Jpn. J. Cancer Res. 88, 414–419, April 1997

Detection of MAGE-4 Protein in Sera of Lung Cancer Patients

Shigeki Shichijo,[1] Tomoaki Hoshino,[1] Kikuo Koufuji,[3] Akihiko Hayashi,[3] Mayumi Kawamoto,[1] Megumi Kikuchi,[1] Takafumi Higuchi,[1] Masao Ichiki,[2] Kotaro Oizumi[2] and Kyogo Itoh[1]

Departments of [1]Immunology, [2]Medicine and [3]Surgery, Kurume University School of Medicine, 67 Asahi-machi, Kurume 830

We investigated the level of MAGE-4 protein in sera of patients with primary lung cancer to understand better the biological roles of the MAGE proteins. MAGE-4 protein was detected as a non-degraded form in both the supernatant of a MAGE-4$^+$ tumor cell line and in a patient's serum. Serum level of the MAGE-4 protein in lung cancer patients ($n=100$, mean$=1.17$ ng/ml) was significantly higher than that in either patients with benign pulmonary diseases ($n=80$, 0.33 ng/ml) or healthy donors ($n=68$, 0.32 ng/ml). It was higher than the cutoff level (1.15 ng/ml) in 34 of 100 cancer patients, but not in anyone in the other groups.

Key words: MAGE-4 protein — Serum — Lung cancer patient — ELISA

The *MAGE-1, -2, -3, -4, -6,* and *-12* genes are frequently expressed in many different cancers, but are not expressed in normal cells or in normal tissues other than testis and placenta.[1–7] MAGE-1 and -3 peptides are currently used as vaccines for cancer patients, and three of 12 HLA-A1 patients with metastatic melanoma responded to MAGE-3 peptide.[8] Therefore, MAGE proteins could be potential vaccines for HLA-A1 cancer patients. However, the biological roles of proteins of the MAGE family remain to be investigated. This is in part due to the lack of antibodies (Abs) to detect the natural forms of MAGE proteins. We have reported a monoclonal Ab (mAb) and a polyclonal Ab recognizing natural MAGE-4 protein.[7,9] In the present study, we investigated whether the MAGE-4 protein is detectable in sera of lung cancer patients by immunoblot analysis and enzyme-linked immunosorbent assay (ELISA) using these antibodies.

MATERIALS AND METHODS

One hundred primary lung cancer patients (75 males, 25 females) were enrolled in this study. Their age ranged from 37 to 83 years, with a mean age of 66.0 years. The tumors were histologically 58 adenocarcinomas, 28 squamous cell carcinomas, 3 large cell carcinomas, and 11 small cell carcinomas. Thirty-six, 8, 27, and 16 patients were respectively stage I, II, III, and IV, and 13 were not classified based upon the 4th edition of the International Union Against Cancer (UICC) TMN classification.[10] Sera were collected from 100 patients with primary lung cancer, 80 patients with benign pulmonary diseases and 68 healthy donors (HD), aliquoted and preserved at -80°C until use. All sera were collected prior to surgery. RPMI-1788 B cell line, from which *MAGE-4* gene was obtained and sequenced,[11] was used as a positive control. The 12 lung cancer cell lines used in this study were A549, 1-87, LK87, 11-18 (adenocarcinomas), LC1-Sq, Sq-1 (squamous cell carcinomas), LC99A (large cell carcinoma), LK79, S-1, S-2, LC65A and KCC-2 (small cell carcinomas). Culture supernatants were collected from these cell lines.

