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ORIGINAL ARTICLE

Expression of FHIT, p16, p53 and EGFR as prognostic markers in thyroid tumors of uncertain malignant potential

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Summary

Purpose: *Thyroid tumors of uncertain malignant potential (TT-UMP) constitute a relatively new diagnosis. The purpose of this study was to analyze the relationship between immunohistochemical panels, prognostic parameters and TT-UMP.*

Methods: *Group I was composed of patients diagnosed as differentiated thyroid carcinoma (DTC) and Group II of patients diagnosed as TT-UMP. The prognostic scores of patients were calculated using data according to the well-known prognostic scoring systems MACIS, AMES, AGES. Evaluations of antibodies were based on the presence of nuclear staining for p16 and p53, membranous and cytoplasmic staining for epidermal growth factor receptor (EGFR) and cytoplasmic staining for fragile histidine triad (FHIT).*

Results: *Statistically significant difference was noted ($p< 0.05$) between Group I and Group II according to MACIS and AMES. No statistical difference was found in terms of immunostaining between groups when stained with p16, p53 and FHIT. On the other hand, in Group II a moderate positive correlation was detected between MACIS and EGFR.*

Conclusion: *According to our findings p53 was not important in tumor genesis at early stages in well-differentiated thyroid carcinomas and p16 loss of expression could be used as a finding to help in difficult microscopic diagnosis. TT-UMP is a gray zone of lesions requiring specific therapeutic procedures and postoperative follow-up. A positive correlation was detected between EGFR and TT-UMP, leading to assume that this situation could be used as a new tool in the follow-up of these patients in the future.*

Key words: *EGFR, FHIT, P16, P53, thyroid tumors, uncertain malignant potential*

Introduction

The most common tumor of the endocrine system is thyroid cancer and it accounts for 90% of all endocrine malignancies [1]. DTC, accounting for the majority of thyroid cancers and having the best prognosis, occurs in two forms: as papillary carcinoma (PC) and follicular carcinoma (FC) [2].

TT-UMP have been a subject in many publications. This new terminology has been suggested not only to avoid a needless surgery, but also to avoid probability of unidentified malignancy [3-5]. The incidence of TT-UMP is not clear yet due to the fact that TT-UMP was recognized late and is not accepted as an international classification by all pathologists. However, the last edition of the WHO publication has formulated the term TT-UMP [4].

In evaluating thyroid nodules, fine needle aspiration (FNA) cytology is still the most valuable and simple diagnostic method. It has been known for decades that conventional histology fails to

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Table 1. Demographic characteristics of the patients

Characteristics	*Group I*	*Group II*	*p value*
Age, years (mean ±SD)	50.34 ± 13.68	50.38 ± 9.60	0.990
Gender (male/female), N (%)	14 (20.3) / 55 (79.7)	3 (14.3) / 18 (85.7)	0.568

Table 2. Similar parameters in scoring systems for thyroid carcinoma

Parameters	*AGES*	*AMES*	*MACIS*
Age	+	+	+
Tumor size	+	+	+
Distant metastasis			+
Gender		+	
Grade	+		
Extension	+	+	
Invasion			+
Complete resection			+

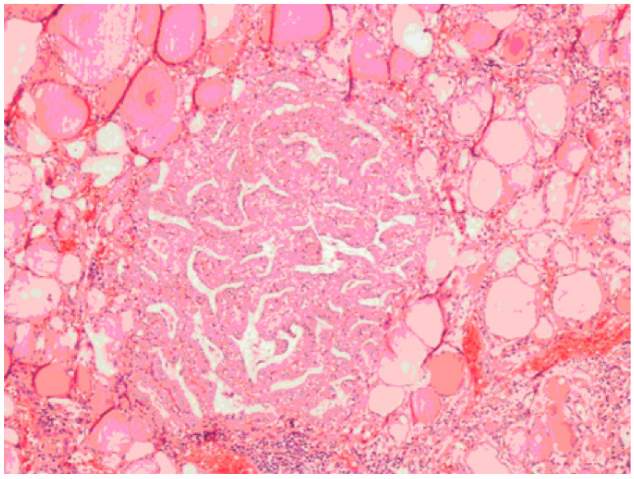

Figure 1. Thyroid papillary carcinoma (H&E x4).

identify some well differentiated tumors as benign or malignant because of overlapping histological features [6-9]. However, routine assessment of thyroid nodules through surgical pathology does not include immunohistochemistry or detection of somatic mutations via genotyping. This problem usually becomes apparent when the lesions show questionable capsular and/or vascular invasion for follicular lesions and absence of nuclear changes in papillary lesions during pathological assessment of thyroid nodules.

