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SIGNIFICANCE OF Ki-67 AND p53 IMMUNOMARKERS IN DIAGNOSING SOLITARY THYROID NODULES AFTER THYROIDECTOMY

Surgery

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ABSTRACT

We have done a prospective observational study using FNAC, follwed by histopathology and immunohistochemistry of the representative tissue from the specimen of excised thyroid gland. Total 50 number of patient who have undergone thyroidectomy were examined and particularly Ki-67 and p53 immunostaining helped a lot in differentiating benign from maignant follicular neoplasm and thus helped in definitive management of thyroid neoplasms.

KEYWORDS

Ki-67, p53, papillary carcinoma thyroid, follicular carcinoma, medullary carcinoma, follicular adenoma, nodular colloid goitre, hyperplastic nodule.

INTRODUCTION

The thyroid gland is one of the major endocrine gland situated in the front of the neck. It plays a very important role throughout life from womb to tomb. Thyroid swelling with or without nodules are encountered during routine medical care.

Prevalence of thyroid nodule increases with age. It estimates 4-7% in adult population. But it is much higher (19-67%) when subclinical nodules are also considered. Thyroid cancer represents 5-24% of all these nodules.

The majority of clinically apparent thyroid neoplasms are primary and epithelial. FNAC is one of the basic tools for differentiating benign from malignant thyroid neoplasms. Result of FNAC depends on the representative material retrieved from the pathological site. It segregates thyroid neoplasms in four categories i.e. Inadequate(sample), Malignant, Intermediate and benign varieties. Inadequate sample needs repeat ultrasound guided FNAC. If it still remains inadequate needs close follow up or surgery. Frank malignant thyroid tumor like papillary cell CA, Medullary cell CA, Anaplastic CA, Metastatic CA, lymphoma of thyroid gland requires surgery and frank benign like colloid, Hurthle cell tumor, teratoma, lipoma needs follow up. Dilemmas remain on indeterminate varieties. Follicular variety, where surgery required following I123 scan or on suspicion for carcinoma considering the finding in clinical examination, USG of thyroid gland etc. In spite of going through a battery of tests, pre surgery diagnosis by FNA changes after post surgery HPE(Histopathological examination) of the excised tissue. However there are situations when thyroid nodule have subtle or attenuated nuclear feature, differentiation between benign (like surcumscribed nodules with follicular architecture) and malignant lesions being difficult in the absence of invasive behavior. In the absence of invasive behavior and / or capsular invasion follicular neoplasms can be differentiated by immunomarkers. Among the proposed markers for differentiating benign from malignant thyroid lesions are p53 and Ki67 markers.

In this study two immunomarkers Ki-67 and p53 were used on histological sections of excised thyroid lesions to evaluate their diagnostic significance in differential diagnosis of thyroid nodules.

In this study we assessed the expression of Ki-67 and p53 in papillary carcinoma, follicular carcinoma, medullary carcinoma, follicular adenoma and nodular hyperplasia and established their utility in differentiating benign from malignant thyroid lesions (derived from follicular cells).

The aim of this study was to establish the correlation of between fine needle aspiration cytology (FNAC) and histopathology of thyroid nodules, and the correlation between histopathology and two immunomarkers Ki-67 and p53 on histological sections of thyroid nodules and assess the significance of Ki-67 and p53 immunostaining to differentiate benign and malignant thyroid lesions.

Method: Study design: Total 50 patients (cases) selected for the present study were collected from those patients with thyroid swelling who were attended the FNAC clinic ,department of pathology and undergone surgical treatment with postsurgical histopathological diagnosis in the same pathology department during a period of one and half years, from February 2013 to July 2014.

23G needle used for FNAC. FNAC followed by surgery and subsequent histopathological examination along with immunohistochemistry with two immunomarkers namely Ki-67 and p53.