MAGE-4 protein was purified from the culture supernatant of the RPMI-1788 B cell line and from serum of a lung cancer patient by using an affinity column that was prepared by coupling R5 mAb (IgG1) or a subclass-matched mAb (601 mAb, anti-bovine conglutinin) to formyl-Cellulofine beads (Seikagaku Kogyo, Tokyo) in the presence of trimethylamine bromide (TMAB, Aldrich, WI) as a reducing agent. Positive fractions were concentrated by ultrafiltration (Centricon, Amicon, MA) for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Detection of the MAGE-4 protein in lung cancer tissue, sera and culture supernatants of tumor cell lines was carried out by sandwich ELISA using a mouse mAb (R5) and a biotinylated polyclonal anti-MAGE-4 affinity-purified IgG as reported.[7,9] Immunoblot analysis for detection of MAGE-4 protein was also previously reported.[7,9,10] The R5 mAb or polyclonal Ab mainly reacted to a single peptide, No.13 (p-13, HFLLRKYRAKELVT, positions 119 to 132) or two peptides (p-13 and p-27, YRQVPGSNPARYEFL, 258 to 272) among a series of 31 different MAGE-4b peptides, respectively.[9] The epitopes recognized by the R5 mAb as well as the polyclonal Ab were specific for MAGE-4 protein.[7] Therefore, this ELISA is expected to detect MAGE-4 protein, but not the other MAGE proteins reported in the literature (MAGE-1, -2, -3, -6, and -12).[7,9] The Mann-Whitney U test was used for the statistical analysis of

serum levels of MAGE-4 protein among HD, patients with benign pulmonary diseases and lung cancer patients. The χ^2 test was used for the statistical analysis of the positive rates among different types of histology.

RESULTS

MAGE-4 protein was detectable in culture supernatants of RPMI-1788 (6.54 ng/ml), S-1 (15.1 ng/ml) and LC1-Sq (2.58 ng/ml) cell lines. These three tumors expressed MAGE-4 mRNA, as reported.[7] In contrast, it was undetectable (less than 0.1 ng/ml) in the supernatants from all of the other 10 lung cancer cell lines that expressed genes of the *MAGE* family other than *MAGE-4* gene. MAGE-4 protein was purified from the culture supernatant of RPMI-1788 cells or serum of a lung cancer patient by the use of an affinity column with the R5 mAb (Fig. 1). Fraction numbers 2 and 3 of the superna-