Some scoring systems such as UICC/AJCC TNM staging system, AMES, AGES, and MACIS have been proposed for DTC in order to identify high-risk cases and to evaluate biological characteristics of the primary tumor [10-13]. All these scoring systems evaluate similar parameters such as tumor size, extra-thyroid extension, lymph node metastasis, distant metastases and patient age [12,14-16].

Several immunohistochemical markers have been studied for the diagnosis and prognosis of thyroid tumors. In a recent study, aberrant FHIT and p53 genes have been studied as possible prognostic markers in highly malignant thyroid lesions [17]. Also, in a Chinese study, an increased EGFR gene copy number has been found in patients with anaplastic thyroid carcinoma [18]. Furthermore, in another study, *de novo* methylation of the 5' CpG island of p16 was common in primary tumors, indicating that the function of this gene could be lost as an epigenetic event during disease progression [19]. However, none of these have shown 100% specificity and sensitivity. In terms of diagnosis and prognosis of TT-UMP, no successful studies, combining immunohistochemical and molecular biological approaches have been published yet.

By evaluating the correlation between the expression of prognostic markers such as FHIT, P16, P53, EGFR and prognostic parameters in borderline cases, the purpose of this study was to introduce an immunohistochemical panel that can be used in daily routine practice.

Methods

The study was performed retrospectively in the departments of general surgery and pathology and approved by the Institutional Ethics Committee of the Medical Faculty of Cumhuriyet University.

Patients

We evaluated all the files of patients who underwent total thyroidectomy in our clinic between 1999 and 2010. In that period, 954 cases of total thyroidec-

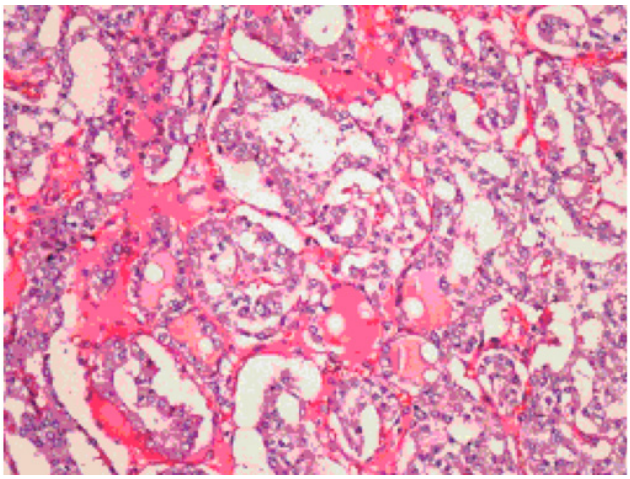

Figure 2. Follicular thyroid carcinoma (H&E x20).

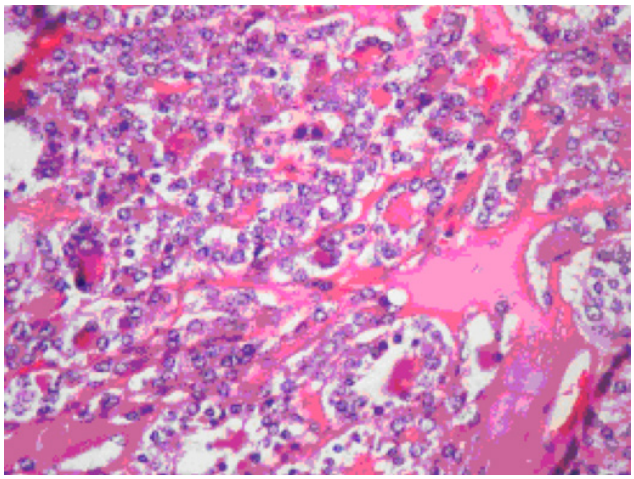

Figure 3. TT-UMP (H&E x40).

tomy were performed and 334 of them were due to malignancy. In the search for the study we detected 21 TT-UMP (2.2% of all thyroid specimens and 6.3% of malignant cases) and allocated them as Group II. Then, to compare it with well differentiated thyroid cancer, we selected 69 (at least 3 control per-case) more cases randomly from the rest of cases to allocate in Group I.

