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Muc-1 Expression May Help Characterize Thyroid Nodules but Does Not Predict Patients' Outcome

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Abstract Our purpose was to evaluate MUC1 clinical utility in the diagnosis and prognosis of thyroid cancer patients. We studied the protein expression of MUC1 in 289 thyroid carcinomas and 121 noncancerous thyroid nodules. There were 41 follicular carcinomas (FC) and 248 papillary thyroid carcinomas (PTC) including 149 classic (CPTC), 20 tall cell (TCPTC) and 79 follicular variants (FVPTC). In addition, we used a quantitative real-time PCR (q-PCR) method to measure *MUC1* mRNA expression levels in 108 carcinomas, 23 hyperplasias, and 19 FA. According to their serum Tg levels and other evidences of recurrence/metastasis, the patients were classified as free-of-disease (185 cases) or bad outcome (56 cases, 10 deaths). MUC1 protein was identified in 80.2% PTC; 48.8% FC; 68.3% FVPTC; 70% TCPTC; 21.8% FA; 30% hyperplasias and 6% normal thyroid tissues. MUC1 distinguished benign from malignant thyroid tissues (sensitivity=89%; specificity=53%). MUC1 also differentiated FC from FA (p=0.0083). q-PCR mRNA expression of *MUC1* also distinguished malignant from benign nodules (Mann–Whitney test, $p<0.0001$). However, neither IHC nor mRNA *MUC1* expression was associated with any clinical or pathological feature of aggressiveness or outcome. We suggest that MUC1 expression may help differentiate follicular patterned thyroid lesions.

Keywords MUC1 · Thyroid cancer · Benign nodules · Follicular carcinomas · Follicular adenomas

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Introduction

Although accounting for no more than 2% of all human malignancies, thyroid cancer has continuously increased in the past decades in many regions of the world [1]. Follicular cell-derived papillary and follicular tumors are both considered well-differentiated thyroid carcinomas (DTC) due to their structural and functional resemblance to normal follicular cells and due to their relatively indolent natural history and good responsiveness to surgery and radioiodine. These characteristics may, however, complicate the diagnostic, especially of the "follicular lesion of undetermined significance". Approximately 20–30% of the nodules submitted to fine needle aspiration biopsy show "indeterminate" cytological findings, a pattern that has remained essentially unchanged over the past two decades [2]. All these patients are currently referred to surgery to definitely reveal the nature of the nodules; however, the vast majority will prove to have undergone an unnecessary procedure as their nodule was benign [2]. Molecular testing using the most frequently mutated genes currently known as markers of thyroid malignancy appear to be very useful in refining follicular lesion of undetermined significance/atypia of undetermined significance cases into high- and

low-risk categories, but are still not able to detect more than 70% of thyroid neoplasia [3].

In addition, the upward trend in thyroid cancer incidence registered all over the world is mainly due to small or very small (micro) papillary thyroid carcinomas (PTC), whose clinical significance remains uncertain and that may account, at least in part, for DTC low mortality [1, 4]. Unfortunately, up to 10–15% of the DTC patients die of the disease, especially in third world countries and some ethnic groups. Even greater proportion faces the morbidity of recurrences, including some small tumors that can exhibit aggressive behavior, characterized by local invasion, distant metastasis, treatment resistance, and mortality [5]. A series of classification systems based on clinical, pathological, and molecular characteristics have been used to predict the prognosis and guide surgical and clinical management strategies. Some molecular markers, especially the *BRAF* mutation, appear very promising in helping to find progression-potent tumors [6]. However, at the present status of knowledge, these markers are not yet powerful enough to predict the need for more aggressive treatment [7].

Thus, there is urgent need to look for genes helpful in the diagnostic and stratification of prognosis with higher accuracy, needed for clinical decisions.

