	18	36
Thyroid profile		
Hyperthyroidism	2	4
Hypothyroidism	4	8
Euthyroid	44	88
U/S		
Suspicious	24	48

Not suspicious	26	52
LNS		
Present	4	8
Absent	46	92
Pathology		
Benign	28	56
Malignant	17	34
Indefinite	5	10
Malignant type (n=17)		
Follicular neoplasm	5	29.4
Papillary carcinoma	12	70.6
Sequencing results (n=47), HRMA results (n=47)		
BRAF v600E Mutation	6/47	12.8
No mutation	41/47	87.2

All patients were subjected to

- Full clinical history including age, sex, past history of head and neck irradiation and positive family history of thyroid cancer.
- Thyroid examination.
- Thyroid profile (FT3, FT4, and TSH).
- High resolution neck ultrasound.
- U/S guided FNAC.
- BRAFV600E analysis.

Ethical considerations

All patients were informed about these types of investigations and for confidentiality their names were omitted and replaced by numerical codes.

Sonographic criteria for thyroid nodules include

Echogenicity, Presence or absence of calcification and its types, A-P to Transverse diameter ratio, Blood flow in the nodule and its patterns whether central or peripheral or both, Solitary or dominant nodules, Presence or absence of halo, Solid, cystic or partially solid and partially cystic, Sharpness of border.

Signs of increase risk of malignancy in U/S include

- Hypo-echogenicity. Micro- or interrupted rim calcifications, Irregular Margins.
- Absence of Halo or incomplete halo.
- Increased Intra-nodular flow.
- Increase antro- posterior to transverse diameter (AP/T) diameter.
- Significant increase in size over time.
- Invasion of anterior strap muscles

- Presence of abnormal cervical lymphadenopathy.

All cytology specimens were obtained under ultrasound guidance using a 21-gauge needle attached to a plastic syringe, part of the smear obtained were wet fixed in 95% ethyl alcohol and stained with hematoxylin and eosin stain for routine cytological evaluation, and the other part put in test tube with saline for genetic examination.

Molecular genetic testing

Specimen collection: Samples were collected from each subject in sterile containers for the genotyping technique. Samples were either processed fresh or were stored at 2-8 °C.

DNA extraction: This was performed using Thermo Scientific Gene JET Whole Blood Genomic DNA Purification Mini Kit supplied by Thermo Scientific Fermentas. The extracted DNA then subjected to the algorithm for molecular detection of BRAFV600E mutation. The aim of the current study is the detection of the most common mutation in the BRAF gene. V600E in DNA samples collected from thyroid nodules.

We used DNA sequencing method for BRAF V600E mutation detection, unfortunately all samples were BRAF V600E mutation negative, so we tried more accurate method (HRMA), then DNA sequencing for validation of the mutant BRAF results had done.

The extracted DNA were conducted to the work as follows

Extracted DNA DNA sequencing of exon 15 HRMA DNA sequencing for validation of the mutant BRAF results

DNA amplification using the polymerase chain reaction

DNA amplification using the PCR: Enzymatic amplification was performed by PCR using HotStarTaq® Master Mix Kit (250 units) supplied by QIAGEN® and BIO RAD T100TM Thermal Cycler*.

Detection of PCR amplification products: Fluorescent stained DNA was detected by using Agarose Gel Electrophoresis and Ultra-Violet Light Trans-illumination.

Purification of PCR product: This was performed using QIAquick® PCR Purification Kit supplied by QIAGEN® (Table 2).

Table 2: U/S Characteristics for the nodules included in the study.

US findings	Frequency (n=50)	%
Nodules Number		
Single	32	64
MNG	18	36
AP/T		
< 1	31	62
≥ 1	19	38
Echogenicity		
Hypoechoic	32	64
Hyperechoic	2	4
Isoechoic	4	8
Heterogeneous	12	24
Halo		
Present	36	72
Interrupted	4	8
Absent	10	20
Border		
Well-defined	36	72
Ill-defined	14	28
Breakdown		
Central	16	32
Peripheral	9	18
Central + Peripheral	5	10
No breakdown	20	40
Calcification		
Present	23	46
Absent	27	54
Calcification type (n=23)		
Micro	20	87
Coarse	2	8.7
Patchy	1	4.3
Blood flow		
Central	12	24
Peripheral	27	54
Central + Peripheral	9	18
No	2	4
LNS		
Present	4	8
Absent	46	92

Sequencing coding sequence and intronic boundaries of Exon 2 and Exon 3 in INS gene Cycle Sequencing: Using the Applied Biosystems* (ABI) PRISM® BigDye® Cycle Sequencing Ready Reaction Kit.