Evaluation of specimens and patients

Patient paraffin blocks were obtained from the pathology archives, and new sections were performed. These sections were stained by using appropriate immunohistochemical staining methods (Avidin-Biotin-Peroxidase method).

PC was accepted as a diagnosis if a tumor of well-differentiated follicular cells exhibited obvious PC-type nuclear changes, regardless of whether capsular invasion was obvious, questionable or absent or whether the pattern of growth was papillary, follicular, or other [12] (Figure 1).

FTC was accepted as a diagnosis if an encapsulated tumor composed of well-differentiated follicular cells showed obvious capsular and/or blood vessel invasion and lacked PC-type nuclear changes and for tumors showing definite capsular invasion and no PC-type nuclear changes [12,20] (Figure 2).

TT-UMP was accepted as a diagnosis if an encapsulated tumor was composed of well-differentiated follicular cells with questionable PC-type nuclear changes, no blood vessel invasion, and capsular invasion that was either absent or questionable. Tumors showing questionable or incomplete capsular invasion were accepted as follicular tumors of uncertain malignant potential (FT-UMP). Also, tumors with absent or questionable PC-type nuclear changes were defined as well-differentiated tumors of uncertain malignant potential (WDT-UMP). According to these pre-definitions WDT-UMP and FT-UMP were classified as TT-UMP [12,20] (Figure 3).

Capsule penetration, macroscopic soft tissue penetration, tumor type, tumor grade, angiolymphatic invasion, microscopic capsule invasion, microscopic invasion of surrounding tissue, multifocality, extra-thyroid tumor tissue, distant metastasis, lymph node involvement, radioactive iodine treatment and remission/recurrence were the parameters evaluated, and prognostic scores of patients were calculated using these data according to the well-known prognostic scoring systems MACIS, AMES, AGES.

Scoring systems determine patients having low and high risk by using similar parameters in order to estimate the life expectancy. Well-known prognostic scoring systems like MACIS, AMES and AGES were used to evaluate the Groups. These scoring systems share similar parameters which are given in Table 2.

MACIS scoring system evaluates 5 parameters (age, tumor size, completeness of surgical resection, extra-thyroid invasion, and distant metastasis).

AMES scoring system evaluates parameters such as age, tumor size, gender and extra-thyroid invasion.

AGES scoring system evaluates parameters such as age, histological grade , extra-thyroid invasion or metastasis and tumor size.

Evaluation of the expression of p16, p53, FHIT and EGFR

The scoring method described by Hermann Brustman for serous ovarian tumors (based on the heterogeneous expression of antibodies) was used to evaluate immune reactivity. Evaluations of antibodies were based on the presence of nuclear staining for p16 and p53, the membranous and cytoplasmic staining for EGFR and the cytoplasmic staining for FHIT (Figure 4a-d).

As positive control in immunohistochemical studies we used paraffin slides of breast tissue positive for FHIT protein, lung and skin squamous cell carcinoma positive for EGFR protein, cervix carcinoma and papillary serous ovarian carcinoma positive for p16 protein and gastric adenocarcinoma positive for p53 protein.

