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RESEARCH ARTICLE

DIAGNOSTIC AND PROGNOSTIC UTILITY OF NRAS MUTATION GENE TESTING IN CYTOLOGICALLY INDETERMINATE THYROID NODULES.

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Abstract

Objective: to evaluate the diagnostic and prognostic impact of NRAS mutational gene testing in fine needle aspiration samples from cytologically indeterminate thyroid nodules.

Background: Fine needle aspiration cytology (F.N.A.C) is the cornerstone of assessment of thyroid nodules, but 15 to 30% of aspirates are indeterminate and are mostly referred for diagnostic surgery; exposing patients to postoperative undue effects. This raises the need to improve preoperative diagnosis of such nodules and here's the role of molecular markers (like RAS) detection in aspirates from these nodules as an adjunct to F.N.A.C.

Patients and Methods: In this study 100 patients with cytologically indeterminate thyroid nodules were subjected to NRAS gene mutation testing at exons 12/13 & 61 by direct sequencing. 90 cases were managed surgically, while 10 cases were followed up by ultrasound (U.S) after 1 year.

Results: The incidence of NRAS mutation was 34%, of which NRAS exon 61 mutations were the commonest (76.4%). In surgery correlation group, 19.6% of the benign cases were +ve for NRAS mutation (all were F.As), while of the malignant cases, 61.5% were +ve for NRAS mutation. The probability of cancer in NRAS +ve cases was 70.6%. The PPV, NPV, sensitivity and specificity for NRAS mutation testing was 70.6%, 73.2%, 61.5% and 80.4% respectively. The accuracy of NRAS mutation testing was 72.2%.

Conclusion: NRAS gene mutational testing represents a promising diagnostic tool for patients with cytologically indeterminate thyroid nodules especially those with follicular patterned thyroid lesions (F.A, F.V.P.T.C & F.T.C) and can be used as a tumor marker in such patients.

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Introduction:-

Thyroid nodules are found in about 50% of individuals during the fifth or sixth decade. Although this high incidence, 5%-15% only of which are cancer. [1]

F.N.A.C is the cornerstone of assessment of thyroid nodules, that shows the cellular morphological characteristics that may not be detected by imaging or clinical assessment. [2] But, 15 to 30% of aspirates give indeterminate cytological diagnosis, which encompass 3 subtypes: "atypia (or follicular lesion) of undetermined significance,"

(AUS/FLUS) “follicular neoplasm or suspicious for follicular neoplasm” (FN/SFN) and “suspicious for malignancy” (SMC). [3]

Cases with cytologically indeterminate nodules are mostly referred for diagnostic surgical excision; however the majority proves to be benign. For such cases, surgery is not necessary, exposing them to a 2 to 10% hazard of surgical complications, and most would require levothyroxine replacement therapy for life. Such data emphasizes the crucial demand of improving the preoperative diagnostic assessment for cases with indeterminate cytological aspirates. [4] So genetic study become a powerful adjunct to microscopic examination, as 60 - 70% of thyroid malignancies have at least 1 known molecular mutation. [5]

The most common genetic alterations in thyroid neoplasia involve RAS and BRAF mutations, PAX8–PPAR γ (peroxisome proliferator–activated receptor gamma 1) and RET/PTC gene rearrangements. [6] Highly homologous 3 human RAS genes, NRAS, KRAS, and HRAS, have been demonstrated. RAS oncogenic alleles carry mutations in specified hotspots of the three genes in codons 12, 13, and 61. [7] NRAS mutation was the most prevalent of RAS mutations in thyroid cancer. [8]

Patients and Methods:-

Study cohort:

This study was performed under Institutional Review Board approval at faculty of medicine, Mansoura University, Egypt. We analyzed prospectively the collected data of 100 patients who had preoperative cytologically indeterminate thyroid nodules by F.N.A.C either solitary or multiple presented to Mansoura university hospitals from May 2015 to May 2017.

