ORIGINAL ARTICLE

EXPRESSION OF CANCER TESTIS ANTIGENS IN HEAD AND NECK SQUAMOUS CELL CARCINOMAS

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Abstract: *Background.* There is considerable interest in the expression of cancer testis (CT) antigens in human cancers, because they may serve as the basis for diagnostic tests or an immunologic approach to therapy, or as prognostic markers.

Methods. On this basis, we evaluated by semiquantitative reverse-transcriptase polymerase chain reaction (RT-PCR) the expression of genes that code for tumor antigens (melanoma antigen-1 [*MAGE*-1], *MAGE*-4, *MAGE*-10, *MAGE*-12, B melanoma antigen, CTL-recognized antigen melanoma antigen (CT antigen 2) [*LAGE*], New York esophageal squamous cell carcinoma antigen (CT antigen 1) [*NYESO*-1], and preferentially expressed antigen of melanoma [*PRAME*]) in surgical samples of the tumors, margins, and lymph nodes (when present) from patients with a diagnosis of head and neck carcinoma. The study was conducted on 33 patients (31 men and two women), aged 31 to 94 years (mean, 56 years), with squamous cell carcinomas located in the mouth (15 cases), larynx (14 cases), and pharynx (four cases).

Results. The findings were compared with the clinical course and laboratory data. Expression of at least one antigen was observed in 66.6% of cases, with different rates of expression according to tumor staging (100% of T4, 57% of T3, 50% of T1 and T2) and smoking habit. There was a significantly higher expression of multiple genes (two or more) in tumors in advanced stages.

Conclusions. We conclude that the tumor-specific antigen genes are expressed in variable frequencies and intensities in the primary lesions of head and neck squamous cell carcinomas and in their metastases, with expression of the *PRAME* gene being always present in the metastastatic lymph nodes. In primary lesions, gene expression correlated with smoking habit and with advanced tumors with a higher malignant potential, with the frequent expression of two or more of these genes.
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Despite the significant advances in the diagnosis and treatment of head and neck cancer, survival has increased by little more than 10% in the past 30 years.[1] Evidence shows that impairment of the

immune responses may play an important role in the onset and propagation of cancer, and the search for immunotherapy specifically directed at the disease has advanced, although there are few studies about head and neck tumors.[2] Within this context, the identification of antigens specifically expressed in tumors may serve as the basis for immunotherapy. Specific antigens are expressed by groups of genes that code for antigenic peptides of tumors presented by class I human leukocyte antigen (HLA) molecules and recognized by cytotoxic T lymphocytes. There are many categories of tumor antigens: those resulting from point mutations or gene rearrangement involved in the oncogenesis, the proteins encoded by the viral oncogenes (such as the papilloma type 16 virus proteins), differentiation antigens encoded by genes that are only expressed in particular types of tissue (many antigens described in melanomas such as Melan-A/MART-1, gp100, and tyrosinase), the proteins that are strongly expressed in the tumor compared with the normal cells (such as cErb2), and the cancer testis (CT) antigens. In normal tissues, these genes are expressed only in cells that do not present HLA genes that therefore cannot present peptides for a T cytotoxic response, such as trophoblasts and male germ cells in the testis.[3–5] A variety of antigens have been described in melanoma, which has been used as a model system for tumor antigen identification, including the first CT antigen MAGE-1.[6]

About 30 CT antigens have been described thus far.[7] In addition to the pattern of expression limited to germ cells and to cancer, other common features have emerged in studies of tumor antigen genes, such as the presence of multigene families, immunogenicity in cancer patients, heterogeneous expression in the various types of cancer, correlation of mRNA expression with tumor progression and with tumors with higher malignant potential, and activation by hypomethylation and/or by histone deacetylase inhibitors.[7–9]

In a search for biologic markers and immunotherapeutic targets for the treatment of head and neck squamous cell carcinomas, we tested samples of tumors of the oral cavity, pharynx, larynx, and in cervical lymph nodes for the expression mRNA of the genes melanoma antigen (*MAGE*)-1, *MAGE*-4, *MAGE*-10, *MAGE*-12, G melanoma antigen (*GAGE*-1/2), CTL-recognized antigen on melanoma (*LAGE*-1), B melanoma antigen (*BAGE*), New York esophageal squamous cell carcinoma antigen (CT antigen 1) (*NYESO*)-1, and preferentially expressed antigen of melanoma (*PRAME*) by reverse-transcriptase polymerase chain reaction (RT-PCR).