The cell immunostaining was evaluated semi-quan-

Table 3. Evaluation of the groups according to scoring systems

	Total N (%)	*Group I* N (%)	*Group II* N (%)	*p value*
MACIS				
LR	77 (85)	56 (81)	21 (100)	0.034
HR	13 (15)	13 (19)	-	
AGES				
LR	84 (93)	63 (91)	21 (100)	0.193
HR	6 (7)	6 (9)	-	
AMES				
LR	73 (81)	52 (75)	21 (100)	0.009
HR	17 (27)	17 (25)	-	

LR: low risk, HR: high risk, N: number of patients

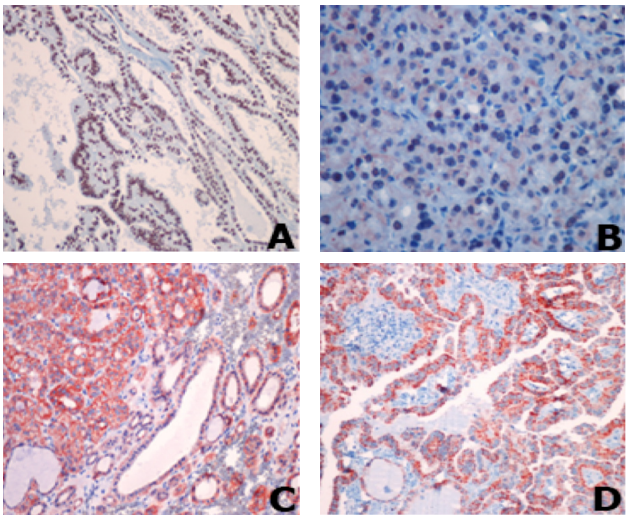

Figure 4. Evaluations of antibodies were based on the presence of nuclear staining for p16 and p53, membranous and cytoplasmic staining for EGFR and cytoplasmic staining for FHIT. **A)** Thyroid papillary carcinoma, p53 positive, score 3 (ABP x40); **B)** Thyroid papillary carcinoma, p16 positive, score 12 (ABP x40); **C)** Thyroid papillary carcinoma, membranous staining for EGFR, score 12 (ABP x20); **D)** Thyroid papillary carcinoma, FHIT antibody positive, score 12 (ABP x20).

titatively, so that 0% stained cells was scored as 0, < 10% stained cells were scored as 1, 10 -50% stained cells as 2, 51 – 80% stained cells as 3, and > 80% stained cells as 4. A weak intensity staining was denoted as (1+), moderate as (2+), and strong as (3+). Values of both parameters for each case (percents of positive cells and predominant intensity of staining) were scored from 0 to 12.

Statistics

The Statistical Package for Social Sciences (SPSS Inc, Chicago, Ill), v.14.0 software was used for statistical analyses. Independent sample t-test, Spearman's correlation test and Fisher's exact test were used to assess variables between groups. Data were expressed as arithmetic mean and standard deviation in Tables and a p value <0.05 was accepted as statistically significant.

Results

Of the 90 patients included in the study 73 (81.1%) were female and 17 (18.9%) male. The mean age was 50 years (range 17-76). Patient characteristics are described in Table 1. There were 69 and 21 patients in Group I and Group II, respectively. Group I was composed of patients diagnosed as PC (N=31; 34%), papillary micro carcinoma (N=27; 30%) and FC and hurthle cell variant (N=11; 12%). Group II was composed of patients diagnosed as TT-UMP (N=21). There was no statistically significant difference between the two Groups in terms of gender and age.

Evaluation of the Groups according to the prognostic scoring systems

According to MACIS in Group I and II, 56 (81%) and 21 (100%) patients were classified as having low risk, while 13 (19%) and 0 (0%) patients as having high risk, respectively (Table 3). The difference was statistically significant (p<0.05).

According to AGES in Group I and II, 63 (91%) and 21 (100%) patients were characterized as having low risk, while 6 (9%) and 0 (0%) patients as having high risk, respectively (p>0.05) (Table 3).

According to AMES in Group I and II, 52 (75%) and 21 (100%) patients were detected to have low risk, while 17 (25%) and 0 (0%) patients were detected to have high risk, respectively (Table 3). A statistically significant difference was noted (p<0.05) between Group I and Group II.

The relationship between expressions of FHIT, p16, p53, EGFR and scoring systems

The relationship between the scoring systemsMACIS, AGES and the expression of FHIT, p16, p53, EGFR were evaluated by using correlation analyses. AMES could not be evaluated because of having no parameters available.

