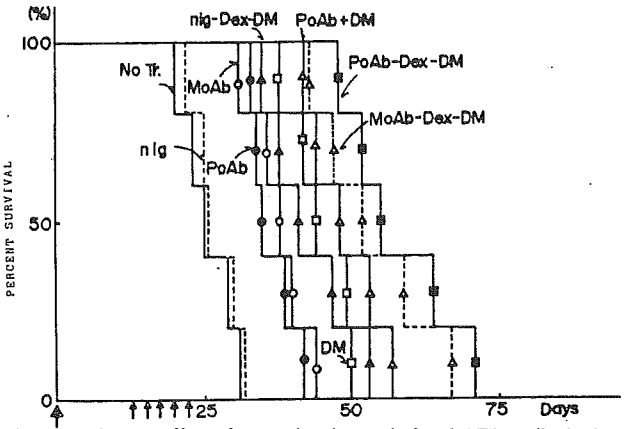

Fig. 1. Antitumor effect of monoclonal or polyclonal AFP antibody-daunomycin conjugate. One million (*large arrow*) of AH66 hepatoma cells were inoculated s.c. at the right thigh of Donryu rats. The tumor cells grew up to the solid tumor of 15–20 mm in diameter with the serum AFP level of 35 μg/ml. The treatment was initiated 14 days after inoculation every other day for five doses (*small arrows*). One dose included 2 mg of purified antibody combined with 200 μg of daunomycin. Five rats were used for each group. ——, nontreated group (*No Tr.*); ----, normal horse immunoglobulin 2 mg (*nIg*); —○—, monoclonal antibody to rat AFP 2 mg (*MoAb*); —●—, polyclonal antibody to rat AFP 2 mg (*PoAb*); —▲—, conjugate of normal horse immunoglobulin (2 mg) and daunomycin (200 μg) (*nIg-Dex-DM*); —□—, daunomycin 200 μg (*DM*); —△—, mixture of polyclonal antibody to rat AFP (2 mg) and daunomycin (200 μg) (*PoAb + DM*); --△--, monoclonal antibody to rat AFP (2 mg) daunomycin (200 μg) conjugate (*MoAb-Dex-DM*); —■—, polyclonal antibody to rat AFP (2 mg) daunomycin (200 μg) conjugate (*PoAb-Dex-DM*).

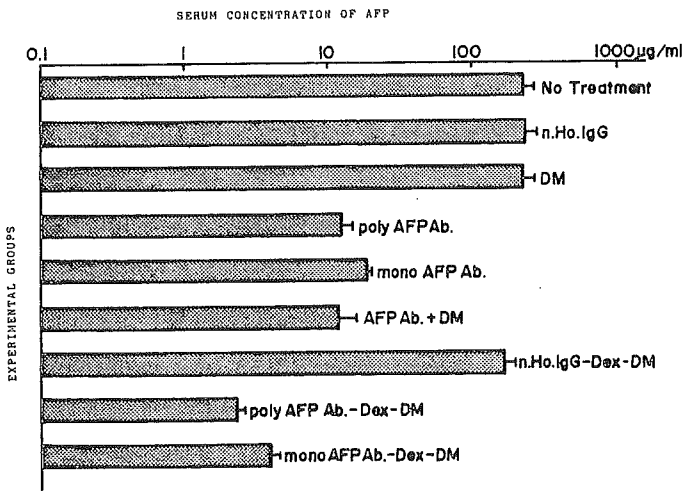

Fig. 2. Serum AFP level at terminal stage. Serum AFP level at terminal stage of rats in Fig. 1 experiment was determined. *n.Ho.IgG*, normal horse immunoglobulin; *DM*, daunomycin; *poly AFP Ab*, polyclonal antibody to rat AFP; *Mono AFP Ab*, monoclonal antibody to rat AFP; *AFP Ab + DM*, mixture of polyclonal antibody to rat AFP and daunomycin; *nHo-IgG-Dex-DM*, conjugate of normal horse immunoglobulin and daunomycin; *poly AFP Ab-Dex-DM*, polyclonal antibody to rat AFP daunomycin conjugate; *mono AFP Ab-Dex-DM*, monoclonal antibody to rat AFP daunomycin conjugate.