Immunohistochemistry were performed in all the 50 cases who were underwent surgical excision, using 3 micron thick sections on poly-l-lysine coated slides. Antigen retrieval was done using microwave in citrate buffer at pH 6. Monoclonal antibodies MIB1 (DAKO M 7240) and p53 (DAKO M 7001) in dilution of 1:70 each were used for antigen detection by standard streptavidinavidin biotin kit (K 0679). Sections from a reactive lymph node were taken as positive control for Ki67 and colon carcinoma for p53, whereas sections treated with tris-buffer solution instead of primary antibody as negative control. Brown nuclear reactivity was considered positive. Ki-67 & p53 was assessed by counting the number of positive and negative cells and the strongest stained tumor areas were chosen to evaluate labelling index (LI) (expressed as percentage of positively stained cells per 100 follicular epithelial cells) after counting at least 100 cells in each case.

After examining several microscopic fields and quantifying immunoreactions, the results were quantitatively expressed according to the percentage of positive tumor cells:

- 0 – positive immunoreaction in less than 5% of tumor cells;
- 1(+) – immunostaining of 5-30% of tumor cells;
- 2(+) – immunostaining of more than 30% of tumor cells.

Score 0 was considered negative and scores 1(+) and 2(+) were considered positive.

RESULTS AND ANALYSIS

Papillary thyroid carcinoma (PC) constituted 18 cases (36%), follicular neoplasm (FN) 12 cases (24%) and suspected neoplasm (SN) 3 cases (6%) and medullary carcinoma (MC) 2 cases (4%) out of a total 50 cases in our study. The other non neoplastic thyroid nodules constituted nodular colloid goiter (NCG) 7 cases (14%) and hyperplastic nodules (HPN) 8 cases (16%) out of 50 cases.

TABLE: 1 Solitary thyroid nodules diagnosed by FNAC (n=50)

FNAC DIAGNOSIS	NUMBER OF CASES	% OF CASES
Nodular colloidgoiter(NCG)	7	14%
Hyperplastic nodule(HPN)	8	16%
Suspected neoplasm(SN)	3	6%
Follicular neoplasm(FN)	12	24%
Papillary carcinoma(PC)	18	36%
Medullary carcinoma(MC)	2	4%
Total	50	100%

Figure: 1 Solitary thyroid nodules diagnosed by FNAC (n=50)

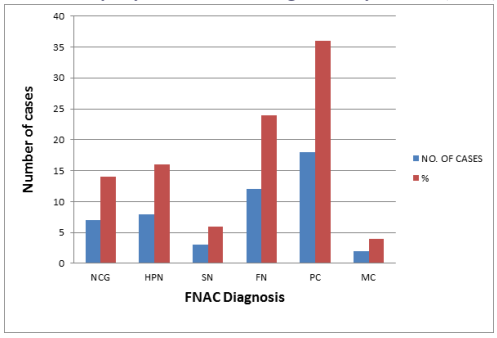


Table: 2 Distribution of patients according to cytological and histological diagnosis

No. of cases	Cytology diagnosis	Histological diagnosis					
		PC	FC	MC	NCG	HPN	FA
7	NCG	1	-	-	3	1	2
8	HPN	1	-	-	1	5	1
3	SN	2	-	-	-	-	-
12	FN	-	1	-	-	1	2
18	PC	16	-	-	-	-	-
2	MC	-	-	2	-	-	-

(PC-Papillary carcinoma, FC-Follicular carcinoma, MC-Medullary carcinoma, NCG-Nodular colloid goiter, SN- suspected neoplasm, HPN-Hyperplastic nodule, FA-Follicular adenoma)

Out of 7 cases of cytologically diagnosed (with FNAC) nodular colloid goiter, the histological findings were consistent in 3 cases (42.85%). Of the rest, 2 cases turned out to follicular adenoma (FA),one turned out to hyperplastic nodule and one as papillary carcinoma.

Out of 8 cases with cytological diagnosis of hyperplastic nodule of thyroid, the histological findings were consistent in 5 cases (62.5%). Among the non consistent cases, one was found to have a focus of papillary carcinoma on histopathology, one nodular colloid goiter and one as follicular adenoma.

Out of 3 cases of suspected neoplasm, two cases diagnosed as papillary carcinoma on histology and in one case even by histology we could not differentiated it between follicular adenoma or follicular carcinoma .In this one case with the help of immunohistochemistry (IHC) it was possible to differentiate between follicular adenoma and follicular carcinoma.