Mucins are multifaceted glycoproteins that provide lubrication of epithelial cell surfaces, prevent tissue dehydration, protect cells from photolytic degradation and constitute a barrier against infection. MUC1 is a transmembrane epithelial cell surface glycoprotein frequently [8]. It belongs to the family of mucin proteins which are expressed by various epithelial cell types over-expressed and aberrantly glycosylated in cancer cells, whereas it is restricted to only the apical surface in normal epithelial cells [9]. In neoplastic tissue, MUC1 is aberrantly underglycosylated, revealing epitopes and permitting the immune system to access the peptide core of MUC1. This feature can help differentiate tumor cells from normal cells and allow for better targeting strategies [9]. The expression of MUC1 has been extensively studied and has been demonstrated to be related to the development of a variety of tumors associated with a worse prognosis including thyroid cancers [10–15]. However, the correlation of MUC1 overexpression with PTC prognosis remains controversial and large series have not proved earlier data indicating MUC1 as a prognosticator of PTC aggressiveness [16, 17].

The aim of this study was to evaluate the clinical utility of MUC1 in the diagnosis and prognosis of thyroid cancer.

Materials and Methods

Patients

This study was approved by the Committee of Ethics in Research of the institutions involved. We investigated 410 patients whose tissue samples were maintained in the tissue bank of the Cancer Hospital A C Camargo, Sao Paulo, Brazil summarized in Table 1. Thyroid carcinoma was diagnosed in 289 patients: 248 with PTC (149 classic form; 79 follicular variant; and 20 tall cell variant), and 41 with follicular carcinomas (28 minimally invasive and 20 frankly invasive). In addition, we obtained 121 normal or benign thyroid tissues including: 16 cases of normal tissue; 50 nodular goiters; and 55 follicular adenomas. Patients' clinical information was obtained from their charts.

Formalin-fixed, paraffin-embedded tissues from all 410 cases were reviewed for diagnostic confirmation and in order to select the most representative areas designed to

Table 1 Immunohistochemical expression of the MUC1 protein classified in percentage of cases identified as positive using visual analysis and using an Automated Cellular Imaging System quantitative analysis according to histopathological diagnosis

Histopathological diagnosis		Number of patients	Gender		Age		Visual analysis		Quantitative analysis	
			M	F	<45	>45	*N*	Positive cases (%)	*N*	Mean value
Non-malignant	Normal thyroid tissue	16	1	14	1	15	16	6.25	–	–
	Nodular goiter	50	8	36	14	30	50	30.0	50	6.5
	Follicular adenoma	55	8	43	18	21	55	21.8	54	3.7
	Total	121								
Malignant	Papillary carcinoma:	248					248	80.2	158	–
	Classical type	149	28	107	81	54	149	87.9	100	76.8
	Follicular variant	79	12	56	29	39	79	68.3	54	45.3
	Tall cell variant	20	5	12	7	10	20	70.0	4	106.5
	Follicular carcinoma	41	9	28	11	26	41	48.8	42	21.6
	Total	289								

See Table 3 for statistical comparisons

build a tissue microarray (TMA, Beecher Instruments®, Silver Springs, MD, USA) for the immunohistochemical detection of MUC1. In addition, we obtained 142 thyroid tissues (including 100 carcinomas, 23 nodular goiters, and 19 follicular adenomas) collected at surgery, immediately snap-frozen in liquid nitrogen, and stored at −80°C for molecular studies.

MUC1 protein immunohistochemical expression was correlated with clinicopathological parameters, such as age, gender, ethnic group, tumor size, multifocality, extra-thyroidal invasion, presence of lymph node metastases, staging and outcome (free of recurrence survival/metastasis), see Table 2.

The patients were followed with periodic total body scans, serum TSH and thyroglobulin (Tg) measurements according to a standard protocol that included X-ray, ultrasonography, computed tomography scan and other eventual procedures to detect distant metastasis for a period for 12 to 298 months (53.8±41 months). Patients presenting or suspicious of high-serum non-stimulated Tg levels (> 2 mg/dl) were submitted to a thorough image search. We defined tumors as recurrent and/or presenting long distance metastasis according to the aforementioned parameters. Thyroid cancer patients were classified as free-of-disease in 185 cases (76.6%), and with recurrence in 56 cases (23.24%, including 10 dead-of-disease). Forty-eight patients could not be classified in either one of these two groups and were further excluded from any analysis involving outcome. Aggressiveness at diagnosis was ascertained using the TNM and stage classification system for differentiated thyroid carcinoma [18].