Written informed consent was obtained from all patients enrolled in the study after explanation and illustration of the study protocol to them. Inclusion criteria included patients with age between 20 and 80 years, both genders, with nodular goiter who had indeterminate cytology using F.N.A.C preoperatively based on the Bethesda system for reporting thyroid cytopathology. Exclusion criteria included patients with diffuse goiter, patients with primary toxic goiter (grave's disease), patients with psychiatric disease & patients' refusal to be included in the study.

After careful history taking, complete clinical examination, thyroid function tests, preoperative neck U.S; U.S guided preoperative F.N.A.C was done based on the ultrasonographic pattern to diagnose thyroid nodules. Gene testing of F.N.A.C samples for NRAS point mutations at exon 12/13 and exon 61 was done reflexively for all patients with indeterminate cytologic diagnosis (AUS/FLUS, FN/SFN & SMC).

Patients were divided into two main groups: Group I: surgery correlation group; this group involved 90 cases in whom surgery was performed: hemithyroidectomy, near total thyroidectomy or total thyroidectomy. In this group L.N dissection was done in cases with suspicious neck L.Ns proved clinically or radiologically (20 cases in our study). Group II: follow up correlation group; this group involved 10 cases presented with small thyroid nodules (less than 1.5 cm) with low or very low suspicious ultrasonographic pattern, indeterminate on F.N.A.C and –ve for NRAS mutation gene testing. These patients were followed up after informing them about the very low probability of malignancy. Follow up in this group was by ultrasound done after 1 year. F.N.A.C was repeated after 1 year during the period of follow up if there was increase in size (at least two nodule dimensions) or development of new suspicious sonographic features.

Postoperative pathology of the removed specimen (in the surgery correlation group) was correlated with results of F.N.A.C and also with results of NRAS mutation gene testing.

Cytological review:

F.N.A.C from suspicious thyroid nodules was done U.S guided in all patients using 23-27 gauge needles attached to 10-20 mL syringe and high resolution U.S with (7.5–15 MHz) linear-array transducer. 3 to 4 entries of the needle were done per nodular lesion, and each nodule was aspirated at least twice. Cytologic examination was done by an expert cytopathologist and results were based on the Bethesda system for reporting thyroid cytopathology.

NRAS Mutation Analysis:

Molecular testing was done for indeterminate aspirates after cytological examination, where the rest of the aspirated material, the remaining material in the needle and the needle washing were directly collected into an eppendorf

containing 400µl of nucleic acids preservative solution (Roche Molecular Biochemicals, Mannheim, Germany), and stored at -20° C for further molecular testing. DNA was extracted from the preoperative aspirated material by F.N.A.C using GeneJET Genomic DNA Purification Kit (Thermo scientific, USA, #K0721). Then the extracted DNA was amplified using Dream Taq PCR master mix (2x) (Thermo scientific, USA, #K1071) and primers (Invetrogen, Thermo scientific, USA): targeting codons: NRAS 61: (Forward: 5'-CCCCTTACCTCCACACC-3' & Reverse: 5'-TGGCAAATACACAGAGGAAGC-3') & NRAS 12/13: (Forward: 5'-ATGACTGAGTCAAACCTGGT-3' & Reverse: 5'-CTCTATGGTGGGATCATATT-3') and thermal cycling was performed using a 9700 thermocycler. Gel electrophoresis was then done and samples with significant PCR product after PCR purification using QIAamp PCR Purification Kit (QIAGEN, USA) and cycle sequencing Using Bigdye Terminator V 3.1 cycle sequencing Kit were subjected to NRAS gene testing using ABI 3500xl DNA Sequencer.

Statistical Analysis:

Data were analyzed with SPSS version 21. The normality of data was first tested with one-sample Kolmogrov-Smirnov test. Qualitative data were described using number and percent. Association between categorical variables was tested using Chi-square test. Continuous variables were presented as mean \pm SD (standard deviation) for parametric data and median for non-parametric data. The threshold of significance is fixed at 5% level (p-value). The results was considered significant when the probability of error is less than 5% ($p \leq 0.05$). The smaller the p-value obtained, the more significant are the results.