Table 4. Correlation between MACIS and FHIT, EGFR, p16, p53

	FHIT	*EGFR*	*p16*	*p53*
Group I	r=-0.19 p= 0.109	r= -0.12 p= 0.308	r= -0.17 p= 0.149	r= 0.20 p= 0.102
Group II	r= 0.11 p= 0.666	r= 0.45 p= 0.041	r= 0.09 p= 0.678	N/A

p values evaluated with Spearman's correlation analysis. N/A: not available

Table 5. Corelation between AGES and FHIT, EGFR, p16, p53

	FHIT	*EGFR*	*p16*	*p53*
Group I	r= -0.19 p= 0.11	r= -0.12 p= 0.332	r= -0.14 p= 0.244	r= 0.22 p= 0.064
Group II	r= -0.20 p= 0.387	r= 0.10 p= 0.638	r= 0.05 p= 0.822	N/A

p values evaluated with Spearman's correlation analysis. N/A: not available

In Group I, the correlation coefficient (r) was very low, indicating only little correlation between MACIS and immunostainings (FHIT, EGFR, p16, p53), and this relationship was without statistical significance (p>0.05) (Table 4). In Group II, the correlation coefficient was very low, indicating little correlation between MACIS and FHIT and p16 and this relationship was without statistical significance (p>0.05) (Table 4). On the other hand, the correlation coefficient was near 1 (r=0.55) between MACIS and EGFR, indicating a moderate positive correlation and this relationship had statistical significance (p=0.041) (Table 4). These results indicated that an increase in EGFR value was likely when an increase occurred in MACIS value. The relationship between p53 and MACIS in Group II could not be evaluated because of having no parameters available.

In both Group I and II, the correlation coefficient was very low, indicating only little correlation between AGES and immunostainings (FHIT, EGFR, p16, p53); this relationship had no statistical significance (p>0.05) (Table 5).

Discussion

Tumors of the thyroid gland show a broad spectrum of neoplastic pathology, ranging from benign colloid adenomas to anaplastic carcinomas [21]. Papillary carcinoma, follicular variant and Hurthle cell carcinoma are the major types of DTC, while some rare subtypes also exist [22].

The difficulty of determining the frequency and prognosis of TT-UMP arises from the fact that these tumors have been newly defined, they lack an international classification and have not been recognized by pathologists [23]. However, the term TT-UMP is clearly mentioned in the latest edition of WHO on Pathology and Genetics of Endocrine Tumors [4]. In surgical pathology, genotypic assessment of these lesions through immunohistochemical somatic mutation studies has not been commonly adopted yet. No successful studies on diagnosis and prognosis of such borderline follicular lesions have been conducted up to date [23].

The present study was undertaken because thyroid tumors with follicular patterns are defined as a subgroup and their malignant or benign potential cannot be easily determined. Their frequency, diagnostic similarities, prognosis, immunohistochemical and molecular genetic properties have not been explored adequately yet. We found that EGFR increased when MACIS score increased in the TT-UMP group, and we believe that EGFR expression has a prognostic value in cases diagnosed as TT-UMP.

In our study, positive staining of p53 was found in only 5 of the cases. In the PC Group, p53 expression increased when MACIS score increased. Based on this finding we believe that cases with p53 positivity should be followed up for metastasis more closely and p53 positivity should be considered as adverse prognostic parameter in routine panel to establish predictions about the prognosis in thyroid pathologies.

It was worth noticing that while p16 showed loss of expression in PC foci, there was a strong positive staining in adjacent normal tissues. Taking all these findings together, we concluded that p53 pathway was not important in tumor genesis at early stages in well-differentiated thyroid carcinomas and loss of p16 expression could help in the diagnosis of cases where microscopic diagnosis was incoclusive.

As a conclusion, TT-UMP are a gray zone of lesions requiring specific therapeutic approach and postoperative follow-up. The moderate positive correlation detected between EGFR and TT-UMP may be used as a new tool in the follow-up of these patients in the future. Needless to say that studies with larger patient numbers and biomarkers revealing the real aggressiveness of these tumors are required. However, to understand whether TT-UMP are malignant tumors capable of metastasing or they are benign tumors imitating DTC is still a difficult question to answer and requires at least 20 years follow- up of these patients.

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