EXPRESSION OF MAGE-A4 AND MAGE-C1 TUMOR-ASSOCIATED ANTIGEN IN BENIGN AND MALIGNANT THYROID DISEASES

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Abstract: *Background.* A subset of thyroid tumors characterized by a follicular growth pattern can represent a serious diagnosis. Immunohistochemistry and molecular pathology for genetic profiling have been used in an attempt to resolve some of these issues.

Methods. Tumor tissue samples of thyroid were obtained from 70 patients who underwent surgical therapy. They were divided into 4 groups: 20 adenomatous goiters, 10 follicular adenomas, 24 papillary carcinomas, and 16 follicular carcinomas. Immunohistochemical analysis was carried out using antibodies for MAGE-A4 (melanoma antigen-encoding gene A4) and MAGE-C1 (melanoma antigen-encoding gene C1).

Results. Standard histologic analysis and immunohistochemistry analysis of MAGE-A4 and MAGE-C1 expression were performed in all patients. The antigens examined were not expressed in any of the tissues.

Conclusions. The malignant degeneration of normal tissues is a multifactorial process, varying considerably both among tumor types and among individual patients. The present study showed that there was no immunolabeling of the MAGE-A4 and MAGE-C1 antigens. © 2011 Wiley Periodicals, Inc. *Head Neck* **33:** 1426–1432, 2011

Keywords: MAGE-C1; MAGE-A4; thyroid diseases; immunohistochemistry; biological tumor markers

Thyroid tumors, characterized by growth from follicular cells, represent the most frequent lesions detected by pathologists, posing a great challenge for histopathologic interpretation. Thyroid tumors of a follicular pattern include lesions ranging from benign (adenomatous goiter, follicular adenoma) to malignant (follicular carcinoma). In addition to these, other types of thyroid tumors belonging to different categories can show a follicular pattern when examined histologically, such as papillary carcinomas of the follicular variant and medullary carcinomas. The histologic characteristics and the diagnostic criteria used to distinguish between these lesions are frequently subtle and subjective.[1–3] Immunohistochemical analysis and molecular biology studies have been used in an attempt to clarify the diagnosis, but have not yet reached an acceptable level of reliability to be introduced in the routine of pathology laboratories.[4,5]

The objective of the present study of benign and malignant follicular diseases of the thyroid gland was to investigate whether the immunohistochemical expression of the antigens MAGE-A4 (melanoma antigen-encoding gene A4) and MAGE-C1 (melanoma antigen-encoding gene C1) can contribute to the differential diagnosis of benign and malignant follicular diseases of the thyroid gland.

MATERIALS AND METHODS

In all, 70 surgical specimens were obtained from surgical patients, 20 of them with colloid goiter, 10 with follicular adenomas, 16 with follicular carcinomas, and 24 with papillary carcinomas. Inclusion criteria

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Table 1. Demographic patient data and tumor characteristics and their relation with the expression of the MAGE-A4 and MAGE-C1 antigens in benign diseases.

Patient	Age, y	Sex	Tumor location	Tumor size, cm	Tumor type	MAGE-A4	MAGE-C1
1	18	F	Right lobe	2.0	Goiter	–	–
2	39	F	Right lobe	1.0	Goiter	–	–
3	58	F	Left lobe	1.5	Adenoma	–	–
4	60	F	Right lobe	3.0	Goiter	–	–
5	29	F	Right lobe	2.0	Goiter	–	–
6	43	F	Right lobe	1.0	Goiter	–	–
7	48	M	Left lobe	2.5	Goiter	–	–
8	32	F	Left lobe	1.0	Adenoma	–	–
9	51	F	Right lobe	2.0	Adenoma	–	–
10	45	F	Right lobe	4.0	Goiter	–	–
11	42	F	Left lobe	5.0	Goiter	–	–
12	46	F	Right lobe	3.0	Adenoma	–	–
13	53	F	Right lobe	2.0	Goiter	–	–
14	28	F	Left lobe	4.0	Goiter	–	–
15	49	F	Right lobe	2.5	Goiter	–	–
16	25	F	Left lobe	4.0	Adenoma	–	–
17	56	M	Right lobe	3.0	Goiter	–	–
18	31	F	Right lobe	2.0	Goiter	–	–
19	38	F	Left lobe	1.5	Adenoma	–	–
20	42	F	Right lobe	2.7	Goiter	–	–
21	36	F	Right lobe	8.0	Adenoma	–	–
22	55	F	Right lobe	5.0	Goiter	–	–
23	43	F	Right lobe	5.0	Goiter	–	–
24	51	F	Right lobe	3.5	Adenoma	–	–
25	42	F	Left lobe	3.0	Goiter	–	–
26	39	F	Right lobe	2.5	Adenoma	–	–
27	43	F	Right lobe	2.0	Goiter	–	–
28	50	M	Right lobe	3.5	Goiter	–	–
29	36	F	Left lobe	3.0	Goiter	–	–
30	38	F	Right lobe	5.0	Adenoma	–	–