Results:-

Demographics and ultrasonographic characteristics:

This study included 100 patients with 78 females (78.0%) and 22 males (22.0%), the age of the studied population ranged from 18 to 78 years, with the mean was 44.92 ± 14.12 . The ultrasonographic features in our study population are illustrated in (Table 1). According to the ultrasonographic characteristics; the ultrasonographic patterns in our study population entailed: high grade suspicious in 24 (24.0%) cases, intermediate grade suspicious in 12 (12.0%) cases, low grade suspicious in 51 (51.0%) cases and very low suspicious in 13 (13.0%) cases.

NRAS mutation results:

Of the 100 cytologically indeterminate aspirates tested for NRAS mutation; 34% were +ve for NRAS, while 66% were -ve for NRAS. Of the 34 +ve cases for NRAS; 8 (23.5%) cases were +ve for exon 12/13 & 26 (76.4%) cases were +ve for exon 61. As regard NRAS 61 mutation, the most common was (CAA {Glutamine aa.} >>> CGA {Arginine aa.}) in 18 (69.2%) out of 26 cases. (Figures 1&2)

Cytological results:

According to the Bethesda system for reporting thyroid cytopathology we had 100 cytologically indeterminate nodules: 59 (59%) cases were Bethesda category III, 31 (31%) cases were Bethesda category IV and 10 (10%) cases were Bethesda category V. When relating NRAS mutational status with the preoperative Bethesda categories in the study population the results were statistically significant (P value = 0.03). We found as regard cases of Bethesda class (III); only 27.1% of cases were +ve for NRAS mutation, 38.7% of class IV cases were +ve for NRAS mutation, while 60% of class V cases were +ve for NRAS mutation.

Surgical results:

Of the 90 cases that underwent surgical treatment; total thyroidectomy was done in 39 (43.3%) cases, near total thyroidectomy was done in 26 (28.9%) cases and hemithyroidectomy was done in 25 (27.8%) cases. Block neck dissection was done in 20 (51.3%) cases out of 39 malignant cases. Postoperatively; 51 (56.7%) cases were proved benign of which 22 (24.4%) cases were colloid goiters, 12 (13.3%) cases were lymphocytic thyroiditis and 17 (18.9%) cases were follicular adenomas. While 39 (43.3%) cases were found malignant on postoperative pathology; of which 18 (20%) cases were P.T.Cs (papillary thyroid carcinomas), 13 (14.4%) cases were F.V.P.T.Cs (follicular variant of papillary thyroid carcinomas) and 8 (8.9%) cases were F.T.Cs (follicular thyroid carcinomas). (Table 2) When comparing NRAS mutation results with the results of postoperative pathology the results were statistically significant (P value < 0.001), as shown in (Table 3)

Follow up correlation group results:

As regard the follow up correlation group; the 10 cases were followed up after 1 year by high resolution U.S, 3 cases showed minimal increase in size with no significant changes in the ultrasonographic characteristics. 5 cases showed

more or less stationary course. While 2 nodules showed regressive course. As regard repeated F.N.A.C; it was done for the 3 cases with increase in size yielding 2 cases with benign cytology, while 1 case remained indeterminate.

Diagnostic value of NRAS mutation analysis in cytologically indeterminate thyroid nodules:

When evaluating the diagnostic significance of NRAS mutation testing in preoperative diagnosis of indeterminate thyroid nodules in this study; it is found that: the TP (true positive) cases were 24 out of 34 +ve cases for NRAS mutation, while the FN (false negative) cases were 15 out of 56 cases –ve for NRAS mutation. The PPV (positive predictive value) for NRAS mutation testing was 70.6%, while the NPV (negative predictive value) was 73.2%. The sensitivity of NRAS mutation testing was 61.5% and the specificity was 80.4%. The accuracy of NRAS mutation testing was 72.2% in this study.