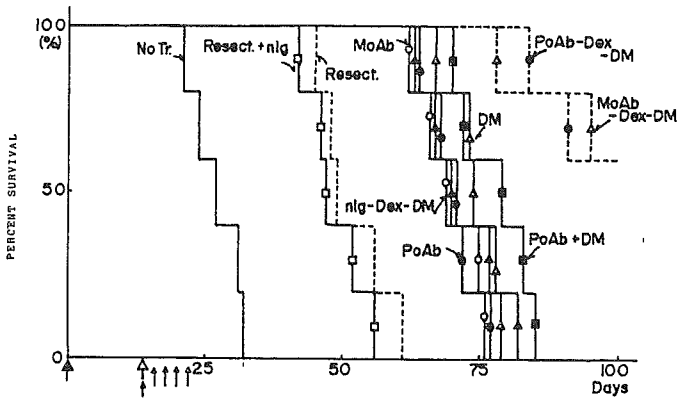

Fig. 3. Antitumor effect of monoclonal or polyclonal AFP antibody daunomycin conjugates after surgical resection of a s.c. tumor mass. One million (*large arrow*) of AH66 hepatoma cells were inoculated subcutaneously at the right thigh of Donryu rats. The tumor masses were excised on day 14 after inoculation. (↑) Antibody treatment was initiated that same day and every other day for 5 doses (*small arrows*). ——, nontreated group (*No Tr.*); ----, resection (*Resect.*); —□—, resection plus normal horse immunoglobulin (*Resect + nIg*); —●—, resection plus monoclonal antibody to rat AFP (*MoAb*); —○—, resection plus polyclonal antibody to rat AFP (*PoAb*); —▲—, resection plus conjugate of normal horse immunoglobulin and daunomycin (*nIg-Dex-DM*); —△—, resection plus daunomycin (*DM*); —■—, resection plus mixture of polyclonal antibody to rat AFP and daunomycin (*PoAb + DM*); --△--, resection plus monoclonal antibody to rat AFP daunomycin conjugate (*MoAb-Dex-DM*); --●--, resection plus polyclonal antibody to rat AFP daunomycin conjugate (*PoAb-Dex-DM*).

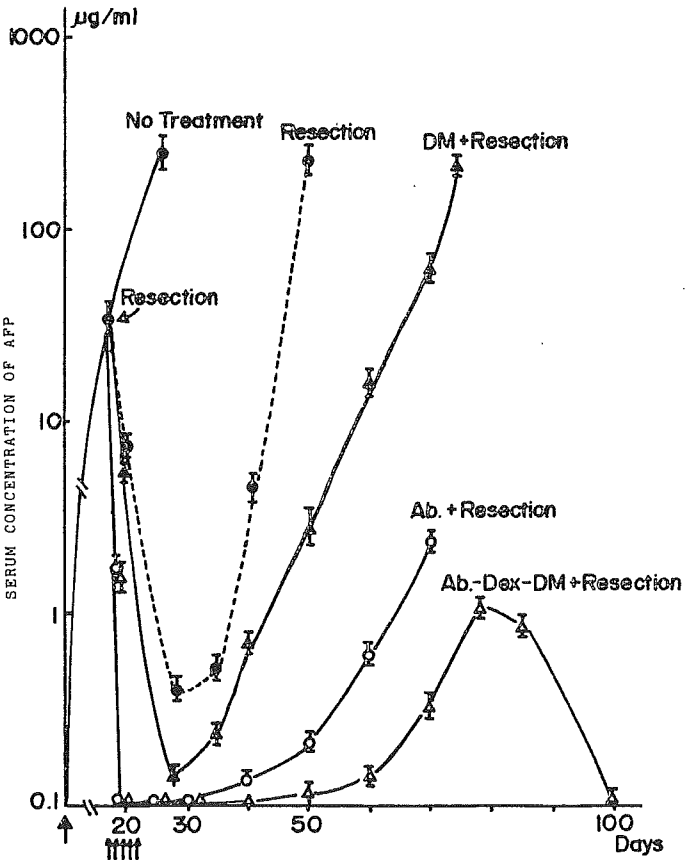

Fig. 4. Change in serum AFP level. Serum AFP level of Fig. 3 experiment during the experimental days was plotted in each group. The blood was collected from the tail vein. For simplicity, serum AFP level of rats received the resection plus conjugate of normal horse immunoglobulin and daunomycin and rats received the resection plus mixture of polyclonal antibody to rat AFP and daunomycin was omitted. Ab includes monoclonal and polyclonal antibodies. Symbols are same as Fig. 1.

administration of normal horse immunoglobulin ($P < 0.05$). There was a striking therapeutic effect of treatment of animals with the monoclonal or polyclonal AFP antibody-daunomycin conjugates in that three of five rats in each group (60%) survived until 100 days after inoculation of tumor. These animals were sacrificed at that time and were found to be tumor free.

The serum AFP concentrations were determined in these resected animals during the experimental period (Fig. 4). Following resection of the primary solid tumor there was an immediate decrease of the serum AFP level and this was followed by a rapid increase. This increase was due chiefly to the production of AFP by the proliferating residual hepatoma cells. The groups which were treated with daunomycin showed a little delay in the second AFP peak. Monoclonal or polyclonal antibody treatment markedly suppressed serum AFP levels after surgical resection of the tumor. A similar but greater suppression of the serum AFP occurred in the rats which were injected

with monoclonal or polyclonal AFP antibody-daunomycin conjugate. It is of note that in those animals which were judged to be tumor free by day 100 there were extremely low levels of AFP at 10 ng/ml.