Second purification: Removal of excess Dye Deoxy™ terminators from sequencing reactions by using Centri-Sep columns (Princeton separations) Terminator v3.1 Completed DNA long Read capillary electrophoresis on the ABI 3500.

Analysis of data

Sequences were compared to the published Reference sequence of Homo sapiens (BRAF), NCBI (National Centre of Biotechnology Information): (NG_007873.3, NM_004333.4, and NP_004324.2) Analysis was done by BLAST (Basic Local Alignment Search Tool) (www.ncbi.nlm.nih.gov) and the CLC-BIO sequence viewer 6 program (www.clcbio.com).

High resolution melt curve analysis: The HRMA reaction was done according to the protocol of the kit MeltDoctor™ HRM Master Mix Applied Biosystems, USA.

DNA sequencing for validation of the BRAF mutant results: The samples with mutant results were processed after HRM analysis by pipetting the reaction from the reaction tubes

followed by purification of the products.

Statistical analysis: Pre-coded data was entered on the computer using "Microsoft Office Excel Software" program (2010) for windows. Data was then transferred to the Statistical Package of Social Science Software program, version 21 (SPSS) to be statistically analyzed.

Data was summarized using range, mean, standard deviation and median for quantitative variables and frequency and percentage for qualitative ones. Comparison between groups was performed using independent sample t-test or Mann Whitney test for quantitative variables and Chi square or Fisher's exact test for qualitative ones. P values less than 0.05 were considered statistically significant, and less than 0.01 were considered highly significant.

Results

There is no statistically significant association between different sex, nodular site, count and thyroid functions with malignancy or BRAFv600E gene mutation (Table 3). Pathology results showed 28 benign nodules, 17 malignant nodule (12 papillary -5 follicular neoplasm) and 5 cases with indefinite nodules. The incidence of BRAF V600E gene mutation in our patients with PTC was 55.6%

Table 3: Association between different variables with malignancy and BRAF v600E mutation.

Pathology					Sequencing or HRMA results					P value
	Malignant (n=17)		Non-malignant (n=33)		P value	Mutation (n=6)		No mutation (n=41)		
	N	%	N	%		N	%	N	%	
Sex										
Male	1	5.9	4	12.1	0.7					
NS	1	16.7	4	9.8	0.5					
Female	16	94.1	29	87.9		5	83.3	37	90.2	NS
Nodule site										
Right lobe	9	52.9	17	51.5	0.5					
NS	2	33.3	22	53.7	0.7					
Left lobe	7	41.2	15	45.5		4	66.7	17	41.5	NS
Ismuth	0	0.0	1	3		0	0.0	1	2.4	
Whole gland	1	5.9	0	0.0		0	0.0	1	2.4	
Count										
Single	12	70.6	20	60.6	0.5					
NS	4	66.7	26	63.4	1					
MNG	5	29.4	13	39.4		2	33.3	15	36.6	NS
Thyroid profile										
Hyperthyroidism	0	0.0	2	6.1	0.5					
NS	0	0.0	2	4.9	0.6					
Hypothyroidism	1	5.9	3	9.1		0	0.0	4	9.8	NS
Euthyroid	16	94.1	28	84.8		6	100	35	85.4	
U/S										

Suspicious	15	88.2	9	27.3	<0.001					
S	5	83.3	16	39	0.08					
Not suspicious	2	11.8	24	72.7		1	16.7	25	61	NS
LNS										
Present	4 23.5	0	0.0	0.001						
S	4	66.7	0	0.0	<0.001					
Absent	13 76.5	33	100		2	33.3	41	100	S	

50 nodules were included but unfortunately because of repetition of genetic analysis for BRAF V600E gene mutations by DNA sequencing then again by HRMA methods that leads to that 3 samples were not enough to complete genetic analysis so BRAF V600E analysis completed only in 47 samples.

We found a positive correlation with significant p value

between some suspicious sonographic criteria and malignancy and some suspicious sonographic criteria and BRAF V600E mutation as increased (AP/T) diameter, absent or interrupted halo, micro-calcifications, increase intra-nodular blood flow, presence of suspicious LNs and irregular border with increased both sensitivity and specificity (Table 4 & 5).

Table 4: BRAF v600E gene mutation among different cytological groups and its p value.

	Sequencing or HRM results				P value
	Mutation (n=6)		No mutation (n=41)		
	N	%	N	%	
Pathology					<0.001 S
Benign	0	0.0	28	68.3	
Malignant	6	100	8	19.5	
Indefinite	0	0	5	12.2	

Table 5: Association between malignancy and BRAF v600E mutation with each parameter of US.