Prognostic utility of NRAS mutation testing:

Relation between NRAS mutation testing and predictors of tumor aggressiveness including; LN (lymph node) metastasis, capsular invasion, extrathyroid extension and lympho/vascular embolization was addressed in this study. (Table 4) LN metastasis was proved in only 35.9% of the malignant cases on postoperative histopathological examination, where 54.2% of NRAS +ve cases had no LN metastasis. Interestingly, the incidence of LN metastasis in our study in follicular patterned carcinomas is low 28.6 % (6 out of 21 cases).

66.7% of malignant cases in our study had capsular invasion, the majority of which (73.1%) were +ve for NRAS mutation. Extrathyroid extension in our study population was detected in 46.2% of malignant cases. 62.5% of NRAS +ve cases had extrathyroid extension, this was statistically significant (P-value=0.034). Lympho/vascular embolization in our study was detected in 41% of malignant cases; of which 62.5% were +ve for NRAS mutation.

Discussion:-

In this study, we evaluated the diagnostic and prognostic validity of detection of RAS mutation represented in our study by NRAS mutational testing (being the most prevalent of RAS mutations in thyroid cancer) in indeterminate thyroid nodules. We aim to show the relation between NRAS genetic mutation and thyroid cancer, and also how this mutational event affects the physical, histopathological and biological features of the tumor. Considering the high cost of screening all possible RAS mutations, this targeted approach in our study was sufficient to acquire diagnostic information on most nodules.

In our study, of the 100 cytologically indeterminate nodules we had 59% Bethesda III, 31% Bethesda IV and 10% Bethesda V. In Nikiforov et al. study, of fifty one cytologically indeterminate specimens; 41.2% were Bethesda III (AUS/FLUS), 45.1% were diagnosed as Bethesda IV (FN or SFN), and 13.7% were Bethesda V (suspicious for malignancy). [9] Differences in percentages compared to ours may be attributed to different sample size in our study.

On direct sequencing of the 100 indeterminate cytologic aspirates for NRAS gene at exon 12/13 and exon 61; 34% were +ve for NRAS. Of these +ve cases for NRAS mutation; the most common was NRAS exon 61 mutation (76.4 %), while NRAS exon 12/13 mutation was detected in only 23.5% of cases. Of NRAS 61 mutant cases, the commonest was (CAA {Glutamine aa.} >>> CGA {Arginine aa.}) in 69.2% of cases. Similar results were detected in a comparative study involving sixty five thyroid nodules with indeterminate cytology, where 38.5% had mutation in RAS gene, of which 70.0% had mutations in NRAS exon 61. The most prevalent mutations were c.182A>G (p.Q61R) in NRAS exon 61, that represent 76.2% (sixteen out of twenty one) of NRAS exon 61 mutations. [10] Schulten et al. showed that 53 % of all found RAS mutations were affecting NRAS codon 61 with only 3 detected mutations of RAS in codons 12 and 13. [11]

In Park et al. study it's recorded that forty five cases were diagnosed as class III Bethesda; of which 33.3% were +ve for RAS mutation. Eight cases diagnosed as class IV Bethesda; of which 37.5% were +ve for RAS mutation. Twenty five cases diagnosed as class V Bethesda; of which 64.0% were +ve for RAS mutation. [12] This agreed with our findings when relating results of NRAS mutational testing with the preoperative Bethesda categories, where we found that 27.1% of Bethesda class (III) cases were NRAS mutation +ve. 38.7% of class IV cases were NRAS mutation +ve. 60% of class V cases were NRAS mutation +ve. These results signified more incidence of NRAS mutation as the Bethesda category increases.

In our study, we found that of the 34 +ve cases for NRAS; 29.4% were benign (all were F.As) & the majority (70.6%) were malignant postoperatively. On the contrary, only 26.8 % of the –ve cases for NRAS mutation were found malignant postoperatively. 92.3% of F.V.P.T.C cases were +ve for NRAS mutation, 75% of F.T.C cases were