Out of 12 cytologically diagnosed follicular neoplasm of thyroid, one case was histologically diagnosed as hyperplastic nodule, 2 cases as follicular adenoma and one case as follicular carcinoma, and in rest of the 8 cases differentiation between follicular adenoma and follicular carcinoma was not possible distinctly even after histology . Immunohistochemistry (IHC) helped a lot to differentiate those 8 cases.

Out of 18 cases of cytologically diagnosed papillary carcinoma, histology were consistent with 16 cases (88.88%) and in two (11.11%) cases even by histology it could not differentiated between follicular adenoma and follicular carcinoma .In these 2 cases IHC helped to differentiate between follicular adenoma and follicular carcinoma.

In 2 cases of medullary carcinoma all 2 cases showed consistent results on histology (100%).

Table: 3 Cytohistological correlation of solitary thyroid nodules

No. of cases	Cytology diagnosis	Histological diagnosis	
		Consistent with cytology diagnosis	In consistent with cytology diagnosis
7	NCG	3(42.85%)	4(57.14%)
8	HPN	5(62.5%)	3(37.5%)
3	SN	-	-
12	FN	-	-
18	PC	16(88.88%)	2(11.11%)
2	MC	2(100%)	-

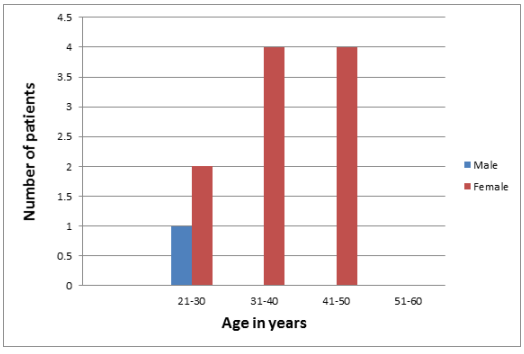
Follicular neoplasms (12 cases) and suspected neoplasm (3 cases) of thyroid could not be classified as benign or malignant lesions by cytology. Therefore, cytohistological correlation in these group was not considered.

Overall cytohistological correlation in our study was found to be 65.38%.

Table: 4 Age and sex distribution in non-neoplastic thyroid nodules (N 11)

No. of cases	Age in yrs	Percentage(%)	Sex	
			Male	Female
3	21-30	27.27	1(33.33%)	2(66.66%)
4	31-40	36.36	0	4(100%)
4	41-50	36.36	0	4(100%)
0	51-60	0	0	0

Figure: 4 Age and sex wise distribution of non-neoplastic thyroid nodules

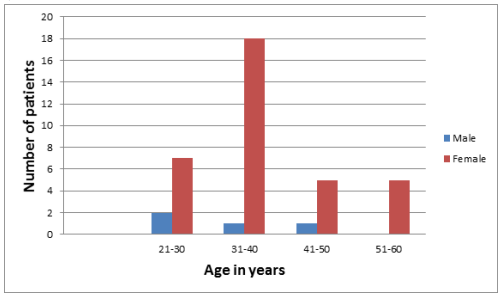


Among the 11 non-neoplastic lesions of thyroid10 were female (90.90%) and one was male (9.09%) (Table-4 & figure-4).The age of the patients ranges from 21 years to 50 years. Maximum number of patients were in the age group is 31 to 40 years (36.36%), and 41 to 50 years (36.36%). Least number of patients (27.27%) was obtained in the age group 20 to 30 years.

Table 5: Age and sex distribution of neoplastic thyroid nodules (n=39)

No. of cases	Age in years	Percentage (%)	Sex	
			Male	Female
9	21-30	23.07	2(22.22%)	7(77.77%)
19	31-40	48.71	1(5.26%)	18(94.73%)
6	41-50	15.38	1(16.66%)	5(83.33%)
5	51-60	12.82	0	5(100%)

Figure: 5 Age and sex distribution of neoplastic thyroid nodules (n=39)



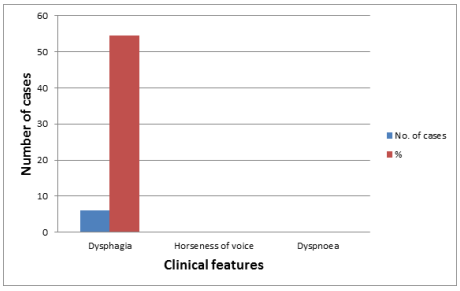
Among the 39 patients 35 patient were female (89.74%) and rest 4 patients were male (10.25%).The age distribution ranges from 21 years to 60 years. Maximum number of the patients were in the age group is 31 to 40 years(48.71%), followed by 21 to 30 years (23.07 %), 41 to 50 years (15.38%) and 51 to 60 years age group (12.82%).