Abbreviation: MAGE, melanoma antigen-encoding gene.

were: available clinical and microscopic data, an adequate representation of the lesion, appropriate histologic processing, and availability of paraffin blocks in good condition. Ten cases of autopsy with disease-free thyroid tissue were used as controls. The study was approved by the Institutional Research Ethics Committee (protocol no. 11.167/2007), and all patients provided informed consent.

The antibodies used for the immunohistochemical reactions were MAGE-A4 (57B, 1:4.000 in 1 m*M* EDTA buffer, pH 8.0; Ludwig Institute, New York, NY) and MAGE-C1 (CT 7-33, 1:32.000 in 10 m*M* citric acid solution, pH 6.0; Ludwig Institute).

Dextran polymer and the NovoLink system of enzymatic amplification (Novocastra Laboratories, Newcastle upon Tyne, UK) were used to prevent an endogenous reaction arising from the presence of biotin and avidin. Tissue sections of 4 μm were submitted to antigen recovery by heating for 3 minutes in a steamer with a 10 m*M* citric acid solution (pH 6.0). Endogenous peroxidase was blocked with an aqueous solution of 6% hydrogen peroxide. Incubation with the primary antibody was performed at 37°C in a moist chamber at 4°C for 12 hours (overnight). Incubation with the secondary antibody diluted in phosphate-buffered saline (PBS) was performed at 37°C for 30 minutes. After being washed in PBS, the sections were incubated with the Novolink amplifying system at 37°C for 30 minutes. Finally, the histologic sections were washed in running water for 10 minutes, counterstained with Harris hematoxylin for 10 seconds, washed again in running water, dehydrated in ethanol, cleared in xylol, and mounted on slides with Permount resin (code S15-100; Fisher Scientific, Fair Lawn, NJ). Readings were performed by 2 independent observers, surgical pathologists with experience in the area, who were unaware of the identity of the cases. Normal tissue samples of testis have been used as positive control displaying strong diffuse, positive reactivity. For statistical analysis, immunolabeling was considered to be positive when >50% of the neoplastic cells were labeled (subjective classification). The relationship between the occurrence of genes MAGE-A4 and MAGE-C1 in neoplasm and the presence of lymphatic infiltration, the histologic type, and the size of the tumor were analyzed and the chi-square test was used for independent samples. On the basis of the contingency table of the observed frequencies, the expected frequencies were calculated. The chi-square test is the sum of all the results that are obtained by dividing the square result of the difference between the observed and expected frequencies by the expected frequency. The obtained value of the chi-square test is compared with the border value for