MATERIALS AND METHODS

This study involved 33 patients (31 men and 2 women) aged 31 to 94 years (mean, 55.5 years) with a diagnosis of head and neck squamous cell carcinoma surgically treated. The study was approved by the Institutional Research Ethics Committee, and all patients provided informed consent. Primary tumors were located in the oral cavity (15 cases), larynx (14 cases), and pharynx (4 cases). On the basis of the TNM System of the Union Internationale Contre le Cancer, eight cases were classified as T1, eight as T2, seven as T3, and 10 as T4.

A 0.5- to 1.0-cm fragment of the tumor and an equivalent fragment from surrounding tissue showing no macroscopic changes were removed intraoperatively during tumor ressection. When a cervical lymph node suspected of malignancy was observed, a lymph node fragment was also extracted at the end of neck dissection. The tissues were immediately immersed into liquid nitrogen and stored at −80°C. Before RNA extraction, a cryostat section was examined under the microscope, and the frozen blocks were dissected by apposition, trimming off nonneoplastic or necrotic areas.

RNA was extracted by the TRIZOL Reagent method (Invitrogen, Carlsbad, CA). The quality of the RNA was assessed by 1.2% agarose gel electrophoresis and ethidium bromide staining and quantitated by spectrophotometry. cDNA synthesis was performed using 2 μg total RNA with 0.5 μ*M* Random Hexamer primer (Amersham Biosciences, Little Chalfont, England) and approximately 7 μl H_2O (70°C for 10 minutes), followed by extension with 200 U of Superscript II enzyme (Invitrogen) at 42°C for 50 minutes and 70°C for 15 minutes. The quality of the synthesized cDNAs was analyzed by PCR using specific primers for the β-actin region (Figure 1). All primer pairs were designed for different exons to prevent the amplification of contaminating DNA.[10] The following cell lines were used as positive controls for the expression of target genes: erythroleukemia cell line K562 (for *MAGE*-1 and *BAGE*), MZ2-MEL (for *MAGE*-2, *MAGE*-10, and *GAGE*-1/2) and *LB373* (for *MAGE*-4, *MAGE*-12, *LAGE*-1, *NYESO*-1, and *PRAME*). A negative (blank) control was included in each reaction; also, we have observed absence of CT antigen expression in leu-

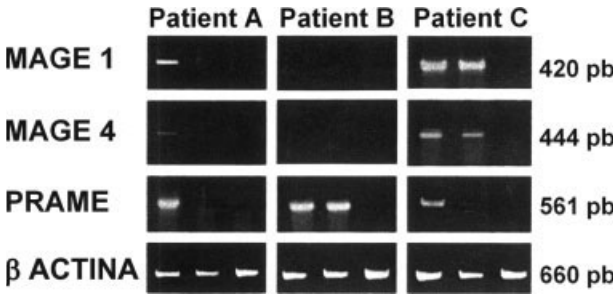

FIGURE 1. Electrophoresis on agarose gel of polymerase chain reaction products for MAGE-1, MAGE-4 and PRAME for samples of three patients, obtained from tumor (T), margin (M), and lymph nodes. N+, metastasis observed on histopathology; N−, nonmetastatic. Amplification of β-actin is shown as reference.

kocytes from 50 healthy blood donors.[11] Samples were then classified as positive for CT antigen expression when the RT-PCR product intensity was similar or higher than that observed for the 1:27 dilution of the positive reference cDNA obtained from cell lines.[10] All PCR reactions were performed in a gene Amp PCR System (Applied Biosystems, Foster City, CA) with 30 cycles for all genes analyzed.

RESULTS

Expression of genes of the family of CT antigens was demonstrated in 66.6% samples of head and neck squamous cell carcinomas located at different sites (Table 1). The significance of the correlation of gene expression with histopathologic and clinical characteristics was analyzed by the Fisher exact test.

Tumor Differentiation. Of the cases studied, 18 (54.5%) had histologic characteristics of well-differentiated tumors, 13 (39.4%) were moderately differentiated, and two (6.1%) were poorly differentiated. No correlation was observed between gene expression and grade of tumor differentiation ($p = .301$).

Staging. All tumors staged as T4 (100%) showed expression of tumor antigen genes as opposed to 50% of T1 and T2 tumors and 57% of T3 tumors. Multiple gene expression (two or more) was significantly higher in tumors in advanced stages ($p = .014$). When considered individually, a statistically significant association with the *MAGE*-10 gene ($p = .021$) and a marginally significant association with the *MAGE*-1 gene ($p = .069$) was observed.