U/S Criteria	Pathology				P value	BRAF v600E analysis				P value
	Malignancy (n=17)		Non-malignant (n=33)			Mutation (n=6)		No mutation (n=41)		
	N	%	N	%		N	%	N	%	
AP/T										
< 1	3	17.6	28	84.8	<0.001 S	0	0.0	30	73.1	<0.001 S
≥1	14	82.4	5	15.2		6	100	11	26.9	
Echogenicity					0.7 NS					0.8 NS
Hypoechoic	11	64.7	21	63.6		4	66.7	26	63.4	
Hyperechoic	0	0.0	2	6.1		0	0.0	2	4.9	
Isoechoic	1	5.9	3	9.1		0	0.0	3	7.3	
Heterogeneous	5	29.4	7	21.2		2	33.3	10	24.4	
Halo					<0.001 S					<0.001 S
Present	5	29.4	31	93.9		1	16.7	35	85.4	
Interrupted	4	23.5	0	0.0		2	33.3	1	2.4	
Absent	8	47.1	2	6.1		3	50.0	5	12.2	

Border					0.047 S					0.004 S
Well-defined	9	52.9	27	81.8		1	16.7	33	80.5	
Ill-defined	8	47.1	6	18.2		5	83.3	8	18.5	
Breakdown					0.8 NS					0.5 NS
Central	4	23.5	12	36.4		3	50.0	12	29.3	
Peripheral	3	17.6	6	18.2		0	0.0	8	19.5	
Central + Peripheral	2	11.8	3	9.1		1	16.7	4	9.8	
No breakdown	8	47.1	12	36.4		2	33.3	17	41.5	
Calcification					0.02 S					0.007 S
Present	12	70.6	11	33.3		6	100	16	39.0	
Absent	5	29.4	22	66.7		0	0.0	25	61.0	
Blood flow					<0.001 S					0.001 S
Central	10	58.8	2	6.1		5	83.3	5	12.2	
Peripheral	3	17.6	24	72.7		0	0.0	27	65.9	
Central + Peripheral	4	23.5	5	15.2		1	16.7	7	17.1	
No	0					0	0.0	2	4.9	
LNS					0.01 S					<0.001 S
Present	4	23.5	0	0		4	66.7	0	0.0	
Absent	13	76.5	33	100		2	33.3	41	100	

Six malignant nodules were found to harbor BRAF V600E gene mutation, five of them were papillary thyroid carcinoma and one was follicular neoplasm by FNAC with P value 0.001 and nothing was found in benign nodules so there was significant

correlation between BRAF V600E gene mutation and papillary thyroid cancer. Overall, sensitivity of ultrasound in detecting malignancy was found to be 88.2%, specificity 72.7%, Sensitivity of US and BRAF V600E mutation combined was 92% (Table 6).

Table 6: Sensitivity and Specificity of each parameter of U/S with malignant Cytology and BRAF v600E mutation.

U/S Parameters	Sensitivity		Specificity		PPV		NPV		Accuracy	
	Malign- ancy	Braf- mutation	Malign- ancy	Braf- mutation	Malign- ancy	Braf- mutation	Malign- ancy	Braf- mutation	Malign- ancy	Braf- mutation
Hypo-echogenicity	66.7%	64.7%	36.6%	36.4%	13.3%	34.4%	88.2%	66.7%	40.4%	46%
Micro-calcification	70.6%	70.6%	61%	66.7%	27.3%	52.2%	100%	81.5%	66%	68%
Irregular margins	83.3%	47.1%	80.5%	81.8%	38.5%	57.1%	97.1%	75%	80.9%	70%
Absence or incomplete halo	83.3%	70.6%	85.4%	93.9%	45.5%	85.7%	97.2%	86.1%	85.1%	86%
++ intra-nodular blood flow	100%	100%	4.9%	6.1%	13.3%	35.4%	100%	100%	17%	38%
AP/T >1	83.3%	52.9%	90.2%	97%	55.6%	90%	97.4%	80%	89.4%	82%
Presence of LNS	66.7%	23.5%	100%	100%	100%	100%	95.3%	71.7%	95.7%	74%

Discussion

It is noticed that thyroid nodules more common finding in females more than males, Framingham survey for thyroid nodules shows that prevalence in females 6.4% and 1.5% in males, and the Whickham study displays (6.6:1 ratio females /males) [21]. This might explain why most of the patients in our study were females representing 90%. The incidence of malignancy in our study was 34% this result is biased because 12 nodules diagnosed as PTC are chosen retrograde after diagnosis of PTC to assess BRAF v600E mutation status.

The incidence of BRAF-V600E gene mutation in our patients with PTC was 55.6%; previous meta-analyses have published values for the overall prevalence of the BRAF-V600E mutation in PTC ranging from 29 to 83%. [10,22,23], this wide range may be due to variations in PTC subtype, subjects' geographical backgrounds, and research methodology.