Immunohistochemical Detection of MUC1

Five-micrometer sections of the TMA were placed on electrically charged slides, deparaffinized, and rehydrated in decreasing concentrations of alcohol. The endogenous peroxide activity was blocked with H_2O_2 for 15 min. All tissue sections were subjected to heat-induced antigen retrieval using 10% citrate buffer (10 mM, pH 6.0) in a steamer (90°C for 30 min). Tissues sections were then incubated overnight at 6°C, with anti-MUC1 mouse monoclonal antibody (clone VU4H5; Santa Cruz Biotechnology, Santa Cruz, CA, USA), diluted at 1:700. The Advance biotin-free polymer detection system was used (DAKO, Carpenteria, CA, USA). DAB (3,3-diaminobenzidine-tetrahydrochloride; Sigma, St Louis, MA, USA) was applied as chromogen for 5 min at room temperature. Sections were counterstained with hematoxylin. Positive and negative controls were run in the same batch of reaction.

Table 2 Clinicopathologic features of differentiated thyroid carcinomas classifying MUC1 immunohistochemical visual expression into negative (0 and 2) or positive (3–7)

Clinicopathological features (Total number of cases)			Scores MUC1 visual expression		*p*
			Negative	Positive	
Age	(257)	<45	80 (31.13%)	48 (18.68%)	0.6441
		≥45	77 (29.96%)	52 (20.2%)	
Ethnic	(148)	White	79 (53.38%)	51 (34.46%)	0.6299
		Non-white	12 (8.11%)	6 (4.05%)	
Sex	(257)	Female	118 (45.91%)	85 (33.07%)	0.0590
		Male	39 (15.18%)	15 (5.84%)	
Tumor size	(145) (cm)	<2	52 (35.86%)	35 (24.14%)	0.7426
		2–4	23 (15.86%)	13 (8.97%)	
		>4	15 (10.34%)	7 (4.83%)	
Extrathyroidal invasion	(131)	Yes	48 (36.64%)	33 (25.19%)	0.9331
		No	30 (22.90%)	20 (15.27%)	
Metastasis on diagnosis	(146)	Yes	35 (23.97%)	19 (13.01%)	0.5461
		No	55 (37.67%)	37 (25.34%)	
Stage	(217)	I	80 (36.87%)	51 (23.50%)	0.1682
		II	13 (5.99%)	4 (1.84%)	
		III	16 (7.37%)	16 (7.37%)	
		IV	18 (8.29%)	19 (8.76%)	
Multifocality	(148)	Yes	39 (26.35%)	18 (12.16%)	0.2140
		No	53 (35.81%)	38 (25.68%)	
Outcome	(222)	Disease free	61 (27.48%)	112 (50.45%)	0.6609
		Recurrence/metastasis	21 (9.46%)	28 (12.61%)	

N was variable as information was not always available for every feature

Immunohistochemical Evaluation

Cells were considered positive for MUC1 when a clear cut brown staining was observed in the cytoplasm as demonstrated in Fig. 1. Visual evaluation was made for each spot of tissue, estimating the percentage of positive tumor cells and the intensity of staining. The percentage of positive cells was graded as: 0=no positive cell; 1=up to 25% positive cells; 2=25 to 50% positive cells; 3=50 to 75% positive cells; 4=more than 75% positive cells. Intensity was graded as: 0=negative; 1=faint staining; 2=moderate staining; 3=strong staining. A final score was calculated adding both, percentage of positive cells and intensity of immunostaining, which ranged from 0, and 2 to 7. For statistical purposes, cases scored as 0 and 2 were grouped as negative and cases scored from 3 to 7 were grouped as positive.