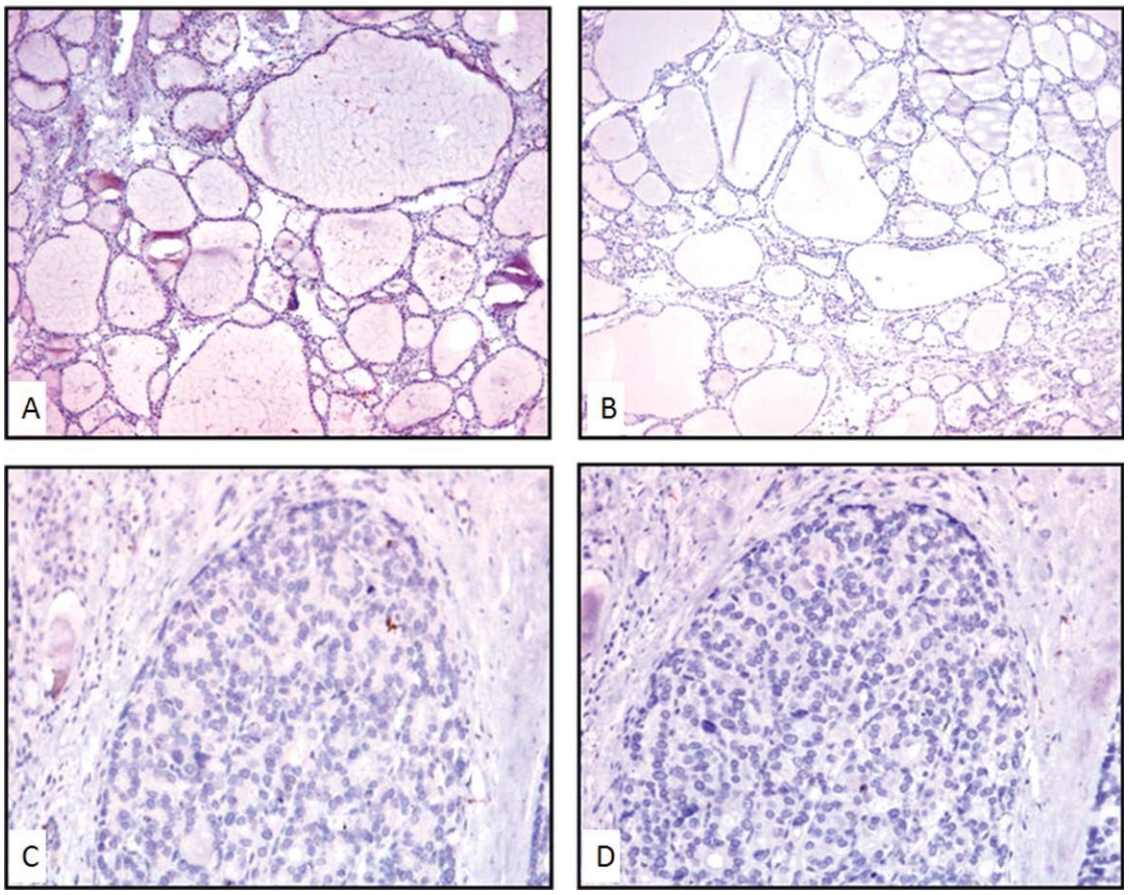

FIGURE 1. Photomicrographs of samples with immunolabeling of the antigens. **(A)** Colloid goiter with MAGE-A4 (melanoma antigen-encoding gene A4) (original magnification, ×100). **(B)** Colloid goiter with MAGE-C1 (melanoma antigen-encoding gene C1) (original magnification, ×100). **(C)** Follicular adenoma with MAGE-A4 (original magnification, ×200). **(D)** Follicular adenoma with MAGE-C1 (original magnification, ×200).

the determined number of the degree of freedom and from the chi-square table, the probability of the zero hypotheses is read. The significance of the correlation of gene expression with histopathologic and clinical characteristics was analyzed by the Fisher exact test.

RESULTS

The 70 patients participating in the study were divided into 2 groups, ie, subjects with benign disease and subjects with malignant disease. The clinical data for the group with benign tumors are presented in Table 1.

Sex distribution revealed that 90% of the patients with benign follicular disease were women, with a 9:1 female:male ratio. The mean (±SD) age of this group was 42.9 ± 9.88 years and the median was 43 years. Mean nodule size by major diameter was 3.0 ± 1.53 cm (1 to 8 cm). Twenty-one patients had tumors located in the right lobe and 9 in the left lobe of the thyroid gland. No immunolabeling of the MAGE-A4 and MAGE-C1 antigens was observed in normal tissue or in benign tumors (see Figure 1).

Table 2 lists the clinical and pathologic parameters of the patients such as age, sex, tumor location, and size observed macroscopically, type of disease, angiolymphatic infiltration, and expression of the MAGE-A4 and MAGE-C1 antigens for the group with malignant tumors.

About 85% of the patients with malignant follicular diseases were women, with a female:male ratio of 8.5:1.5. Mean (±SD) age of patient with these diseases was 48.45 ± 17.0 years and the median was 48 years. Mean nodule size as a function of the major diameter was 3.63 ± 1.73 cm (0.6 to 8 cm). In the group with follicular carcinomas, the tumor was predominantly located in the right lobe, 1.5:1 in relation to the left lobe. Among the cases of papillary carcinoma, the tumor was located in the left lobe in 25 patients and in the right lobe in 15 patients. Angiolymphatic invasion was observed in 37.5% of the patients with follicular carcinomas and in 50% of patients with papillary

Table 2. Demographic patient data and tumor characteristics and their relation with the expression of the MAGE-A4 and MAGE-C1 antigens in malignant diseases.