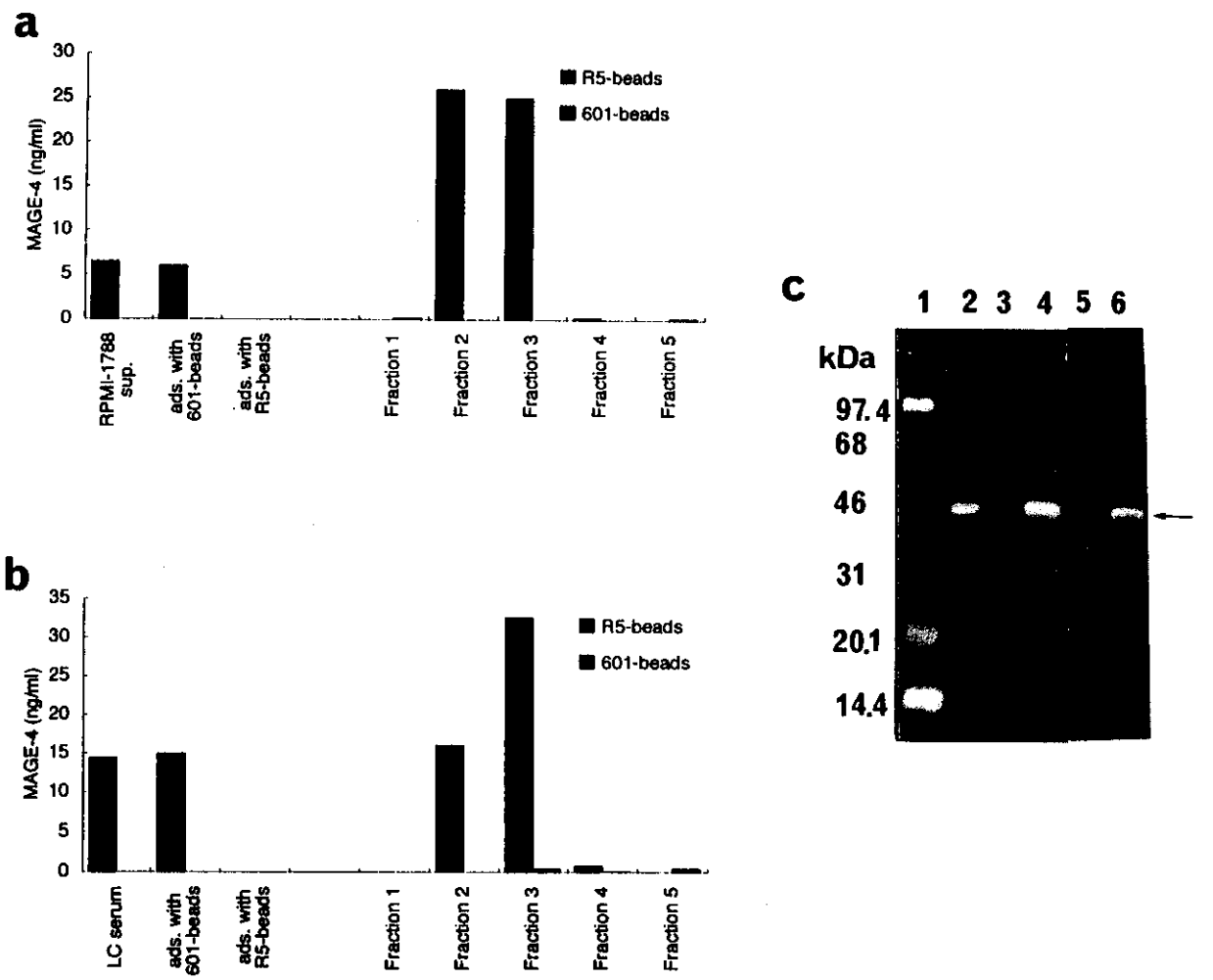

Fig. 1. Detection of MAGE-4 protein. MAGE-4 protein was purified from 7 ml of the culture supernatant of RPMI-1788 cell line (7 ng/ml of MAGE-4) and from 7 ml of serum of a patient with lung cancer (13.8 ng/ml). Control (601) mAb-coupling beads were added to each sample and mixed for 2 days at 4°C. The supernatant was then mixed with the R5 mAb-coupling beads for 2 days at 4°C. The beads were washed and eluted with citrate buffer (pH 3.5, 0.2 *M*). Levels of MAGE-4 protein of these fractions were measured by ELISA (a, culture supernatant; b, serum). Fractions 2 and 3 from each sample were pooled, concentrated by ultrafiltration and subjected to SDS-PAGE. MAGE-4 protein was detected by immunoblot analysis with anti-MAGE-4 rabbit IgG, as reported[7] (c). The band with a molecular weight of about 45,000 daltons was observed in the cell lysate of RPMI-1788 cells (a positive control, lane 2), the culture supernatant of RPMI-1788 cells (lane 4) and the patient's serum (lane 6). In contrast, that band was not observed in either sample purified by the control mAb-affinity column. The weak bands over 45,000 daltons were evaluated as non-specific, as reported.[7]

tant (Fig. 1a) or the serum (Fig. 1b) were concentrated and used for immunoblot analysis. The molecular weight of MAGE-4 protein was about 45,000 daltons (lane 4 or 6 for the supernatant or the serum, respectively, Fig. 1c), which was identical to that of cellular MAGE-4 protein of RPMI-1788 cells (lane 2). In contrast, it was undetectable in the control mAb-affinity-purified supernatant or serum (lane 3 or 5). Therefore, soluble MAGE-4 protein in both the supernatant of the tumor cell line and the patient's serum was present as a non-degraded form.[6,7,9] MAGE-4 protein was detected in culture supernatants of tumor cell lines expressing *MAGE-4* gene, but not the genes of other *MAGE* family members, as reported.[7] These results suggest that MAGE-4 protein is released as a non-degraded form in the supernatant from the MAGE-4 protein$^+$ tumors.