In addition to visual evaluation we also analyzed the immunohistochemical expression of MUC1 using the ACIS-III Automated Cellular Imaging System (Chroma Vision Medical Systems, Inc, DAKO). Briefly, each tissue spot was digitalized to the system software and a numerical value proportional to the intensity and extension of brown staining was attributed by the computer analysis.

Quantitative Real-Time q-PCR

Total RNA was extracted from pulverized frozen thyroid tissues using the Trizol reagent (Invitrogen Life Technologies Inc., Carlsbad, CA, USA), according to the manufacturer's instructions. The samples were digested with DNAse I Amplification Grade (Life Technologies, Rockville, MD, USA) and reverse-transcribed using SuperScript III® reverse-transcriptase (Invitrogen Life Technologies Inc). The assays were carried with an aliquot of 25 ng cDNA used in a PCR reaction containing Maxima SYBR Green/ROX qPCR Master Mix (Fermentas Life Sciences, Glen Burnie, Maryland/USA); 50 ng of each specific primer for the *MUC1* gene and *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase), which was used as endogenous control. The sequences of *MUC1*-primers were (MWG Oligo Synthesis Report, Miami, Florida, USA):

MUC1 forward: 5′ ACA ATT GAC TCT GGC CTT CC 3′
MUC1 reverse: 5′ CAG GTT ATA TCG AGA GGC TGC 3′

As housekeeping gene, we used *GAPDH* primers (Invitrogen Custom Primers):

GAPDH forward: 5′ GAA GGT GAA GGT CGG AGT C 3′
GAPDH reverse: 5′ GAA GAT GGT GAT GGG ATT 3′.

Analysis was performed with a 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using a four-stage program: 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 s, and 60°C for 1 min. Each sample was assayed in triplicate. Threshold cycle

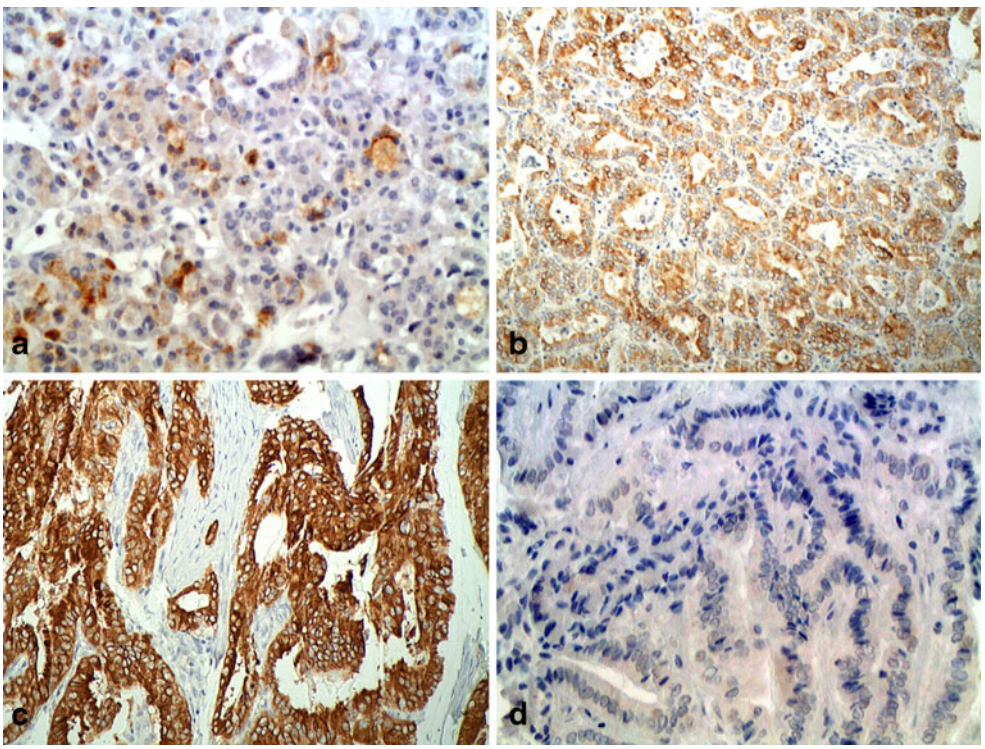

Fig. 1 Immunohistochemical expression of MUC1 in thyroid lesions. **a** Only rare cells are positive in one case of adenoma (×500); **b** follicular carcinoma showing moderate diffuse cytoplasmic expression of MUC1 (×400); **c** and **d** papillary carcinoma with strong diffuse expression of MUC1 **c**, and negative for this marker **d** (×640)

(Ct) was obtained using the Sequence Detection Software (Applied Biosystems).