Patient	Age, y	Sex	Tumor location	Tumor size, cm	Tumor type	Agiolymphatic infiltration	MAGE-A4	MAGE-C1
1	51	F	Right lobe	4.5	Follicular	–	–	–
2	39	F	Right lobe	5.8	Follicular	–	–	–
3	28	F	Left lobe	1.7	Follicular	+	–	–
4	71	F	Left lobe	2.5	Follicular	–	–	–
5	37	F	Right lobe	1.8	Follicular	+	–	–
6	55	M	Left lobe	5.0	Follicular	+	–	–
7	43	F	Right lobe	8.0	Follicular	–	–	–
8	48	M	Right lobe	2.5	Follicular	+	–	–
9	49	F	Left lobe	3.5	Follicular	+	–	–
10	45	F	Right lobe	6.0	Follicular	–	–	–
11	64	M	Right lobe	4.0	Follicular	+	–	–
12	42	F	Left lobe	3.0	Follicular	–	–	–
13	71	F	Right lobe	2.5	Follicular	–	–	–
14	53	F	Left lobe	4.0	Follicular	–	–	–
15	20	F	Left lobe	4.0	Follicular	–	–	–
16	50	F	Right lobe	3.5	Follicular	–	–	–
17	44	F	Right lobe	0.6	Papillary	–	–	–
18	78	F	Right lobe	4.2	Papillary	+	–	–
19	56	F	Left lobe	5.0	Papillary	–	–	–
20	46	F	Right lobe	6.0	Papillary	+	–	–
21	62	F	Right lobe	2.8	Papillary	–	–	–
22	75	M	Left lobe	3.5	Papillary	+	–	–
23	39	F	Right lobe	3.0	Papillary	+	–	–
24	29	F	Right lobe	2.5	Papillary	+	–	–
25	55	F	Right lobe	6.0	Papillary	–	–	–
26	62	F	Left lobe	1.5	Papillary	–	–	–
27	39	F	Right lobe	1.5	Papillary	–	–	–
28	58	F	Right lobe	2.7	Papillary	+	–	–
29	24	M	Right lobe	3.6	Papillary	+	–	–
30	34	F	Left lobe	3.0	Papillary	+	–	–
31	23	F	Left lobe	1.2	Papillary	+	–	–
32	40	F	Right lobe	1.6	Papillary	–	–	–
33	21	F	Left lobe	5.0	Papillary	–	–	–
34	69	F	Right lobe	3.2	Papillary	–	–	–
35	74	F	Right lobe	2.3	Papillary	–	–	–
36	78	F	Left lobe	3.9	Papillary	–	–	–
37	26	F	Right lobe	1.6	Papillary	+	–	–
38	21	F	Left lobe	3.8	Papillary	–	–	–
39	60	F	Left lobe	4.5	Papillary	+	–	–
40	59	M	Right lobe	8.0	Papillary	+	–	–

Abbreviation: MAGE, melanoma antigen-encoding gene.

carcinomas. Tumor classification, in accord with the 2004 American Joint Committee on Cancer (AJCC) classification, was T1N0 in 10% of patients, T1N1 in 10% of patients, T2N0 in 25% of patients, T2N1 in 20% of patients, T3N0 in 20% of patients, T3N1 in 5% of patients, T4N0 in 5% of patients, and 5% of patients were labeled as T4N1.

For malignant tumors, the study of the expression of the MAGE-A4 and MAGE-C1 antigen was negative with the method used (see Figure 2). Positive immunolabeling was not observed, even in advanced cases with angiolymphatic invasion.

DISCUSSION

In view of their pattern of expression, cancer/testis antigens (CTAs) have attracted attention as potential targets for tumor immunotherapy,[6] but few reports are available in the literature about their expression, especially regarding the MAGE family in head and neck cancers. Although there are reports about expression of MAGE gene family in thyroid cancers, the expression of proteins coded by MAGE-A4 and MAGE-C1 had not been previously investigated and the present study is the first description in malignant follicular neoplasias of the thyroid.[7–10] For papillary carcinomas of the thyroid, Ruschenburg et al[7] demonstrated that expression of the MAGE-A1 and MAGE-1/2 antigens detected in fine-needle aspiration biopsies (FNABs) by reverse transcriptase–polymerase chain reaction (RT-PCR) could facilitate the diagnosis of thyroid nodules by eliminating the pitfalls of cytologic differentiation between thyroid carcinoma and adenomatous goiter. However, in the assessment of the 30 cases studied here no expression of the MAGE-A4 or MAGE-C1 antigens was observed in benign diseases. Similarly, no expression of these