MAGE-4 protein was measured by ELISA in sera from patients with primary lung cancer ($n=100$), benign pulmonary diseases ($n=80$) and from HD ($n=68$). Representative values are plotted in Fig. 2a. Mean concentrations$\pm$SD of MAGE-4 protein in sera from these three groups were 1.17 ± 2.03 ng/ml ($P=0.0013$ or $P=0.0021$ versus patients with benign pulmonary diseases or HD, respectively), 0.33 ± 0.30 ng/ml, or 0.32 ± 0.28 ng/ml, respectively. Based on these results, the cutoff level was set at 1.15 ng/ml of MAGE-4 (mean level+3 SD in HD)

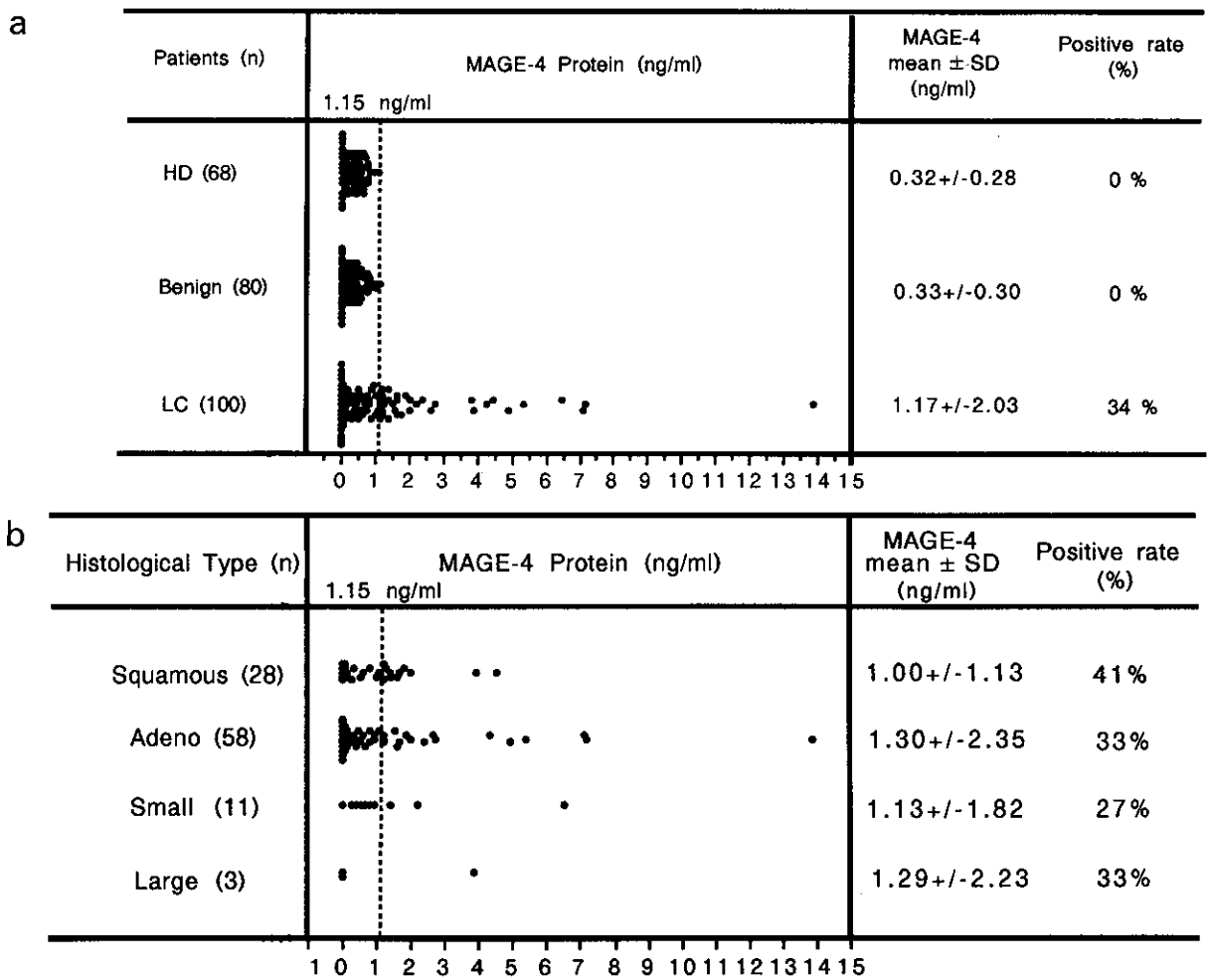

Fig. 2. MAGE-4 protein in sera of patients. The distribution of the serum level of MAGE-4 protein (ng/ml) in sera of lung cancer patients ($n=100$, mean$=1.17$ ng/ml), patients with benign pulmonary diseases ($n=80$, 0.33 ng/ml) or HD ($n=68$, 0.32 ng/ml) is shown (a). The MAGE-4 protein level in sera of lung cancer patients was significantly higher than that in sera of patients with benign diseases or HD ($P=0.0013$ or 0.0021 vs. benign diseases or HD, respectively, by the Mann-Whitney U-test). No apparent difference was observed among four different histological types of lung cancers (b). The distribution of the

in this study. Under these conditions, MAGE-4 protein in sera from 34 of 100 cancer patients, 0 of 80 patients with benign diseases and 0 of 68 HD exceeded the cutoff level (1.15 ng/ml). No apparent difference was observed among MAGE-4 protein-positive cases in four different histological types of lung cancers, although only 3 cases of large cell carcinomas were studied (Fig. 2b). These results indicate that MAGE-4 protein is elevated in sera from a significant proportion of lung cancer patients.