Statistical Analysis

The statistical analysis was carried out using the SAS System for Windows (Statistical Analysis System, version 9.1.3, Service Pack 3. SAS Institute Inc, 2002–2003, Cary, NC, USA). Recurrence-free survival was calculated using Kaplan–Meier survival curves with log-rank comparison. Nonparametric analysis was performed using either the chi-square or Fisher's exact tests as indicated. A multivariate logistic regression model was applied using type of tumor (papillary or follicular carcinoma) as dependent variables and protein expression, genotype and clinical risk factors, including gender and age as explicative variables. The Mann–Whitney tests were used to compare continuous or arranged measures between two groups; Kruskal–Wallis test was used to compare three or more groups. The predictive accuracy of *MUC1* mRNA expression to predict malignancy was evaluated using receiver operating curve (ROC) analysis based on predicted probabilities from logistic regression models. All tests were conducted at the significance level $p=0.05$.

Results

Demographic characteristics of the 410 patients are summarized in Table 1. As expected, DTC patients were predominantly females (80.8%) with a mean age at diagnosis of 44.1±16.1 years (range, 7 to 88 years). DTC group did not differ from the individuals with normal thyroid or with benign thyroid diseases regarding gender or age at diagnosis.

MUC1 expression was positive in 219 (75.8%) out of the 289 cases of thyroid carcinomas and 28 (23.1%) out of the non-malignant thyroid samples analyzed ($p<0.0001$). Table 3 summarizes the positivity for MUC1 in benign and malignant cases and Fig. 1 illustrates the immunohistochemical staining patterns obtained in different follicular patterned thyroid lesions.

Table 2 summarizes clinicopathological features of the patients classified as negative, weak positive or clearly positive for MUC1 protein expression. We were not able to find any significant association between the expression of MUC1 and any clinical or histological parameter of aggressiveness at diagnosis or follow-up.

MUC1 expression distinguished benign from malignant thyroid tissues with a sensitivity of 89%; specificity of 52%; predictive positive value=75%; predictive negative value=74%. MUC1 also differentiated FC from FA ($p=0.0083$); FVPTC from FA ($p<0.0001$); FVPTC from nodular goiter ($p<0.0001$); CPTC from FA ($p<0.0001$). MUC1 was also differentially expressed among PTC histological variants: 131 (87.9%) classical PTCs were positive, whereas 54 (68.3%) follicular variants and 14 (70%) tall cell variant were MUC1 positive ($p=0.0008$). Table 3 summarizes the significant relationships found among different histopathological diagnosis.

Immunohistochemical expression of MUC1 using the quantitative analysis method (ACIS-III) confirmed visual

Table 3 Relationships found among different histopathological diagnoses, according to visual (semi-quantitative) and quantitative immunohistochemical (IHC) analyses of MUC1 and to quantitative mRNA analysis by real-time polymerase chain reaction (q-PCR)