+ve for NRAS mutation, while only 33.3% of P.T.C cases were +ve for NRAS mutation. The incidence of NRAS mutation among follicular patterned tumors (F.A, F.V.P.T.C&F.T.C) was 73.7% & the incidence of NRAS mutation among follicular patterned carcinomas was 85.7%. No NRAS mutation detected in neither colloid goiter nor lymphocytic thyroiditis in our study. The series showed near similar result to ours, with overall incidences of RAS mutation detected in about forty eight percent of benign follicular adenomas (F.A), sixty seven percent of F.T.C, and twenty eight percent of P.T.C. [8] Similarly Schulten et al. detected that RAS mutation was most commonly detected in neoplasms with follicular patterned histopathology involving F.As and F.V.P.T.Cs. [11] RAS mutation, and especially NRAS exon 61 mutation, in P.T.C is usually linked to the follicular variant. [13]

Nikiforov et al. assessed a large series of F.N.A.B samples that are cytologically indeterminate and detected that RAS was the commonest mutation identified. Also, in that series, the possibility of malignancy associated with RAS +ve mutational status was about eighty five percent, this is higher than the results of our study where the probability of malignancy in NRAS +ve indeterminate nodules was 70.6%; this may be due to that study tested for all RAS isoforms not only for NRAS like our series, but our results are more or less concordant with other reports in literature ranging from seventy percent to eighty eight percent. [6]

In Nikiforov et al. study, follow up of 147 patients was done by annual ultrasound observation with no detected changes in the nodule status in 124 patients, while 23 patients showed changes in nodular size/ultrasonographic characteristics, in these cases repeated FNAC was applied with 17 cases proved having benign cytology and 6 cases remained indeterminate. [9] This agreed with the results of our follow up correlation group; in which high resolution U.S was done for the 10 cases after 1 year, where 30% showed minimal increase in size with no added suspicious ultrasonographic features. 50% showed more or less stationary course. While 20% showed regressive course. As regard repeated F.N.A.C; it was done for the 3 cases with increase in size giving 2 (66.7%) cases with benign cytology, while 1 (33.3%) case remained indeterminate. These results emphasized the decision of follow up in such patients avoiding the undue effects of surgical treatment.

The relation between RAS mutation and progressive tumour characteristics involving: L.N metastasis, capsular invasion, extrathyroid extension and lymphovascular embolization is controversially discussed in literature. While a number of series detected correlation of RAS mutation with tumour progression in F.T.C and P.T.C. [14] [15], other series correlated RAS mutation with encapsulated rather than with infiltrative FVPTC. [12]

In our study population, 54.2% of NRAS +ve cases had no L.N metastasis denoting less incidence of L.N metastasis in NRAS +ve malignant thyroid nodules. The incidence of L.N metastasis in our study in follicular patterned carcinomas is low 28.6 %. Capsular invasion was detected in 73.1% of NRAS mutation +ve cases. These results refer to higher incidence of capsular invasion in malignant thyroid nodules +ve for NRAS mutation. As regard extrathyroid extension in our study population, for NRAS +ve cases; there was extrathyroid extension in 62.5% of cases. These results signify more incidence of extrathyroid extension in NRAS +ve malignant thyroid nodules. As regard lympho/vascular embolization in our study group, 58.3% of NRAS +ve cases had no lympho/vascular embolization.

In a study of the impact of NRAS mutations on the diagnosis and prognosis of follicular neoplasm of the thyroid; capsular invasion was detected in 9 (40.9 %) out of 22 F.N cases +ve for NRAS mutation. 9 cases had vascular invasion; of which 2 (22.2 %) cases were +ve for NRAS mutation. Lymph node metastasis was detected in 9 (40.9%) out of 22 F.N cases +ve for NRAS mutation, while L.N metastasis was detected in 13 out of 25 cases -ve for NRAS. [16] In another study, of 21 +ve cases for NRAS; 5 (23.8%) cases have extrathyroidal extension. [17] So our results refer to higher incidence of L.N metastasis ,capsular invasion and extrathyroid extension in NRAS +ve malignant thyroid nodules than reported in literature this discrepancy may be attributed to different sample size, different detection methods of NRAS point mutation used, selection bias concerning on ultrasonographic suspicious cases& including Bethesda V category cases which have high malignant potential.