Gene Expression in Lymph Nodes. Lymph nodes were available for analysis in 15 cases, and only five of them were found to contain carcinoma metastases on microdissection. None of the lymph nodes free of pathologic changes presented expression of the tumor antigen genes, whereas four of five metastatic lymph nodes did present it, with the *PRAME* gene being expressed in all of them (Table 2). Among the cases with gene expression in metastatic lymph nodes, three also had a lymph node without pathologic changes on microdissection, which was negative in terms of gene expression.

Gene Expression in the Margins. In 19 cases (58%), we studied gene expression in the apparently disease-free margins, which was positive in a single case.

Clinical Characteristics. An association was detected between the number of cigarettes smoked per day and frequency of gene expression ($p = .051$). No relationship was observed between gene expression and locoregional recurrence, distant metastases, or death caused by the malignancy.

DISCUSSION

In this study, the expression of at least one of the CT antigen genes was demonstrated in 66.6% of patients with squamous cell carcinoma at various sites in the head and neck. This prevalence is similar to data reported in other studies[12–14] for smaller sets of markers. Studies of expression of these genes in head and neck tumors or in other

Table 1. Frequency of cases positive for the expression of each of the tumor antigens.

Antigen gene	No. of cases	(%)
MAGE-1	10	(30.3)
MAGE-4	17	(51.5)
MAGE-10	7	(21.2)
MAGE-12	3	(9.1)
BAGE	3	(9.1)
GAGE-1	5	(15.2)
LAGE-1	3	(9.1)
NYESO-1	4	(12.1)
PRAME	14	(42.4)

Abbreviations: MAGE, melanoma antigen; BAGE, B melanoma antigen; GAGE-1/2, G melanoma antigen; LAGE-1, CTL-recognized antigen on melanoma; NYESO-1, New York esophageal squamous cell carcinoma antigen; PRAME, preferentially expressed antigen of melanoma.

Table 2. Expression of tumor antigens in lymph nodes with or without metastases, compared with gene expression in the primary tumor, in the five patients who had histologically confirmed metastases in the lymph nodes.

	Patient 1			Patient 2			Patient 3			Patient 4		Patient 5	
Antigen	T	N+	N−	T	N+	N−	T	N+	N−	T	N+	T	N+
MAGE-1	−	−	−	+	+	−	−	−	−	−	−	−	−
MAGE-4	−	−	−	+	+	−	−	−	−	−	−	−	−
MAGE-10	−	−	−	+	+	−	−	−	−	−	−	−	−
MAGE-12	−	−	−	+	+	−	−	−	−	−	−	−	−
BAGE	−	−	−	+	−	−	−	−	−	−	−	−	−
GAGE-1/2	−	−	−	+	−	−	−	−	−	−	−	−	−
LAGE	−	−	−	−	−	−	−	−	−	−	−	−	−
NYESO-1	−	−	−	−	−	−	−	−	−	−	−	−	−
PRAME	−	+	−	+	+	−	+	+	−	−	+	−	−

Abbreviations: T, primary tumor; N+, lymph node with a metastasis detected histologically; N−, lymph node without metastasis; MAGE, melanoma antigen; BAGE, B melanoma antigen; GAGE-1/2, G melanoma antigen; LAGE, CTL-recognized antigen on melanoma; NYESO-1, New York esophageal squamous cell carcinoma antigen (cancer testis antigen 1); PRAME, preferentially expressed antigen of melanoma.

tumors such as melanoma, hematologic neoplasias, or carcinomas of other locations were limited to the analysis of the expression of one or a few of these genes. To our knowledge, this is the first study in which the expression of this large set of genes (*MAGE*-1, -4, -10, -12, *BAGE, GAGE*-1/2, *LAGE*-1, *PRAME*, and *NYESO*-1) was investigated in head and neck carcinomas, and for genes *MAGE*-12, *LAGE*-1, and *PRAME,* this is the first report.