No difference in BRAFV600E gene mutation incidence between different age groups and this shows agreement with Ah Young Park et al. [24], but regarding our result of absence of significant association of BRAFV600E gene mutation with special sex that shows disagreement with Ah Young Park et al. study that shows increase mutation in male sex, this may be due to the small number of cases involved in our study, also 90% of our cases are females that cannot reflect the actual prevalence [24].

Our study contained five nodules (10%) with undetermined cytology, four of them have suspicious US features and all were negative regarding BRAF-V600E gene mutation, we can't exclude malignancy in these cases by absence of BRAF-V600E gene mutation alone as BRAF-V600E mutation is found only in nearly 40% of PTC cases [25].

The presence of BRAF-V600E mutation in one case of follicular neoplasm out of five cases shows agreement with only one case report Pennelli et al. [26], this conflicting result may be due to inadequate cytological diagnosis and interpretation as follicular variant of papillary cancer may be interpreted as a micro follicular lesion in FNA [27], so this case need follow up and pathological confirmation post thyroidectomy to make sure of this result.

In this present study BRAF-V600E mutation was negative in benign cases, this result shows agreement with other studies [10,11] and BRAF-V600E mutation is believed to be specific for PTC because no report has been issued of this mutation in benign nodules [28].

The sensitivity of BRAF-V600E gene mutation in detecting PTC was 42.9%, specificity was 100%, positive predictive value 100%, and accuracy 83%, this result shows agreement with Kim et al that shows BRAF-V600E mutation alone has a specificity of 100% and a positive predictive value of 100% for diagnosing malignancy in PTC [9,29].

Nam et al. [30] reported significant improvements in the sensitivity, negative predictive value, and diagnostic accuracy of FNA cytology for diagnosing malignancy by adding BRAF-V600E analysis to both US and FNAC [30].

Several studies have found a positive correlation between the BRAF-V600E mutation and suspicious sonographic features of thyroid nodules [7], other studies show inconsistent results; Kwak, et al. found that only marked hypo echogenicity was associated with BRAF-V600E positivity, Hwang et al. found that only calcification was associated with BRAF-V600E positivity [29,31] both these studies predominantly evaluated small papillary thyroid micro carcinomas, with mean tumor sizes of 6-9mm³, the small size was likely a significant confounding factor in characterizing these lesions by preoperative US.

Our study shows positive correlation between most suspicious US findings and BRAF-V600E gene mutation and shows that AP/T \geq 1, absent or incomplete halo, irregular border, micro-calcifications and Increase intra-nodular blood flow have positive correlation with positivity of BRAF-V600E mutation these results show agreement with Kabaker et al. [32] that showed association of BRAF-V600E positivity with most known suspicious U/S finding including taller than wide shape, ill-defined margin, hypo-echogenicity, micro-calcifications and absent halo (p value<0.001) [32].

This study also showed that BRAF-V600E gene mutation is significantly present in cases with cervical lymphadenopathy this result shows agreement with many studies that correlate BRAFV600E gene mutation with the frequency of lymph node metastases [10,22] Park et al. [24] study concluded that the BRAF-V600E mutation was associated extra-thyroidal extension, central and lateral lymph node metastasis, and advanced tumor stage (P<0.0001, so BRAFV600E analysis preoperative may be of value as prognostic marker and affect surgical decision regarding type of surgery.

DNA sequencing method shows high cost, time consuming and low sensitivity for the clinical screening of BRAF V600E mutation and this shows agreement with other study [33] that shows accuracy of DNA sequencing is low when dealing with cytological specimens where the cells in question may be a minor population among the vast majority of background non-neoplastic cells, Furthermore, this method needs expensive equipment which may not be economical for all the patients [34], In contrast HRMA has been proved to be applicable, cost-efficient and very sensitive scanning method that allows rapid detection of DNA sequence variations without cumbersome post-polymerase chain reaction (PCR) methods, which is not achievable by direct sequencing [35]. Because targeted therapy for thyroid cancers with multikinase inhibitors is under active development [36,37], the detection of mutations in the FNAC material may be helpful in the future to guide mutation-specific targeted therapies that can be initiated preoperatively or in

those patients who are not surgical candidates. This study has some limitations as small number of patients included, some genetic mutations should be included as RAS and RET and RET/PTC rearrangement.

Conclusion

BRAFV600E positivity was associated with most known suspicious U/S finding, BRAFV600E gene mutation analysis is a new hopeful diagnostic and prognostic tool that helps to overcome limitations of high resolution neck ultrasonography and fine needle aspiration biopsy, Adding BRAF-V600E analysis to U/S and FNA would increase the sensitivity of preoperative diagnosis of PTC especially in cases of indefinite nodules. HRMA is an excellent, simple, low cost tool for BRAF-V600E analysis in comparison to DNA sequencing method.

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