DISCUSSION

Specific antiserum to AFP has an inhibitory effect of the growth of AFP-producing tumor cells (1). This has been confirmed by Mizejewski *et al.* using mouse hepatoma cell lines (12, 13). Affinity-purified monoclonal and polyclonal antibodies to AFP have been utilized and proved to be equally effective as whole antiserum in inhibiting the growth of tumor cells (4). Conjugation methods were developed which made it possible to use intermediate macromolecules as carriers so that anticancer drugs could be conjugated to affinity-purified antibodies (14, 15–19). Successful therapeutic efforts have been made to target chemotherapy by utilizing conjugating purified antibodies with chemotherapeutic agents in rat and mouse systems (3, 4, 14) and more recently with human tumor cells lines transplanted in nude mice (20, 21).

In the experiments described in this paper we have used the conjugate of specific antibodies with daunomycin via a dextran bridge and studied what effect this treatment has on a solid tumor model which has micrometastasis. The administration of either monoclonal or polyclonal antibody to AFP-daunomycin conjugates to animals having a s.c. tumor significantly prolonged survival when compared to the polyclonal antibody to AFP and daunomycin. The median survival time of rats receiving the conjugate was 2-fold that of the nontreated group. In a second study, groups of rats were first treated with surgical resection and then treated with various combinations of antibody or antibody drug conjugates. Six of 10 rats injected with the monoclonal or polyclonal antibodies conjugated with daunomycin were "cured" of their residual micrometastatic disease as they were found to be microscopically tumor free at day 100. Since we had previously shown that 100% of the animals at 14 days had microscopic metastases in their lungs (5), these experiments mean that the remaining small foci of lung metastases were effectively killed by the antibody drug treatment. This latter study shows that the optimal time for antibody drug conjugate treatment may be immediately postoperatively when there is minimal residual tumor burden. Serum studies in which the concentration of AFP was quantitated showed that the optimal therapeutic effect was obtained in those animals treated with the antibody-daunomycin conjugates.

ACKNOWLEDGMENTS

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Monoclonal Antibody, MAb 12C3, Is a Sensitive Immunohistochemical Marker of Early Malignant Change in Epithelial Ovarian Tumors

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Original Article

Monoclonal Antibody, MAb 12C3, Is a Sensitive Immunohistochemical Marker of Early Malignant Change in Epithelial Ovarian Tumors

KYOSUKE YAMADA, MD,[1] KIYOSHI OHKAWA, MD, PHD,[2] AND KENSUKE JOH, MD[3]

A murine monoclonal antibody (MAb 12C3) that is specific to human ovarian carcinomas was generated by immunizing mice with a human ovarian germinoma cell line (JOHYC-2). The antigen distribution that was defined by MAb 12C3 in normal and malignant human tissues was analyzed by immunohistochemistry on paraffin-embedded and frozen sections. The antibody reacted with 67.7% (21 of 31 cases) of epithelial ovarian carcinomas (6 of 12 cases of serous cystadenocarcinoma, 5 of 7 cases of mucinous cystadenocarcinoma, 7 of 9 cases of clear cell carcinoma, 3 of 3 cases of endometrioid adenocarcinoma), but did not react with any of the benign epithelial ovarian adenomas tested. Partial regions of borderline ovarian malignancies that exhibited marked papillary projection of the lining cells with cellular atypia reacted positively with MAb 12C3 in 14 of 25 cases (56.0%). The histologic features of regional early malignant change corresponded to the expression of the MAb 12C3 epitope in the borderline malignant tumor cells. There was a low frequency of reaction (4.3%) between the antibody and other gynecologic and nongynecologic malignancies (46 cases of 12 tissues). In normal tissues, the antibody reacted positively with only three tissues, including corpora lutein cells, excretory ducts in the submandibular gland, and basal cells of the sebaceous glands. The antigen epitope defined by MAb 12C3 was present on a glycoprotein with a molecular mass of 200 kDa and did not exhibit any cross-reactivity with other well-known tumor markers. These data suggest that MAb 12C3 may be a useful tool for the immunohistologic detection of early malignant changes in epithelial ovarian tumors. In addition, MAb 12C3 also may facilitate a differential diagnosis between benign and borderline malignancies. (Key words: Monoclonal antibody; Human ovarian borderline malignancy; Immunohistochemistry) Am J Clin Pathol 1995;103:288–294.