The expression of different antigens in a single type of tumor is highly variable.[7] In this study, 57.1% of the cases expressed at least one gene of the MAGE family (*MAGE*-1, *MAGE*-4, *MAGE*-10, and *MAGE*-12), with lower rate of expression of the *BAGE* and *GAGE* genes, in agreement with previous data.[12–15] Studies on the expression of the *PRAME* gene are more scarce, most of them in hematologic neoplasias.[16–20] In our study, the first conducted on head and neck squamous cell carcinomas for *PRAME*, this was the second most frequently expressed gene (42.2%), similar to the results obtained in renal cell carcinoma.[21]

Our results show an association between tumor progression and the expression of CT antigen genes ($p = .014$, Table 3) and also report that the expression of these genes is often simultaneous. Studies have shown that methylation probably is the primary mechanism of inactivation of these genes,[7,15,22,23] and that global DNA hypomethylation and gene-specific hypomethylation occurs in tumors associated with tumor progression,[24,25] suggesting that demethylation may be the common mechanism of activation and coexpression and the association with tumor progression. These results support the idea of new therapeutic options based on the use of demethylating agents and of specific immunotherapy.[26,27] Recently, a new member of the CT antigen gene family, BORIS, has been linked with molecular mechanisms of epigenetic reprogramming in normal male germ cell development and in cancer.[28] In this study, we analyzed for the first time the expression of these CT antigens in cervical lymph nodes from 15 patients, although expression of other antigens has been previously reported.[29,30] *PRAME* was expressed in all four cases that were positive for CT antigens among the five that had metastatic squamous cell carcinoma. None of 13 negative nodes (10 patients without metastasis and three cases with both positive and negative nodes; see Table 2) were positive for CT antigens. The finding of gene expression in cervical metastases of head and neck squamous cell carcinoma is

Table 3. Expression of tumor antigens in head and neck carcinoma of different T classifications (Fisher exact test, $p = .014$).

	No. of cases by tumor antigen expression			
T classification	Negative	+	++	Total
T1	4	3	1	8
T2	4	2	2	8
T3	3	2	2	7
T4	0	1	9	10
Total	11	8	14	33

+, expression of one antigen; ++, expression of two or more antigens.

of special interest, because occult cervical metastases occur in 15% to 60% of cases, and the treatment of an N0 patient (with no clinical metastases) has been recommended when the risk of occult metastasis is approximately 30% or more.[31] Yoshioka et al,[29] recently reported the clinical application of RT-PCR for intraoperative diagnosis of lymph node micrometastasis. The finding obtained in this study justifies investigations on larger samples of the *PRAME* gene in metastases of head and neck squamous cell carcinoma in view of the possibility of using the expression of tumor gene in the search for sentinel lymph nodes.

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. CT antigen genes 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.[9] Our results show a frequent expression of these genes in head and neck squamous cell carcinomas, mainly in advanced tumors that have a poor prognosis with the treatments currently available. This finding suggests that this patient population is a potential target for immunotherapy, either in the form of cancer "vaccines" or by the use of antibodies directed against the antigens carried specifically by the tumor cells. The results obtained here on the basis of mRNA expression should be further analyzed on the basis of protein expression.