When evaluating the diagnostic significance of NRAS mutation testing in preoperative diagnosis of indeterminate thyroid nodules in this study; it is found that: the PPV for NRAS mutation testing was 70.6%, while the NPV was 73.2%. The sensitivity of NRAS mutation testing was 61.5% and the specificity was 80.4%. The accuracy of NRAS mutation testing was 72.2% in this study. In a comparative study, the sensitivity, specificity, positive predictive value, and negative predictive value of NRAS mutation +ve specimens to suspect malignant nodules were 37%, 79%, 67%, and 58%, respectively. [16] Our results showed better sensitivity, specificity, PPV& NPV this may be

explained by better detection method used in our series which is direct sequencing which is considered the gold standard in point mutation detected nowadays.

Most series suggest that RAS mutation is specific but not sensitive for cancer and carries only a PPV of 78.0% and a NPV of 64.0%, so the impact of testing of indeterminate cytology nodular lesions for RAS mutation alone is controversial. So a growing body of literature is evaluating the impact of testing multiple markers simultaneously aiming at improving sensitivity. [18] Nikiforov et al showed that a panel involving point mutations in RAS & BRAF, and also gene rearrangements such as RET/PTC1 or RET/PTC3 & PAX8/PPAR γ , is providing test specificity & sensitivity of 98% & 61%, respectively. [6]

Conclusion:-

NRAS gene mutation testing is of great value in early diagnosis and treatment of patients with follicular patterned thyroid lesions (F.A, F.V.P.T.C & F.T.C) and can be used as a tumor marker in such patients. NRAS mutation (mainly exon 61) was significantly associated with a high malignant rate in thyroid nodules. In patients having small sized nodules less than 1.5 cm having low or very low suspicious ultrasonographic pattern, cytologically indeterminate and -ve for NRAS mutation; there's no need for diagnostic thyroid surgery and just follow up by ultrasound and repeated F.N.A.C when indicated, is the preferred plan for management. As regard the prognostic utility of NRAS mutation testing, in our study, NRAS mutation positivity was linked with extrathyroid extension significantly, but less significantly related with L.N metastasis, capsular invasion and lymphovascular embolization. Further investigations are needed to ensure the prognostic utility of RAS mutation.

Recommendations

As regard the clinical use of RAS mutational analysis as diagnostic and prognostic genetic marker the following recommendations may be considered: 1) NRAS exon 61 is the most common mutation site to be searched for in RAS gene mutational analysis. 2) RAS mutation has a fundamental diagnostic impact as a part of **a molecular marker panel**. 3) In RAS mutation +ve cytologically indeterminate thyroid nodular lesions there is relatively high probability of malignancy so should be treated surgically in the appropriate clinical settings. 4) As incidence of L.N metastasis among the follicular patterned carcinomas (F.V.P.T.C & F.T.C) is relatively low, such patients usually will not benefit from more extensive surgical treatment, such as a prophylactic central compartment L.N.D unless gross disease is evident either by presurgical neck ultrasonography or based on intraoperative findings.

	Study group (n=100)
Number	
Single	36 (36%)
Multiple	64 (64%)
Size (L) Median (Min-Max)	3.00 (1.00-11.00)
Size (w) Median (Min-Max)	2.50 (0.70-7.00)
Margin	
Regular	59 (59%)
Irregular	41 (41%)
Border	
Well	67 (67%)
Ill	33 (33%)
Homogeneity	
Homogenous	44 (44%)
Heterogeneous	56 (56%)
Consistency	
Solid	62 (62%)
Cystic	2 (2%)
Mixed	35 (35%)
Spongiform	1 (1%)
Echogenicity	
Hypoechoic	48 (48.0%)
Hyperechoic	18 (18%)

Isoechoic	26 (26%)
Mixed	8 (8%)
Calcifications	
Yes	36 (36%)
No	64 (64%)
Cystic degeneration	
Yes	47 (47%)
No	53 (53%)
Halo sign	
Yes	27 (27%)
No	73 (73%)
Vascularity	
Int. Vascularity	38 (38%)
Normal	62 (62%)
LN	
Yes significant	14 (14%)
Yes insignificant	33 (33%)
No	53 (53%)