Histopathologic diagnosis of ovarian tumors with borderline malignancy has been difficult because ovarian lesions are otherwise overtly malignant or unquestionably benign.[1–3] The prognosis for patients with borderline malignancy is good, but it is essential to diagnose malignant change in the tumor cells histologically and to accurately follow the clinical course. There have been few reports dealing with the diagnosis of borderline malignancy using MAbs,[4] although many reports[5–23] have described murine monoclonal antibodies (MAbs) as potent tools for diagnosing malignant ovarian tumors. We attempted to develop a MAb that can differentiate cells with early malignant change from adjacent benign tumor cells in cases of borderline malignancy. In this article, we describe the immunohistologic expression of MAb 12C3-defined antigen in specimens from various ovarian epithelial tumors, other gynecologic and non-gynecologic tumors, and normal tissues. We also discuss some biochemical properties of the antigen recognized by MAb 12C3.

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MATERIALS AND METHODS

Cell Line

The human ovarian germinoma cell line, JOHYC-2,[24] was maintained in 10% newborn calf serum and 5% fetal bovine serum in Earle's minimum essential medium (Nissui, Tokyo, Japan) under conventional culture conditions.

Production of MAbs

BALB/c male mice were immunized three times at 2-week intervals with intraperitoneal injections of 5×10^6 JOHYC-2 cells. Four weeks later, the mice were given booster intraperitoneal injections of the same cells (5×10^6). Three days after the booster, the immune spleen cells were fused with P3 X 63 Ag8 U1 myeloma cells using the previously described method.[6] Supernatants with growing hybridomas were initially screened for reactivity against JOHYC-2 cells using a cell-target enzyme-linked immunosorbent assay (cell ELISA) as previously reported.[6] Briefly, JOHYC-2 cells cultured in tissue culture microplates (Falcon No. 3072; Becton Dickinson, Lincoln Park,

TABLE 1. SUMMARY OF MONOCLONAL ANTIBODY 12C3 REACTIVITY AGAINST MALIGNANT TUMORS

Tissue	*Specimens Stained/Total (n)*
Ovarian carcinoma	21/31*
Lung carcinoma	0/5
Breast carcinoma	0/4†
Gastric carcinoma	0/5‡
Hepatocellular carcinoma	0/4
Carcinoma of the gall bladder	0/4
Pancreatic carcinoma	0/4
Thyroid carcinoma	0/4
Renal cell carcinoma	1/2
Bladder carcinoma	0/3
Prostatic carcinoma	0/2
Uterine cervical carcinoma	0/7
Endometrial carcinoma	1/2

* Three cases, † one case, and ‡ two cases of frozen specimens were additionally stained with monoclonal antibody 12C3, and the results were identical to those obtained from paraffin-embedded specimens.

NJ) were fixed *in situ* with 0.25% glutaraldehyde, then blocked with 1% bovine serum albumin (BSA; Sigma, St. Louis, MO), and 0.1 M glycine in Dulbecco's phosphate buffered saline (PBS). After blocking the activity of endogenous peroxidase, the wells were further blocked with 20 mM Tris-HCl, pH 7.6, 0.5 M NaCl (TBS) containing 10% skimmed milk (Difco Laboratories, Detroit, MI). Fifty μL of hybridoma supernatants were added to the cell-coated wells and incubated overnight at 4 °C. For the detection of bound antibodies, horseradish peroxidase (HRP)-labeled goat anti-mouse immunoglobulin (Ig) (Cappel, Malvern, PA) was used as the second antibody with O-phenylenediamine as the substrate. Absorbance at 492 nm was determined with a microplate reader (TOHSO, Tokyo, Japan). The positive hybridomas were cloned twice, expanded in tissue culture, and grown in ascites in pristane-primed mice. Ascites fluid that was diluted 400-fold with 1% BSA in TBS was used as the antibody source.

Immunoperoxidase-staining

Fresh, normal tissues (20 organs) and neoplastic tissues (77 cases from 13 tissues) were obtained from surgical and autopsy materials. The tissues were fixed with 10% buffered-formalin and embedded in paraffin. They were used for histologic diagnosis and immunohistochemical studies. Borderline malignancy (25 cases) was diagnosed according to the World Health Organization's diagnostic criteria[1] as follows: stratification of epithelial cells (2-3 layers), apparent detachment of cellular clusters from their sites of origin, and mitotic activity (more than $^2/_{10}$ high-power fields) and nuclear abnormalities without obvious invasion of the adjacent stroma. Several tissues, including normal kidney, liver, colon, and ovary, as well as carcinomas of the ovary (3 samples), stomach (2 samples), and breast (1 sample), were snap-frozen in liquid nitrogen and stored at −80 °C. Formalin-fixed, dewaxed 5-μm-thick sections were stained with avidin-biotinylated enzyme complex (ABC) method (Vector, Burlingame, CA) using MAb 12C3 according to the method previously described.[6] The reaction was visualized using 3,3'-diaminobenzidine 4HCl (DAB) as the substrate. As a negative control, preimmune mouse serum (diluted 100-fold with 1% BSA in TBS) was used instead of MAb. Serial sections of borderline malignancies (23 cases) were stained with the same method using MAb OC125[5] (MAb against CA125, diluted 200-fold with 1% BSA in TBS [Centocor, Malvern, PA]). To confirm the specificity of immunohistochemical staining, acetone-fixed cryostat sections were also stained by the ABC method.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDSPAGE) and Western Blot Analysis