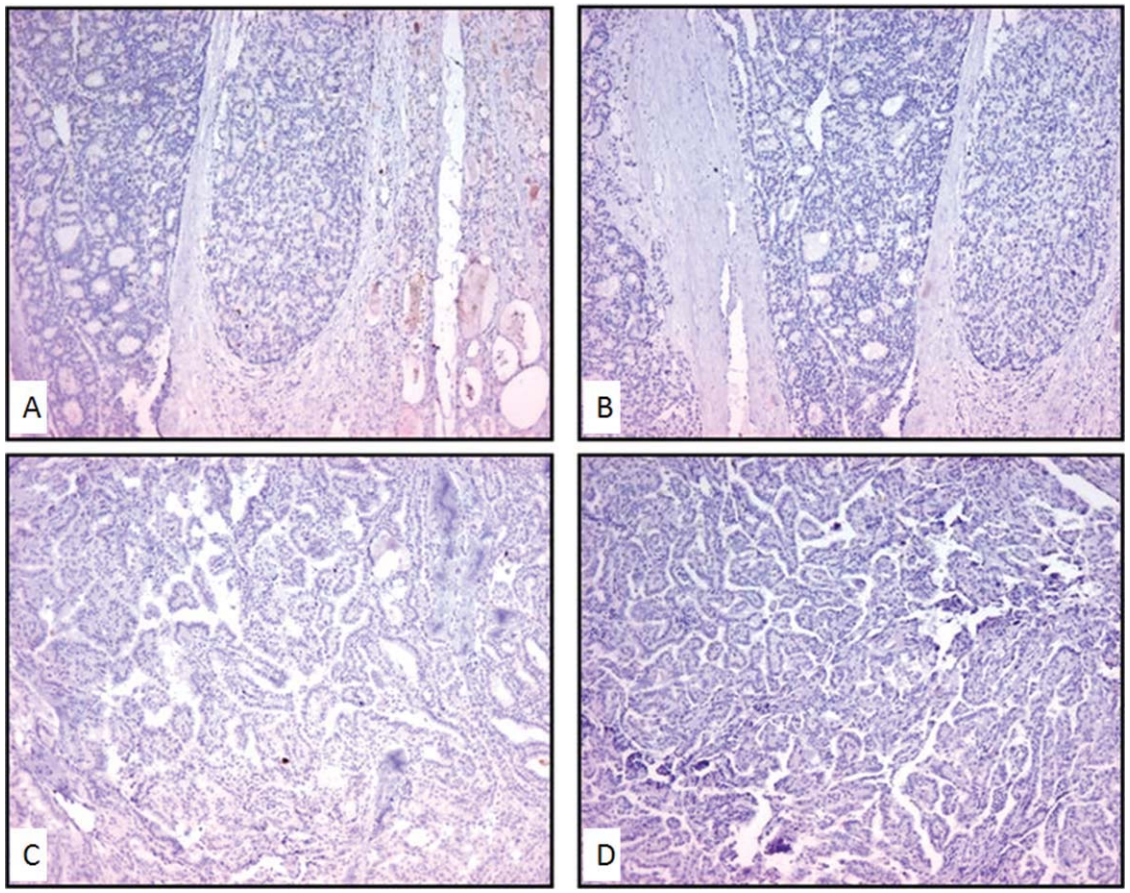

FIGURE 2. Photomicrograph of samples with antigen immunolabeling. **(A)** Follicular carcinoma with MAGE-A4 (original magnification, ×100). **(B)** Follicular carcinoma with MAGE-C1 (original magnification, ×100). **(C)** Papillary carcinoma with MAGE-A4 (original magnification, ×100). **(D)** Papillary carcinoma with MAGE-C1 (original magnification, ×100).

antigens was observed in the 40 cases of malignant disease, including those with clinical and anatomopathologic evidence of aggressiveness (angiolymphatic invasion). Thus, in the present study, differentiation between benign and malignant lesions based on the presence of MAGE-A4 and MAGE-C1 was not possible.