Groups analyzed	*p* value (IHC semi-quantitative)	*IHC semi-quantitative%*		Predictive value%		*p* value (IHC quantitative)	*p* value (q-PCR)	q-PCR%	
		Sensitivity	Specificity	Positive	Negative			Sensitivity	Specitivity
Malignant vs benign	<0.0001	89.0	52.7	75.7	74.2	<0.0001	<0.0001	83.1	95.2
PTC vs goiter	<0.0001	92.9	41.6	80.2	70.0	<0.0001	<0.0001	–[a]	–[a]
CPTC vs goiter	<0.0001	89.7	66.0	87.9	70.0	<0.0001	<0.0001	–[a]	–[a]
FVPTC vs goiter	<0.0001	78.2	58.3	68.3	70.0	–[a]	<0.0001	–[a]	–[a]
TCVPTC vs goiter	0.0031	48.2	85.3	70.0	70.0	–[a]	–[a]	–[a]	–[a]
FC vs goiter	NS	NS	NS	NS	NS	<0.0001	–[a]	–[a]	–[a]
PTC vs FA	<0.0001	94.3	46.7	80.2	78.1	<0.0001	<0.0001	–[a]	–[a]
CPTC vs FA	<0.0001	91.6	70.4	87.9	78.1	<0.0001	<0.0001	–[a]	–[a]
FVPTC vs FA	<0.0001	81.2	63.4	68.3	78.1	–[a]	<0.0001	–[a]	–[a]
TCVPTC vs FA	0.0002	53.8	87.7	70.0	78.1	–[a]	–[a]	–[a]	–[a]
FC vs FA	0.0083	62.5	67.1	48.7	78.1	<0.0001	–[a]	–[a]	–[a]

PTC papillary thyroid carcinoma, *CPTC* classic type PTC; *FVPTC* follicular variant PTC, *TCVPTC* tall cell variant PTC, *FC* follicular carcinoma, *NS* not statistically significant

[a] No sufficient cases for statistical analysis

findings and was closely associated to the semi-quantitative evaluation (Spearman; r=0.8635, p<0.0001), but did not bring any new information (Table 1 and Fig. 2).

MUC1 gene mRNA expression was analyzed in 142 cases of thyroid tissues. *MUC1* mRNA expression distinguished benign from malignant thyroid tumors (Mann–Whitney; p<0.0001), as seen in Fig. 3. A ROC curve analysis performed to determine the optimal cut off value of quantitative *MUC1* mRNA indices for detection of malignancy indicated that a cut off of 81,138 RQ (relative quantification score) sorted out malignant nodules with 94.5% accuracy; 83.1% sensitivity; 95.2% specificity; 98.3% positive predictive value; and 62.5% negative predictive value (data not shown).

Univariate analysis did not show any significant correlation between MUC1 protein expression and overall survival rates (data not shown). Also, there was no correlation between protein expression and mRNA expression.

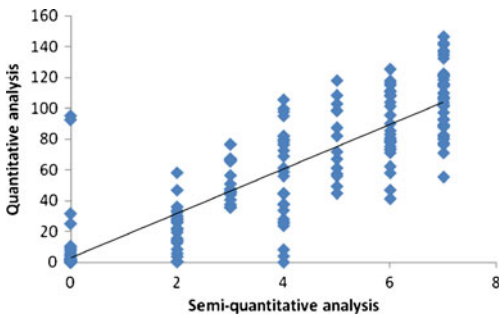

Fig. 2 Linear correlation between semi-quantitative and quantitative analysis of MUC1 protein expression

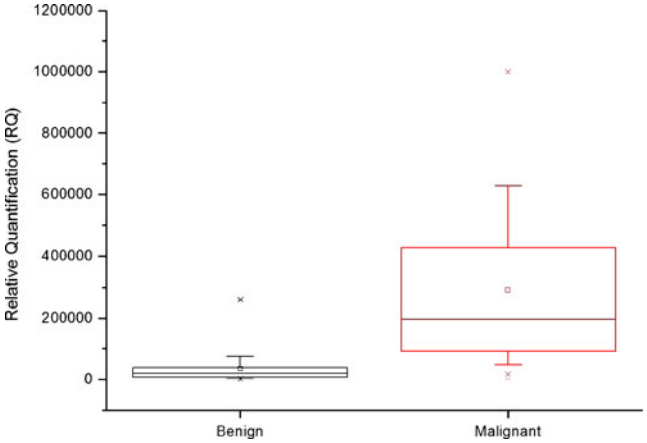

Fig. 3 *MUC1* mRNA expression assessed by quantitative RT-PCR in benign and malignant thyroid tumors