All procedures were carried out as previously reported.[6] Briefly, SDSPAGE was performed by Laemmli's system[25] using 10% acrylamide or 3% to 30% acrylamide gradient gels under reducing conditions. Western blot analysis was performed by the method of Towbin and colleagues.[26] The blotted nitrocellulose paper was incubated first with MAb 12C3 and then with HRP-conjugated goat anti-mouse Ig antibody (1:1000). After the blot was sufficiently washed with 0.05% Tween 20 containing TBS (T-TBS), the bands were detected by exposing the membrane to 200 μg/mL of DAB and 0.01% H_2O_2. The molecular size markers were obtained from Amersham Japan (Rainbow prestained marker; Amersham, Tokyo, Japan).

Gel Filtration

Superose 12, HR 10/30, column in FPLC system (Pharmacia, Uppsala, Sweden) equilibrated with TBS was used. The cell extract was prepared as follows: JOHYC-2 cells (10% weight/volume) suspended in 10 mM Tris HCl, pH 7.2, 0.15 M NaCl, 0.02% NaN_3, 1 mM phenylmethylsulphonylfluoride, and 0.5% Nonidet P-40, were sonicated, incubated for 20 minute on ice, and centrifuged for 20 minutes at 23,000 g and 0 °C. The supernatant was collected and used as the cell extract. Protein concentrations were assayed using a Bio-Rad protein assay kit (Bio-Rad, Richmond, CA). Antigen distributions were determined by Western blot analysis of each eluted fraction using MAb 12C3 as the first antibody. The intensity of the reactive bands was determined with a densitometer (Flying-Spot CS-9000, Shimadzu, Tokyo, Japan) at 492 nm. The molecular

TABLE 2. IMMUNOREACTIVITY OF MONOCLONAL ANTIBODY 12C3 WITH EPITHELIAL OVARIAN TUMORS

Tumors	*Reactivity**	*Intensity†* 1+	2+	3+	%
Malignant	21/31	5	10	6	67.7
Serous	6/12	2	2	2	50.0
Mucinous	5/7	1	3	1	71.4
Clear cell	7/9	1	3	3	77.8
Endometrioid	3/3	1	2	0	100
Benign	0/30				0
Serous	0/9				0
Mucinous	0/21				0
Borderline malignancy	14/25	6	8	0	56.0
Serous	4/10	2	2	0	40.0
Mucinous	10/15	4	6	0	66.7

* Number of positive cases/total number of cases tested.
† Staining intensity: 1+ = a few cells were positive; 2+ = less than half of the cells were positive; 3+ = more than half of the cells were positive.