Table 1:-The ultrasonographic characteristics of the studied group of patients. (N=number, L= length, W=width, LN= lymph node)

Diagnosis	Study group (n=50)
postoperative pathology	
Benign (51)	
colloid goiter	22(24.4%)
lymphocytic thyroiditis	12(13.3%)
follicular adenoma	17(18.9%)
Malignant (39)	
PTC	18(20.0%)
FVPTC	13(14.4%)
F.C	8(8.9%)

Table 2:-Results of postoperative pathology in the study population.

Post-operative	NRAS mutation			χ^2	p-value
	Positive exon 12/13 only	Positive exon 61 only	Both Negative		
colloid goiter	0(0%)	0(0%)	22(39.3%)	54.81	<0.001*
lymphocytic thyroiditis	0(0%)	0(0%)	12(21.4%)		
follicular adenoma	2(25.0%)	8(30.8%)	7(12.5%)		
PTC	4(50.0%)	2(7.7%)	12(21.4%)		
FVPTC	1(12.5%)	11(42.3%)	1(1.8%)		
F.C	1(12.5%)	5(19.2%)	2(3.6%)		
Benign	2(25%)	8(30.8%)	41(73.2%)	16.61	<0.001*
Malignant	6(75%)	18(69.2%)	15(26.8%)		

Table 3:-Relation between NRAS mutation results & postoperative pathology results.

L.N metastasis	NRAS mutation			χ^2	p-value
	Positive exon	Positive exon 61	Both Negative		

	12/13				
Yes	4(66.7%)	7(38.9%)	3(20.0%)	4.18	0.123
No	2(33.3%)	11(61.1%)	12(80.0%)		
Capsular invasion	NRAS mutation			χ^2	p-value
	Positive 12/13	Positive 61	Both Negative		
Yes	4(66.7%)	15(83.3%)	7(46.7%)	4.95	0.084
No	2(33.3%)	3(16.7%)	8(53.3%)		
Extrathyroid extension	NRAS mutation			χ^2	p-value
	Positive 12/13	Positive 61	Both Negative		
Yes	4(66.7%)	11(61.1%)	3(20.0%)	6.76	0.034*
No	2(33.3%)	7(38.9%)	12(80.0%)		
Lympho/vascular invasion	NRAS mutation			χ^2	p-value
	Positive 12/13	Positive 61	Both Negative		
Yes	2(33.3%)	8(44.4%)	6(40%)	0.24	0.887
No	4(66.7%)	10(55.6%)	9(60%)		

Table 4:-Relation of NRAS mutation and predictors of tumor aggressiveness (combined).