Presenting complains

Table-6: Clinical features of non-neoplastic thyroid nodules

Presenting complains	Number of cases(n=11)	%
Dysphagia	6	54.54
Horseness of voice	0	0
Dyspnoea	0	0

Figure: 6 Clinical features of non-neoplastic thyroid nodules

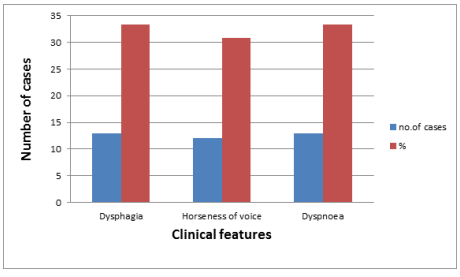


All 11 patients of non-neoplastic lesions presented with neck swelling in thyroid region, were gradual in onset in all cases and 6 patients (54.54%) had complain of dysphagia.

Table: 7 Clinical features of neoplastic thyroid nodules

Presenting complains	No. of cases(n=39)	%
Dysphagia	13	33.33
Horseness of voice	12	30.76
Dyspnoea	13	33.33

Figure: 7 Clinical features of neoplastic thyroid nodules



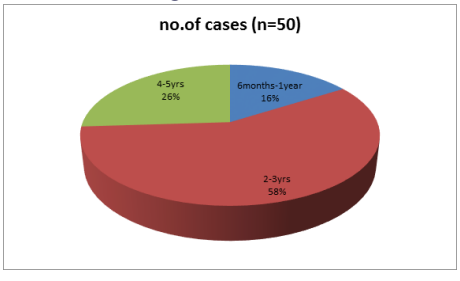
All 39 patients of neoplastic lesion presented with neck swelling in thyroid region. The mode of onset was gradual in all cases. 13 patients (33.33%) presented with dysphagia, 12 patients(30.76%) presented with horseness of voice and 13 patients (33.33%) presented with dyspnoea.

The duration of swelling ranged from 6 months to 5 years. Among them29 patients (58%) were suffering for 2-3 years, 13 patients (26%) were for 4-5 years and 8 patients (16%) were for 6 months to 1 year.

Table: 8 Duration of swelling

Duration	No. Of cases (n=50)	Percentage (%)
6 months-1 year	8	16
2-3 yrs	29	58
4-5 yrs	13	26

Figure: 8 Duration of swelling



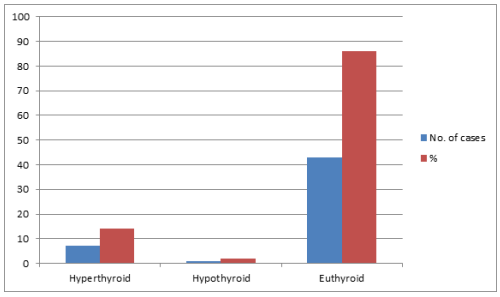
Thyroid hormone profile studies:

Lab values of biochemical investigations like T3 (Tri-iodothyronine), T4 (Tyroxine) and TSH (Thyroid stimulating hormone) were compared with clinical history and clinical findings. (Table 9 & figure 9)

Table: 9 Thyroid hormone profile (n=50)

Thyroid hormone	TSH		T3		T4	
	NO.	%	NO.	%	NO.	%
Increased	1	2	7	14	7	14
Decreased	7	14	0	0	0	0
Normal	42	84	43	86	43	86

Figure: 9 Thyroid hormone profile (n=50)