The TNM international staging system was used for 86 patients with non-small cell lung cancer to investigate the correlation between clinical stages and serum levels of MAGE-4 protein. There was no statistically significant difference in positive rates among any classification and stage (Fig. 2c) except for the N3 classification (Fig. 2d), in which sera from 5 of 6 patients (83%) exceeded the cutoff level ($P=0.009$ vs. N0 classification <15 of 51, $29\%>$, $P=0.027$ vs. N1 classification <3 of 11, $27\%>$, and $P=0.022$ vs. N2 classification <5 of 17, $29\%>$ by χ^2 test).

DISCUSSION

The results of this study suggest that non-degraded MAGE-4 protein is released from MAGE-4-positive lung cancers. In contrast, none of the sera from HD or

c

Stage (n)	MAGE-4 Protein (ng/ml) (1.15 ng/ml)	MAGE-4 mean ± SD (ng/ml)	Positive rate (%)
I (36)		0.91+/-1.37	25%
II (8)		1.05+/-1.34	25%
III (27)		0.91+/-1.06	41%
IV (16)		2.58+/-3.65	50%

0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

d

Classification (n)	MAGE-4 Protein (ng/ml) (1.15 ng/ml)	mean ± SD	Positive rate (%)
N0(51)		0.90+/-1.31	29%
N1(11)		0.98+/-1.41	27%
N2(17)		1.10+/-1.67	29%
N3(6)		2.26+/-2.26	83%

0 1 2 3 4 5 6 7 8

serum level of MAGE-4 protein (ng/ml) in patients with lung cancer according to clinical stage and N classification (TNM international staging system) is shown in c and d, respectively. There was no statistically significant difference in the distribution of the concentration of MAGE-4 protein (c and d) or in positive rates among any stage (c), except for N3 classification (d), in which sera from 5 of 6 patients (83%) exceeded the cutoff level ($P=0.009$ vs. N0 classification <15 of 51, $29\%>$, $P=0.027$ vs. N1 classification <3 of 11, $27\%>$, and $P=0.022$ vs. N2 classification <5 of 17, $29\%>$ by the χ^2 test).