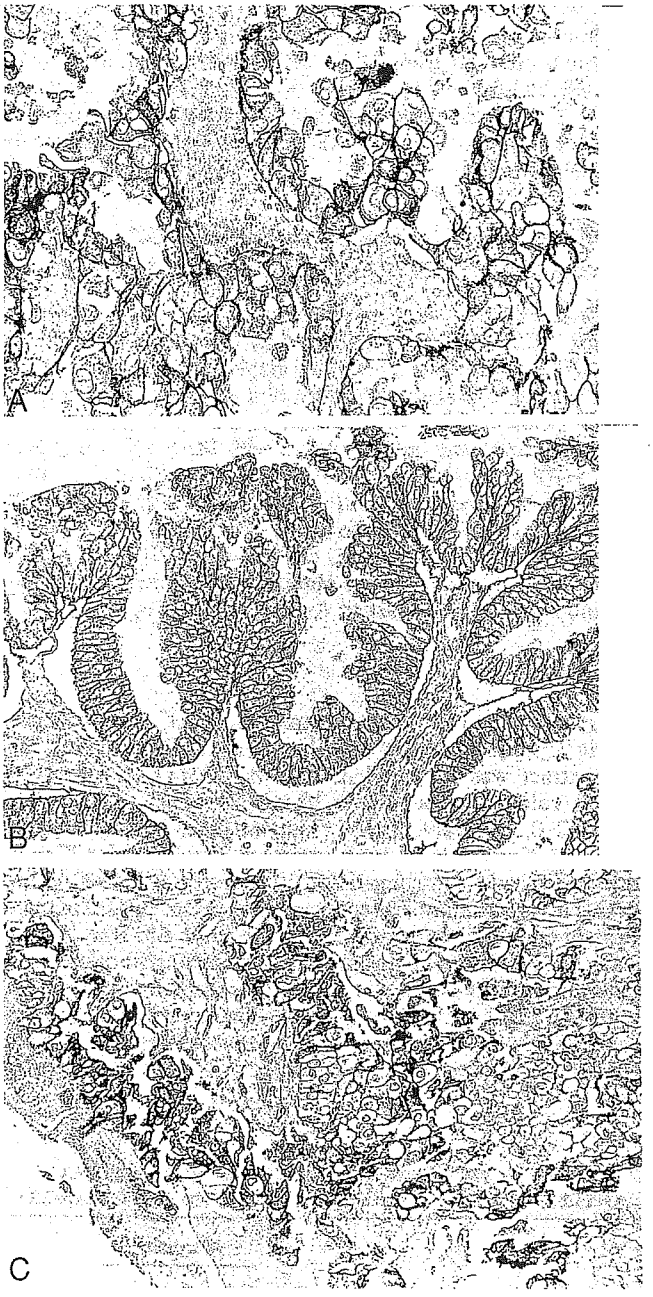

FIG. 1. Immunoperoxidase staining of serous (A) mucinous (B), and clear cell (C) carcinoma of the ovary with MAb 12C3 showing the positive staining of the cytoplasmic membranes.

size markers used were blue dextran, horse IgG, and BSA (Pharmacia, Piscataway, NJ).

Enzyme and Reagent Treatment

All treatments with enzymes and reagents were performed in Eppendorf microtubes. Each tube contained 30 mg of packed JOHYC-2 cells (approximately 5×10^6 cells) that had been detached from a culture dish by treatment with 0.02% ethylenediaminetetraacetate. The cells in these tubes were treated with either enzymes or reagents. For $NaIO_4$ treatment, the packed cells were treated with 15 mM $NaIO_4$ in a 10-mM acetate buffer, pH 4.5, for 15 hours at room temperature in the dark. For neuraminidase digestion, the cells were incubated with 0.1 U/mL of neuraminidase (*Streptococcus* sp. IID 6646, Seikagaku Kogyo, Tokyo, Japan) in a 10 mM-acetate buffer, pH 6.5, containing 10 mM of $CaCl_2$ for 2 hours at 37 °C. For trypsin digestion, the cells were incubated for 4 hours at 37 °C with 0.125% trypsin (Difco) in PBS. Proteinase K (1 mg/mL in PBS [Boehringer Mannheim, Penzberg, Germany]) digestion was performed for 1 hour of incubation with the cells at 37 °C. Control incubations were also carried out in the same buffer under the same conditions without the addition of any enzymes or reagents. The reaction was terminated by the addition of SDS containing PAGE sample buffer followed by 5 minutes of incubation at 100 °C. After centrifugation, the supernatants were analyzed by gradient gel SDSPAGE and Western blot, as previously described.

Purification and Biotinylation of MAb 12C3

The ascites fluid was dialyzed against 0.1 M Tris-HCl, pH 7.4, and applied to a DEAE cellulose column (Whatman, Maidstone, Kent, England) equilibrated with the same buffer. Flowed-through fractions containing Ig were collected and precipitated by the addition of an equal volume of saturated ammonium sulfate. The precipitate was dissolved and dialyzed against TBS. One mL of purified Ig (1 mg/mL) in 0.1 M $NaHCO_3$ was mixed with 100 μL of N-hydroxysuccinimidobiotin (Pierce, Rockford, IL) dissolved in dimethylsulfoxide (1 mg/mL). The mixture was incubated for 4 hours at room temperature, then dialyzed extensively against TBS overnight at 4 °C.

Competitive ELISA to Determine Cross-Reactivity Between MAb 12C3 and Well-Known Tumor Markers

Each antibody (15 μg/50 μL) against carcinoembryonic antigen (CEA),[7] CA125,[5] CA19-9,[8] CA72-4,[10-13] and ABH blood group antigens or MAb 12C3 (50 μg/50 μL) was added to JO-

FIG. 2. Immunoperoxidase staining of a serous ovarian tumor with borderline malignancy using MAb 12C3. Positive reactions were observed only in the cells of the suspected malignant lesion (Bottom right).