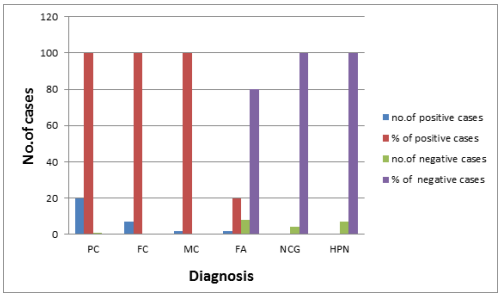
Out of 50 patients 7 (14%) were hyperthyroid with decreased TSH and increased in T3&T4 levels. One case had features of mild hypothyroidism with mild increased TSH level. Rest of the 42 patient was found essentially euthyroid.

According to the result of immunoreaction (positive or negative), the studied lesions were then divided into 2 groups: the "negative" group and the "positive group".

Table: 10 Expression of p53 immunomarker

Diagnosis	Positivity score of p53			No. of cases& percentage			
	0	1	2	Positive	%	Negative	%
PC(n=20)	0	1	19	20	100	0	0
FC(n=7)	0	2	5	7	100	0	0
MC(n=2)	0	0	2	2	100	0	0
FA(n=10)	8	2	0	2	20	7	80
HPN(n=7)	7	0	0	0	0	7	100
NCG(n=4)	4	0	0	0	0	4	100

Figure: 10 Expression of p53immunomarker

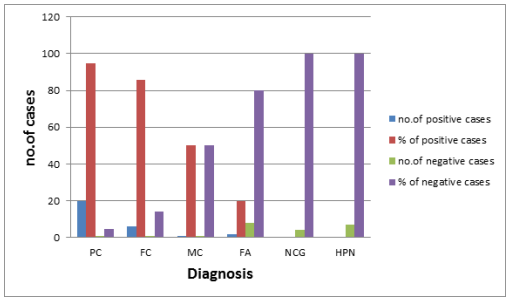


Positive expression of p53 protein were seen in 20 cases (100%) of papillary carcinoma, in 7 cases (100%) of follicular carcinoma, in 2 cases (100%) of medullary carcinoma and 2 (20%) out of 10 cases of follicular adenoma and it was negative in all (100%) cases of nodular colloid goiter and hyperplastic nodule .

Table: 11 Expression of Ki-67 immunomarker

Diagnosis	Positivity score of Ki-67			No. of cases & percentage			
	0	1	2	Positive	%	Negative	%
PC(n=20)	1	19	0	19	95	1	5
FC(n=7)	1	6	0	6	85.71	1	14.28
MC(n=2)	1	1	0	1	50	1	50
FA(n=10)	8	2	0	2	20	8	80
HPN(n=7)	7	0	0	0	0	7	100
NCG(n=4)	4	0	0	0	0	4	100

Figure: 11 Expression of Ki-67 immunomarker



Positively expressed Ki-67 antigen were found in 19 (95%) of 20 cases of papillary carcinoma, in 6 (85.71%) of 7 cases of follicular carcinoma, in one case (50%) of medullary carcinoma and 2 (20%) of 10 cases of follicular adenoma. The result was negative in all (100%) of nodular colloid goiter and hyperplastic nodule.

DISCUSSION

A number of important facts had been revealed from the present study of 50 cases of solitary thyroid nodules.

Age & Sex: The age of the patients in our study ranged from 21 years to 60 years with a female to male ratio of 9:1. In male maximum number of cases were found in the age group was 21-30 years and in female it was 31-40 years. In the non-neoplastic thyroid nodules (11/50=22%), maximum number of the patients found in the age group of 31-40 years and 41-50 years. In case of neoplastic thyroid nodules (39/50=78%), maximum number of the patients found in the age group of 31-40 years and least number of the patients found in the age group of 51-60 years. (Table 4&5)

In the study of Islam et al. 2009, showed the majority of the patients were within 21-40 yrs of age.

In one study solitary thyroid nodules were more common in female, where male female ratio was 1:2.2. This female preponderance is reflected in all studies including the present.

Despite the fact that thyroid disorders are more common in females, the probability of a male patient harbouring a malignancy in a nodule is three to five times higher.