patients with benign pulmonary diseases was positive. MAGE-1 and -4 proteins were detected in the spermatogonia and early stages of primary spermatocytes, but not in any other normal cells or tissues.[11] Therefore, it is likely that antigen-presenting cells of the MAGE-4-positive cancer patients present MAGE-4 protein or its peptides as antigens to T cells, which differentiate into cytotoxic T lymphocyte (CTL) or helper T cells reacting to the MAGE-4 protein. Subsequently, anti-MAGE-4 antibody might be raised in these cancer patients. The Ab might mediate Ab-dependent-cell-mediated cytotoxicity against MAGE-4-positive tumor cells, although it is not clear whether the MAGE-4 protein is expressed on the cell membrane. On the other hand, either the MAGE-4 protein or the Ab might inhibit CTL activity against MAGE-4-positive tumor cells. The biological roles of cancer-testis antigens (MAGE, BAGE, GAGE) in cancer patients have not yet been identified, although some of them have been used as cancer vaccines.[8] The existence of non-degraded MAGE-4 protein in sera of lung cancer patients might be a clue to the better understanding of the roles of MAGE-4 protein and possibly the other cancer-testis antigens in cancer patients.

Higher levels of serum MAGE-4 protein were associated with lung cancer patients with N3 classification, but were not associated with any other classification or clinical stage. However, there was a clear trend between stage and MAGE-4 positivity (25% in stages I and II, 41% for stage III and 50% for stage IV). The lack of statistical power is due to the relatively small number of samples. No apparent difference was observed among MAGE-4-positive cases in four different histological types of lung cancers. However, because only 3 cases of large cell carcinoma were studied, this issue also needs to be further investigated.

We have previously reported that MAGE-4 mRNA was expressed in lung cancer tissue in only 13% ($n=53$) of patients.[7] The expression of MAGE-4 and -41 mRNA in human head and neck squamous cell carcinoma was positive in 22% and 16% of patients ($n=83$), respectively.[12] On the other hand, MAGE-4 protein was detected in 13 of 44 samples (30%) of lung cancer tissue.[7] The positive rate of serum MAGE-4 protein was also higher than the expression of MAGE-4 mRNA. These results suggest that the ELISA employed in this study is more sensitive than the reverse transcriptase-polymerase chain reaction (RT-PCR) method for measuring the expression of *MAGE-4* gene in lung cancer tissues. Further, from the viewpoint of clinical utility as a tumor marker, ELISA using patients' sera could be superior to the RT-PCR method primarily because of its simplicity and the easiness of repeated sampling. Serial study is also feasible. Indeed, a serial study of patients with head and neck squamous cell carcinoma showed that the serum levels of MAGE-4 protein in all 7 patients whose levels were >1.15 ng/ml before operation quickly decreased after removal of the tumors, and that re-increase was associated with recurrence (Iwamoto *et al.*, unpublished results). Similarly, MAGE-4 protein was detected in sera of a proportion of hepatocellular carcinoma patients, as well as esophageal cancer patients (data not shown). Therefore, this ELISA might be a useful tool for determining MAGE-4 protein expression in lung cancer and also other cancers.

ACKNOWLEDGMENTS

This study was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture, Japan, as well as grants from Yasuda Medical Research Foundation, the Japanese Foundation for Multidisciplinary Treatment of Cancer, and the Fukuoka Cancer Society.