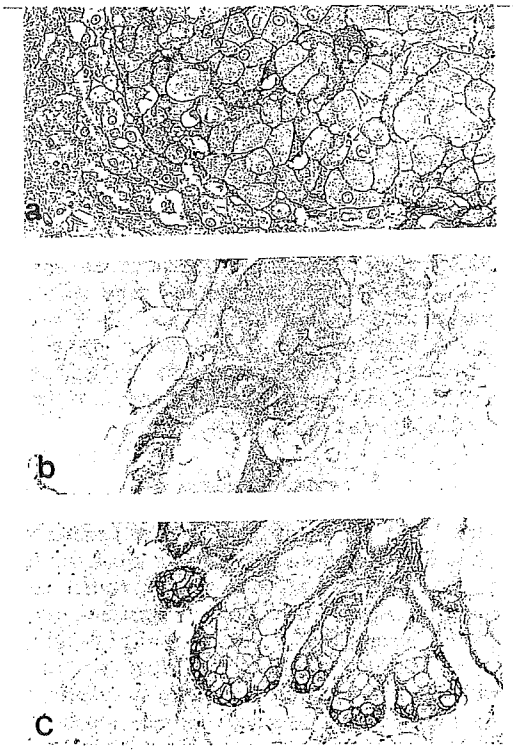

FIG. 3. Immunoperoxidase staining of normal tissue sections, corpus luteum (a), submandibular gland (b), and sebaceous gland (c) with MAb 12C3, showing strong reactivity with sebaceous gland and moderate to weak reactivity with corpus luteum and submandibular gland.

HYC-2-coated wells and incubated for 2 hours at 37 °C. After washing the wells, 50 μL of biotinylated MAb 12C3 (1 μg/mL in T-TBS) was added and incubated for 2 hours at 37 °C. After washing, 50 μL of ABC complex was added to each well and incubated for another 30 minutes at room temperature. The color was developed and determined according to the method previously described.

Type of Immunoglobin (Ig)

The subclass of MAb was determined by the Ouchterlony test with rabbit IgG against different mouse Ig subclass (ICN Biomedicals, Lisle, IL).

RESULTS

Establishment of MAb 12C3 and Reactivity Using Immunohistochemistry

The supernatant solutions from the 150 wells containing growing hybridomas were initially screened by cell-ELISA, followed by immunohistochemical examination. One hybridoma, MAb 12C3 (IgG1, κ), was selected and cloned. In the immunohistochemical staining using MAb 12C3, the localization of the defined antigen on the paraffin-embedded sections was the same as that on the frozen sections. The germ cell tumors of the ovary were stained weakly but positively with MAb 12C3 in one of four cases of dysgerminoma and none of four cases of yolk sac tumor (data not shown). As shown in Table 1, MAb 12C3 reacted with 21 of 31 ovarian carcinomas (67.7%), but with only 2 of 46 cases of the other types of carcinomas tested (4.3%). The reactivity of the antibody against epithelial ovarian tumors is presented in detail (Table 2). MAb 12C3 reacted strongly in 21 of 31 cases (67.7%) of ovarian carcinomas (serous: 6 of 12, or 50.0%; mucinous: 5 of 7 or 71.4%; clear cell: 7 of 9 or 77.8%; endometrioid: 3 of 3 or 100%), but did not react with serous (0 of 9 cases) and mucinous (0 of 21 cases) adenomas, nor with adjacent nonmalignant tissues. Ovarian carcinomas were positively stained mainly on the cell surfaces, but not in the cytoplasm (Figs. 1A, 1B, 1C). In the 25 cases of borderline malignancy, MAb 12C3 reacted with 14 specimens (56.0%). The positive staining reactions were localized on the cell membrane in the suspected malignant lesions that showed marked projection into the lumen without invasion. There was no reactivity in the adjacent adenomatous tissues (Fig. 2). In normal adult tissues, MAb 12C3 reacted only with three tissues in the same manner as it reacted with malignant cells: corpora lutein cells (7 of 8 cases); some epithelial cells of the secretory ducts of the submandibular gland (1 of 1 case); and basal cells of the sebaceous glands (2 of 2 cases) (Figs. 3A, 3B, 3C, Table 3). MAb 12C3 exhibited no reactivity with any other normal tissues tested. The differences in reactivity between MAb 12C3 and MAb OC125 with benign and suspected malignant cells of borderline malignancy are summarized in Table 4. Also included in Table 4 is a direct comparison with serial sections. The antigen detected by MAb 12C3 was expressed strictly in the suspected malignant lesions in 14 of 25 cases (56.0%), but in none of the 25 cases (0%) in adjacent benign tissues. By contrast, MAb OC125 labeled not only 13 of the 23 suspected malignant lesions (56.5%), but also 11 of 23 adjacent benign tissues (47.8%).

TABLE 3. IMMUNOREACTIVITY OF MONOCLONAL ANTIBODY 12C3 WITH NORMAL TISSUES

Tissue	*Specimens Stained/Total (n)*
Esophagus	0/2
Stomach	0/3
Lung	0/3
Small intestine	0/2
Kidney	0/3
Urinary bladder	0/1
Colon	0/2
Liver	0/3
Gall bladder	0/1
Pancreas	0/2
Spleen	0/1
Thyroid gland	0/1
Parotid gland	0/1
Submandibular gland	1/1*
Cardiac muscle	0/2
Skin	2/2†
Adrenal gland	0/1
Uterine cervix	0/2
Fallopian tube	0/1
Ovary	7/8‡

* Some lining cells of secretory ducts.
† Basal cells of sebaceous gland.
‡ Corpora lutein cells.