Clinical features: Neck swelling was the commonest complain of the patients in our study. In the non-neoplastic thyroid nodules group, 6 patients (54.54%) out of 11 patients presented with dysphagia compared to 13 patients (33.33%) out of 39 patients neoplastic thyroid nodules group. 12 patients (30.76%) presented with hoarseness of voice and 13 (33.33%) patients presented with dyspnoea. (Table 6&7)

Duration of complains: Maximum duration of complains of 4-5 years found in 13 (26%) patients and maximum patients with solitary thyroid nodule had a duration of complains of 2-3 years were 20 patients (58%). Only 8 patients (16%) came within 6 months to 1 year after the onset of swelling. (Table 8)

Hormone profile: In our study, most of the patients (42 out of 50 patients) of solitary thyroid nodules were found to be euthyroid (84%), 7 patients (14%) were found to be hyperthyroid ,one patient (2%) was mild hypothyroidism. (Table 9)

In a study of 35 subjects with toxic adenoma, Hamburger found 16 with elevations in both T3 and T4 levels, 16 with elevations of only T3, 3with isolated T4 excess, and 7 with subclinical hyperthyroidism.

Fine needle aspiration cytology was established as a valuable and safe test in diagnosis of various thyroid diseases. Advantage and limitation of FNAC should be recognized and the procedure should be knowledgably applied to the evaluation of the thyroid diseases.

In the present study out of 50 cases of solitary thyroid nodules diagnosed by FNAC before surgery.(Table 1) Papillary thyroid carcinoma found in 18 cases (36%), follicular neoplasm in 12 cases (24%) and suspected neoplasm in 3 cases (6%) and medullary carcinoma in 2 cases (4%) ,other non neoplastic thyroid nodules constituted nodular colloid goiter in 7 cases (14%) and hyperplastic nodules in 8 cases (16%). (Table 2)

In the present study out of 7 cases with cytological diagnosis of nodular colloid goiter, the histologic finding was consistent in 3 cases (42.85%). In the remaining 4 cases, 2 cases turned out to be follicular adenoma, one case turned to be hyperplastic nodule and one as papillary carcinoma.

Histologic finding of 5 cases (62.5%) out of eight cases were consistent with cytologic diagnosis of hyperplastic nodule of thyroid. Among the remaining 3 cases which were non consistent, 1 case was found to have a focus of papillary carcinoma on histopathology, 1 case as nodular colloid goiter and one case as follicular adenoma.
Out of 3 cases of suspected neoplasm, 2 cases were diagnosed as papillary carcinoma by histology and the other1 case could not be differentiated even by histology between follicular adenoma and follicular carcinoma. IHC it is possible to differentiated that into follicular carcinoma which was impossible to diagnosis even after FNAC and histology.

Out of 12 presurgically diagnosed follicular neoplasm of thyroid, 1 case turned out as hyperplastic nodule on histology, 2 cases as follicular adenoma and 1 case as follicular carcinoma, and rest 8 cases could not differentiate whether it is follicular adenoma or follicular carcinoma. IHC differentiated 4 of them into follicular adenoma and rest 4 into follicular carcinoma.

Out of 18 cases of papillary carcinoma 16 cases were diagnosed as papillary carcinoma by histology and in rest two cases could not differentiated even by histology between follicular adenoma and follicular carcinoma. IHC differentiated 1 case into follicular adenoma and the Suspected neoplasm could not be classified as benign or malignant lesions by cytology. Therefore, cytohistological correlation in this group was not considered.

Follicular neoplasms could not be classified as benign or malignant lesions by cytology alone. Therefore, cytohistologic correlation in this group was not considered Overall, the cytohistological correlation, in our study, was found to be 65.38%.

This value is lower than figure reported by Das *et al*. in which case it was 88.9%.

p53 protein and Ki-67 antigen are the proposed markers for the differentiation of benign from malignant thyroid nodules.

During the last decade researchers studied to find additional criteria to make accurate diagnosis of thyroid nodules.

Present study highlights the use of Ki-67 and p53 to differentiate benign and malignant thyroid lesions.

p53 is a tumor suppressor gene localized on chromosome 17p13.1, with a role in cell cycle and in the initiation of apoptosis as a response to DNA alteration. Mutations of p53 gene had been reported in over 50% of human tumors.