(Received November 11, 1996/Accepted January 27, 1997)

REFERENCES

1) van der Bruggen, P., Traversari, C., Chomez, P., Lurquin, C., De Plaen, E., van den Eynde, B., Knuth, A. and Boon, T. A gene encoding an antigen recognized by cytolytic T lymphocytes on a human melanoma. *Science*, **254**, 1643–1647 (1991).
2) Traversari, C., van der Bruggen, P., Luescher, I. F., Lurquin, C., Chomez, P., van Pel, A., Plaen, E. D., Amar-Costesec, A. and Boon, T. A nonapeptide encoded by human gene MAGE-1 is recognized on HLA-A1 by cytolytic T lymphocytes directed against tumor antigen MZ2-E. *J. Exp. Med.*, **176**, 1453–1457 (1992).
3) Gaugler, B., van den Eynde, B., van der Bruggen, P., Romero, P., Gaforio, J. J., De Plaen, E., Lethe, B., Brasseur, F. and Boon, T. Human gene *MAGE-3* codes for an antigen recognized on a melanoma by autologous cytolytic T lymphocytes. *J. Exp. Med.*, **179**, 921–930 (1994).
4) van der Bruggen, P., Szikora, J.-P., Boel, P., Wildmann, C., Somville, M., Sensi, M. and Boon, T. Autologous cytolytic T lymphocytes recognize a MAGE-1 nonapeptide on melanomas expressing HLA-Cw 1601. *Eur. J. Immunol.*, **24**, 2134–2140 (1994).
5) van der Bruggen, P., Bastin, J., Gajewski, T., Coulie, P. G., Boel, P., De Smet, C., Traversari, C., Townsend, A. and Boon, T. A peptide encoded by human gene MAGE-3 and presented by HLA-A2 induces cytotoxic T lymphocytes that recognize tumor cells expressing MAGE-3. *Eur. J. Immunol.*, **24**, 3038–3043 (1994).
6) De Plaen, E., Arden, K., Traversari, C., Gaforio, J. J.,

Szikora, J.-P., De Smet, C., Brasseur, F., van der Bruggen, P., Lethe, B., Lurquin, C., Brasseur, R., Chomez, P., De Backer, O., Cavenee, W. and Boon, T. Structure, chromosomal localization, and expression of 12 genes of the MAGE family. *Immunogenetics,* **40,** 360–369 (1994).

7) Shichijo, S., Hayashi, A., Takamori, S., Tsunosue, R., Hoshino, T., Sakata, M., Kuramoto, T., Oizumi, K. and Itoh, K. Detection of MAGE-4 protein in lung cancers. *Int. J. Cancer,* **64,** 158–165 (1995).
8) Marchand, M. P., Weynants, E., Rankin, F., Arienti, F., Belli, G., Parmiani, N., Cascinelli, N., Bourlond, A., Vanwijck, R., Humblet, Y., Canon, J.-L., Laurent, C., Naeyaert, J.-M., Plagne, R., Deraemaeker, R., Knuth, A., Jager, E., Brausseur, F., Herman, J., Coulie, P. G. and Boon, T. Tumor regression responses in melanoma patients treated with a peptide encoded by gene MAGE-3. *Int. J. Cancer,* **63,** 883–885 (1995).
9) Shichijo, S., Tsunosue, R., Kubo, K., Kuramoto, T., Tanaka, Y., Hayashi, A. and Itoh, K. Establishment of an enzyme-linked immunosorbent assay (ELISA) for measuring cellular MAGE-4 protein on human cancers. *J. Immunol. Methods,* **186,** 137–149 (1995).
10) Ginsberg, R. J., Kris, M. G. and Arnstrong, J. G. Cancer of the lung. *In* "Cancer: Principles and Practice of Oncology," ed. V. T. DeVita, S. Helleman and S. A. Rosenberg, pp. 673–723 (1993). Lippincott, Philadelphia.
11) Takahashi, K., Shichijo, S., Noguchi, M., Hirohata, M. and Itoh, K. Identification of MAGE-1 and -4 proteins in spermatogonia and primary spermatocytes of testis. *Cancer Res.,* **55,** 3478–3492 (1995).
12) Eura, M., Ogi, K., Chikamatsu, K., Lee, K. D., Nakano, K., Masuyama, K., Itoh, K. and Ishikawa, T. Expression of the MAGE gene family in human head- and neck-squamous-cell carcinomas. *Int. J. Cancer,* **64,** 304–308 (1995).