Discussion

MUC1 is a heavily glycosylated transmembrane epithelial cell surface glycoprotein. Numerous studies have shown that it is widely expressed in normal epithelial tissues where its expression is restricted to the luminal membrane which is inaccessible to antibodies present in the blood or to active immunocytes. MUC1 becomes aberrantly glycosylated in tumor cells that tend to lose cellular polarity revealing epitopes and permitting the immune system to access the peptide core of MUC1. This fact is the reason of the conversion of MUC1 into a tumor antigen and a potential target for cancer immunotherapy [8, 9]. The protein is frequently over-expressed in the cytoplasm of cancer cells, a feature that can help differentiate tumor cells from normal cells [19].

MUC genes have consistently been found to be associated to thyroid carcinogenesis by different techniques [15, 20]. Analysis of gene expression using a quantitative RT-PCR in cytological samples demonstrated that *MUC1* could reliably distinguish goiters (n=5) from FA (n=7), FC (n=2) and PTC (n=4) [21]. Weiss et al. also used RT-PCR to demonstrate over-expression of *MUC*1 in six out of eight PTC [22]. Employing immunohistochemistry, Biéche et al. found MUC1 in 6 out of 11 PTC but none of the 10 adenomas [23]. The authors described MUC1 as an irregular thin and/or thick lining at the apical domain of tumor cells and intracytoplasmic staining in five of the six samples with *MUC1* mRNA over-expression. Moreover, they found that intracytoplasmic MUC1 staining occurred in 75% of "high-risk" papillary thyroid carcinoma (PTC; PTC with extrathyroid extension at initial diagnosis and/or lymph node involvement), and in only 28.5% of "low-risk" purely intrathyroidal carcinomas (PTC) suggesting that MUC could be associated to more aggressive tumors [23]. Indeed, comparing classic PTC to tall cell variants, Wreesman et al. demonstrated over-expression of the gene in 97.5% of the nine tall cell PTC variants compared to 35% of the 14 cases of classic PTC he examined [15]. Baek also found *MUC1* mRNA over-expressed in 15 PTC in contrast to 22 normal thyroid tissues and 22 follicular adenomas [24]. The positive MUC1 expression rate of the PTC patients with lymph node metastasis was 68.4% (26/38); significantly higher than that of the patients without lymph node metastasis (26.7%, 4/15, P<0.05), however, there were no significant differences in the MUC1 expression rate among papillary thyroid carcinoma patients differing in gender, age, and tumor size (all P>0.05). We were not able to find any correlation between protein expression and mRNA expression. Other authors also have reported no correlation between protein and mRNA on NIS and other thyroid markers [25, 26]. It is possible that the abnormal metabolism of the cancer cell impairs the transcription/translation rate.

More recently, a Chinese group reported MUC1 expression significantly higher and positive in 56.6% of 53 PTC

tissues compared to 20 nodular goiters and 20 controls [27]. Immunohistochemical MUC1 expression rate was higher in PTC patients with lymph node metastasis 68.4% (26/38), than that of the patients without lymph node metastasis (26.7%, 4/15, $P<0.05$), however, there were no significant differences in MUC1 expression rate among the PTC patients differing in gender, age, and tumor size (all $P>0.05$). Confirming our own results, using a larger sample of 209 PTC of different histotypes, sizes, and morphological features of aggressiveness, Abrosimov et al. found MUC1 expression to vary broadly in different histological variants, however the authors were not able to demonstrate its utility as a prognosticator [16]. Furthermore, MUC1 was not able to predict lymph node metastasis in a large series of 198 papillary thyroid microcarcinomas [17].

In summary, using a large series of 410 thyroid tissues including 289 thyroid carcinomas managed according to a standard protocol in the same institution, we demonstrated that neither immunohistochemical nor mRNA *MUC1* expression was associated with tumor stage, grade of differentiation or any clinical or pathological feature of aggressiveness or outcome. We suggest that MUC1 expression it is not a reliable marker of thyroid cancer patients' prognostic, but may help differentiate follicular patterned thyroid lesions and characterize thyroid carcinomas.

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