Mutations of p53 represent a late genetic event in thyroid carcinogenesis. As a result, p53 accumulation can be immunohistochemically (IHC) detected especially in anaplastic and poorly differentiated thyroid carcinomas and rarely in papillary and follicular well differentiated carcinomas, as well as in medullary carcinomas. Positive p53immunoreactivity is an independent prognostic factor for the survival of patients with thyroid cancer.

In our study, the IHC expression of p53 in 50 cases of solitary thyroid lesions was shown and it helped us to differentiate between benign and malignant thyroid lesions.

We found p53 positivity in 20 cases (100%) of papillary carcinoma, 7 cases (100%) of follicular carcinoma and 2 cases (100%) of medullary carcinoma. Weak immunoreactivity for p53 was observed in 2 of 10 cases (20%) of follicular adenoma and p53 expression was absent in hyperplastic nodules and nodular colloid goiter. (Table10)

Nasir *et al* noted a 15% positivity of p53 in their follicular adenomas.

Nasir et al. pointed out the presence of p53 mutations in 90% of FC with intense nuclear positive p53 expression and weak positive p53immunostaining expressed in only 15% of FA, thus showing that IHC detection of p53 protein can be useful in differentiating FA from FC.

Moon et al. detected positive p53 immunoreaction in 9.1% of FA and in 16.7% of FC, with moderate to intense immunostaining, the results being statistically insignificant.

Ki-67 is a nuclear protein expressed by cells in proliferative phases G1, G2, M and S, being known that there is a correlation between Ki-67 immunoreaction and mitotic activity.

Ki-67 is recognized with monoclonal antibody MIB1. It is expressed in active phase of cell cycle and it rapidly degrades as the cell enters non

proliferative state. Various workers have successfully used MIB1 antibodies for separating benign and malignant thyroid tumor.

In our study we found Ki-67 weak immunoreactivity in 19 cases (95%) of papillary carcinoma, 6 cases (85.71%) of follicular carcinoma and 1 case (50%) of medullary carcinoma and 2 of 10 cases (20%) of follicular adenoma whereas ki67 expression was absent in hyperplastic nodules and nodular colloid goiter. (Table11)

Muller-Hocker and Augustynowicz *et al*. reported that proliferative activity, as measured by Ki-67, was much higher in oncocytic carcinoma than in oncocytic adenoma.

Advantages of immunohistochemistry (IHC)

- The technique is performed on paraffin-embedded tissue sections, which allows preservation of cellular details in a better way compared to frozen sections.
- IHC may show positivity in decalcified or totally necrotic material
- It can be used upon specimens stored for a long duration and previously stained tissue sections.
- IHC has an edge over routine enzymatic tissue staining by its ability to identify wider arrays of tissue antigens.

Disadvantages of immunohistochemistry (IHC)

- False positive and false negative results.
- Non-specific staining as well as aberrant immunoreactivity are the technical snags associated with IHC. Hence, diagnosis by IHC should always be supplemented by some confirmatory method.
- The chromogen DAB imparts a brown color to the tissue section creating confusion with melanin pigment. Hence, for evaluation of melanin containing lesions, other chromogens are to be used.

The limitations of the present study are-

- The sample collection being small, single institute based and conducted over a short period (one and half year) not be truly representative of the general population.
- Overlap of cytological patterns between neoplastic and non-neoplastic lesions.
- Overlap of cytological features between various neoplasms.
- Coexistence of non-neoplastic and neoplastic processes and multiple malignancies in the same gland.

Recommendations:

- Large scale control studies and longitudinal follow-up studies would be very useful as it would provide a more detailed, vivid and true picture of the population.
- More studies are needed to confirm this observation.

CONCLUSION

We conclude that, Ki-67 and p53 are reliable markers for differential diagnosis between benign and malignant thyroid lesion, these markers significantly help the clinicians to separate follicular thyroid carcinoma from follicular thyroid adenoma post operatively.

However, larger studies are needed to confirm this observation as well as to assign the cut-off value for differentiation between benign and malignant thyroid nodules.

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