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REFERENCES

1. Jernal A, Thomas A, Murray T, et al. Cancer statistics 2002. CA Cancer J Clin 2002;52:23–47.
2. Resser JR, Carbone D. Immunotherapy of head and neck cancer. Curr Opin Oncol 1998;10:226–232.
3. Boon T, Cerottini JC, van den Eynde B, et al. Tumor antigens recognized by T lymphocytes. Annu Rev Immunol 1994;12:337–365.
4. Van den Eynde B, Peeters O, De Backer O, et al. A new family of genes coding for an antigen recognized by autologous cytolytic T lymphocytes on a human melanoma. J Exp Med 1995;182:689–698.
5. Janeway CA, Travers P, Walport M, Shlomchik M. Immunobiology,5th ed. New York and London: Garland Publishing; 2001.
6. van der Bruggen P, Traversari C, Chomez P, et al. A gene encoding an antigen recognized by cytolytic T lymphocytes on a human melanoma. Science 1991;254: 1643–1647.
7. Scanlan MJ, Gure AO, Jungbluth AA, et al. Cancer/testis antigens: an expanding family of targets for cancer immunotherapy. Immunol Rev 2002;188:22–32.
8. Old LJ. Cancer/testis (CT) antigens—a new link between gametogenesis and cancer. Cancer Immunity 2001;1:1–7.
9. Old LJI. Cancer vaccine collaborative 2002: opening address. Cancer Immunity 2003;3S1:1–9.
10. Van Baren N, Brasseur F, Godelaine D, et al. Genes encoding tumor-specific antigens are expressed in human myeloma cells. Blood 1999;94:1156–2264.
11. Proto-Siqueira R, Falcao RP, Souza CA, et al. The expression of PRAME in chronic lymphoproliferative disorders. Leukemia Res 2003;27:393–396.
12. Kienstra MA, Neel BH, Strome SE, et al. Identification of NYESO 1, MAGE-1, and MAGE-3 in head and neck squamous cell carcinoma. Head Neck 2003;25:457–463.
13. Park JW, Kwon TK, Kim IH, et al. A new strategy for the diagnosis of MAGE-expressing cancers. J Immunol Methods 2002;266:79–86.
14. Eura M, Ogi K, Chikamatsu K, et al. Expression of the MAGE gene family in human head and neck squamous cell carcinomas. Int J Cancer 1995;64:304–308.
15. De Backer O, Arden KC, Boretti M, et al. Characterization of the GAGE genes that are expressed in various human cancers in normal testis. Cancer Res 1999;59: 3157–3165.
16. Van Baren N, Chambost H, Ferrant A, et al. PRAME, a gene encoding an antigen recognized on a human melanoma by cytolytic T cells, is expressed in acute leukaemia cells. Br J Haematol 1998;102:1376–1379.
17. Steinbach D, Hermann J, Viehmann S, et al. Clinical implications of PRAME gene expression in childhood acute myeloid leukemia. Cancer Genet Cytogenet 2002; 133:118–123.
18. Steinbach D, Viehmann S, Zintl F, et al. PRAME expression in childhood acute lymphoblastic leukemia. Cancer Genet Cytogenet 2002;138:89–91.
19. Pellat-Deceunyunck C, Mellerin MP, Labarriere N, et al. The cancer germ-line genes MAGE-1, MAGE-3 and PRAME are commonly expressed by human myeloma cells. Eur J Immunol 2000;30:803–809.
20. Matsushita M, Ikeda H, Kizaki M, et al. Quantitative monitoring of the PRAME gene for the detection of minimal residual disease in leukaemia. Br J Haematol 2001; 112:916–926.
21. Neumann E, Engelsberg A, Decker J, et al. Heterogeneous expression of the tumor-associated antigens RAGE-1, PRAME, and glycoprotein 75 in human renal cell carcinoma: candidates for T-cell-based immunotherapies? Cancer Res 1998;58:4090–4095.
22. De Smet C, De Backer O, Faraoni L, et al. The activation of human gene MAGE-1 in tumor cells is correlated with genome-wide demethylation. Proc Natl Acad Sci U S A 1996;93:7149–7153.
23. Weber J, Salgaller M, Samid D, et al. Expression of the MAGE1 tumor antigen is up regulated by the demethylating agent 5-aza-2′-deoxycytidine. Cancer Res 1994;54: 1766–1771.
24. Diala ES, Chea MSC, Rotwitch D, et al. Extent of methylation in human tumor cells. J Natl Cancer Inst 1983; 71:755–764.
25. Gama-Sosa MA, Slagel VA, Trewyn RW, et al. The 5-methylcytosine content of DNA from human tumors. Nucleic Acids Res 1983;11:6883–6894.
26. Coral S, Sigalotti L, Altomonte M, et al. 5-aza-2′-Deoxycytidine-induced expression of functional cancer testis antigens in human renal cell carcinoma: immunotherapeutic implications. Clin Cancer Res 2002;8:2690–2695.

27. Weiser TS, Guo ZS, Ohnmacht GA, et al. Sequential 5-aza-2-deoxycytidine-depsipeptide FR901228 treatment induces apoptosis preferentially in cancer cells and facilitates their recognition by cytolytic T lymphocytes specific for NY-ESO-1. J Immunother 2001;24:151–161.
28. Loukinov DI, Pugacheva E, Vatolin S, et al. BORIS, a novel male germ-line-specific protein associated with epigenetic reprogramming events, shares the same 11-zinc-finger domain with CTCF, the insulator protein involved in reading imprinting marks in the soma. Proc Nat Acad Sci U S A 2002;99:6806–6811.
29. Yoshiota S, Fujiwara Y, Sugita Y, et al. Real-time rapid reverse transcriptase-polymerase chain reaction for intraoperative diagnosis of lymph node micrometastasis: clinical application for cervical lymph node dissection in esophageal cancers. Surgery 2002;132:34–40.
30. Strome SE, Savva A, Brissett AE, et al. Squamous cell carcinoma of the tonsils: a molecular analysis of HPV associations. Clin Cancer Res 2002;8:1093–1100.
31. Alex JC, Sasaki CT, Krag DN, et al. Sentinel lymph node radiolocalization in head and neck squamous cell carcinoma. Laryngoscope 2000;110:198–203.