The expression of MAGE-A4 in head and neck cancer reported by Iwamoto et al,[11] Prasad et al,[12] and Ries et al[13] was not observed in the present study because this antigen is not expressed in the thyroid gland, as suggested by Maio et al[8] in a study on medullary carcinoma of the thyroid and Cheng et al[10] in papillary thyroid carcinomas. The same can be said about the MAGE-C1 gene, which was also not expressed, as suggested by Jungbluth et al[14] when they obtained weak expression in head and neck squamous cell carcinomas.

The mechanisms involved in tumor pathogenesis are obscure and are still being studied experimentally, but malignant tissue degeneration is a specific process for each tissue, with different genes participating in it in a complex chain of successive alterations. Based on the present results, it is possible that MAGEs have no function in the genesis of benign or malignant tumors of the thyroid. It should be remembered, however, that the lack of expression occurs either because expression is not detected by the method used or because this expression depends on stimuli that were not present in the sample under study.

Most of the studies cited have analyzed antigen expression by PCR, which is a technique of very good quality and reliability, but this technique is also labor intensive and requires a specially equipped laboratory. Thus, its result is time consuming, delaying the application of immediate actions to the patient. Switching from RT-PCR to immunohistochemistry cannot be accounted for the lack of detection of the expression of these CTAs, since Jungbluth et al[14] and Sharma et al[15] previously demonstrated that immunohistochemistry and PCR provide similar results regarding gene expression. The hypothesis that expression was not detected because the validity of the antibodies used had expired cannot be accepted because the antibodies were obtained from a qualified company, the expiration date was not exceeded, in other experiments there was

immunolabeling, and the testis samples used as positive controls were positive for these antigens.

The malignant degeneration of tissues is a multifactorial process involving genetic and environmental elements whose contribution varies widely among races, among different types of tumors, and even from an individual to another.[16,17] Rigopoulou et al[18] investigated the presence of class I (-A, -B, -C) and class II (-DR, -DQ) human leukocyte antigens (HLAs) in thyroid cancer, whose presence was a matter of controversy in various studies. These investigators observed factors of susceptibility to papillary carcinoma in both class I and class II and that their presence varied in accord with the population studied.[18] They also observed that subtypes B35 and DR11 were the most frequent among Spaniards, in agreement with the non-Arabic population of North Africa and in contrast to Italians (B35 and DR1), Americans (DR7), and Germans. Among Germans there was a predominance of 3 subtypes (B62, DR5, and DR6). These observations suggest that environmental (geographic), ethnic, and/or genetic factors may influence the genetic structure of molecules. This suggests that, even though the MAGE-A4 and -C1 genes were not expressed in the population studied here, they might be identified in others.

The advances of modern molecular biology regarding thyroid diseases may identify markers that will allow an accurate characterization of the types and subtypes of benign and malignant diseases in histologic samples and cells obtained by FNAB. CTAs, promising genes for this function, are expressed in small groups of human tumors. In some cases, attempts should be made to identify other CTAs attributed to the phenomena to which they are subjected, ie, heterogeneity, coexpression, and interaction between genes.[6] Heterogeneity may explain the absence of expression in our patients. In the majority of cancers, the pattern of CTA expression is heterogeneous, and homogeneous antigen expression throughout the tumor mass is the exception. This applies to a variety of tumors, such as head and neck carcinoma, hepatocellular carcinomas, mammary carcinomas, urothelial carcinomas, and ovarian carcinomas. Even in melanomas, a tumor type with a high frequency of CTA expression, the pattern of antigen expression is mostly heterogeneous. The basis for this striking heterogeneity in CTA expression is unknown.[6]

The successful development of antigen-specific vaccines against cancer depends on the identification of appropriate target antigens on the establishment of effective immunization strategies and on the ability to create methods that will "deceive" the escape mechanism of evolving tumors from the immune system. CTAs currently are of great interest for the development of a vaccine against cancer and will probably be a focal point of cancer vaccine research in the near future.[19,20]

Although the incidence of many malignant neoplasias of the head and neck region is declining, thyroid cancers have been found to be increasing.[21] Although the increased incidence of malignant diseases of the thyroid may be related to detection of nodules with the common use of ultrasound, the combination of an early detection and the probability of a cancer diagnosis in alterations of apparently low importance support the need for nonsurgical methods for the differentiation between benign and malignant diseases and the detection of cancers with a high risk of recurrence and metastasis.

The present study of benign and malignant follicular diseases of the thyroid showed that there was no immunolabeling of the MAGE-A4 and MAGE-C1 antigens.

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