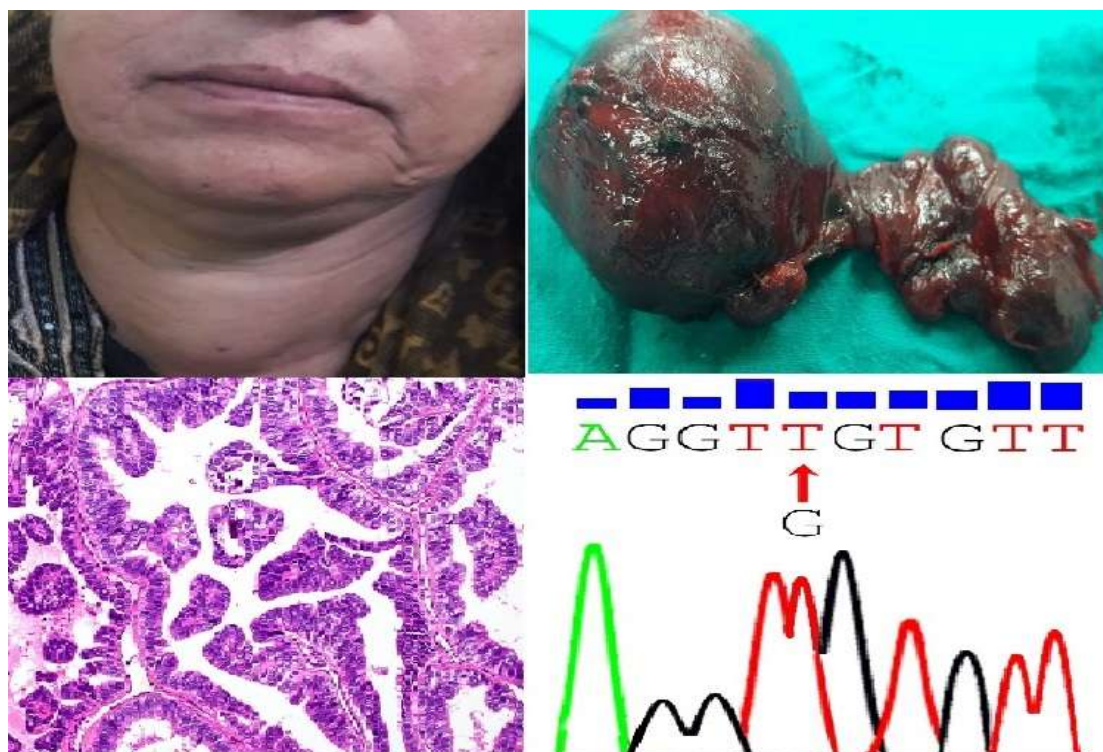


Image 1:-Showing female patient with Rt. STN, Removed specimen after total thyroidectomy, Histopathological examination Showing PTC with molecular testing for NRAS mutation showing +ve result at exon 12/13 with substitution of T instead of G (GGT {glycine aa.} >>>TGT {Cysteine aa.}).

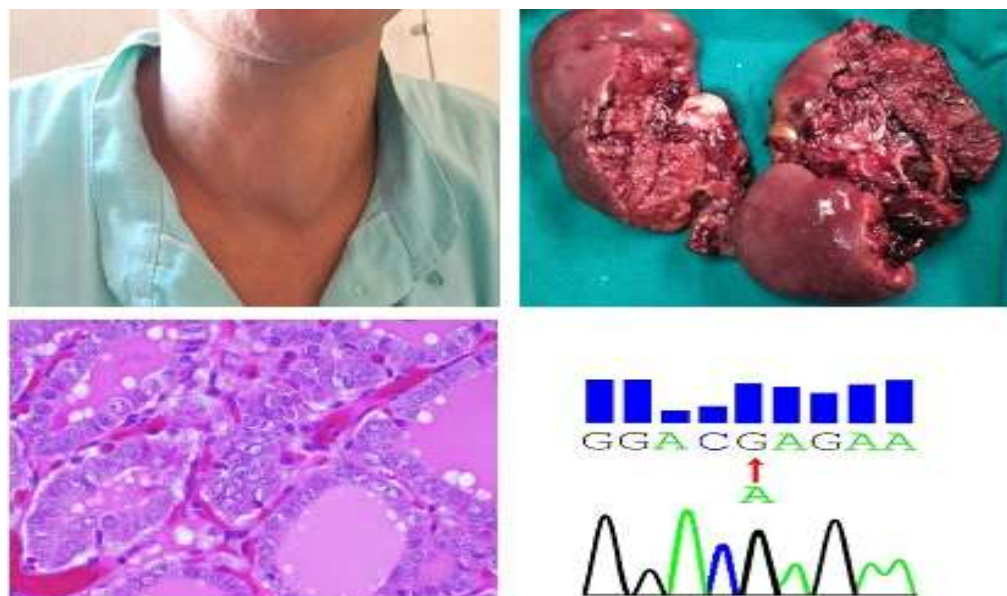


Image 2:-Showing male patient with MNG, the removed specimen after total thyroidectomy, histopathological examination of the specimen Showing FVPTC with molecular testing for NRAS mutation showing +ve result at exon 61 with substitution of G instead of A (CAA { Glutamine aa. } >>>CGA { Arginine aa. }).

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