TABLE 4. DIFFERENCE IN REACTIVITY BETWEEN MONOCLONAL ANTIBODY 12C3 AND A MONOCLONAL ANTIBODY SPECIFIC TO CA125 AGAINST BORDERLINE TUMOR CELLS

Monoclonal Antibody	*Lesion**	*Specimens Positively Stained/Total (%)*
12C3	Benign	0/25 (0)
	Malignancy suspected	14/25 (56.0)
OC125	Benign	11/23† (47.8)
	Malignancy suspected	13/23 (56.5)

* MAb reactions to benign or suspected malignant lesions in the same specimens with a direct comparison with serial sections.

† Two cases were not examined using the monoclonal antibody specific to CA125.

Biochemical Properties of MAb 12C3 Antigen

Figure 4 shows the Western blot analysis of cell extracts of JOHYC-2. MAb 12C3 detected several bands with an apparent molecular mass of 48-55 kDa and one band of 35 kDa. By contrast, Lane 2 displayed no reactive band recognized by CA72-4 as a negative control (Fig. 4).By gel filtration analysis of the extract from JOHYC-2 cells, the MAb 12C3-reactive bands found by Western blot were detected at fractions eluting just between the elution volume of IgG and void, and the native molecular mass was estimated to be 200 kDa (Fig. 5). By contrast, the antibody did not react with the eluate from spent culture media from JOHYC-2 nor with the serum from a patient with ovarian carcinoma. The antibody also did not react with the tissue sections that were positively stained by MAb 12C3 (one case of clear cell carcinoma in Table 2). This result indicated that the MAb 12C3 antigen is not a shed antigen.

The effect of the treatment with chemical reagents or enzymes on the antigen-MAb 12C3 reaction is shown in Fig. 6. After treatment with $NaIO_4$, the MAb 12C3-reactive bands decreased in intensity. In contrast, after neuraminidase treatment, these bands maintained their reactivity with slightly increased migration. After treatment with proteinase K and trypsin, the MAb 12C3-defined antigen exhibited extremely reduced antigenicity and MAb 12C3 recognized weak, but detectable reactive bands (probably smaller peptides with antigenicity) with molecular masses of 31 kDa and 24 kDa, respectively. The results indicated that the antigenic sites recognized by MAb 12C3 must reside in a glycoprotein. No cross-reactivity of MAb 12C3 with ABH antigens and other well-recognized tumor marker antigens, such as CA125, CA19-9, and CA72-4, was observed. Preincubation of the purified MAb 12C3 with JOHYC-2 coated on the microtiter wells showed complete absorption of the ELISA signal using biotinylated MAb 12C3 as a detector antibody (data not shown).

DISCUSSION

Although MAb 12C3 was generated by immunizing mice with a cell line derived from a human germ cell tumor, the antibody reacted with human ovarian carcinomas rather than with germ cell tumors. When tested on paraffin-embedded tissues, MAb 12C3 stained 67.7% of ovarian epithelial malignancies, but exhibited an extremely low frequency of reactivity (4.3%) with other gynecologic and nongynecologic malignancies. MAb 12C3 showed no reactivity with ovarian adenomas of any of the histologic types tested. The epitope was also expressed in only three normal tissues: (1) in some lining cells of the secretory duct of the submandibular gland; (2) in the basal cells of the sebaceous glands; and (3) in the corpora lutein cells. No report has demonstrated that the MAbs generated against ovarian carcinoma react with corpora lutein cells. Because of the extremely low reactivity with ovarian adenomas, we studied whether MAb 12C3 might identify early histologic malignant transformation of ovarian tumors, especially in cases of borderline malignancy. Interestingly, in 56% of the specimens of borderline malignancy, MAb 12C3 reacted exclusively with the cells of the suspected malignant foci, which were papillary

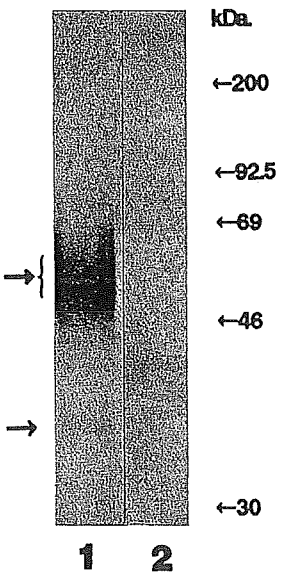

FIG. 4. Western blot analysis of MAb 12C3 and MAb CA72-4 against JOHYC-2 cell extract separated by SDSPAGE. Lane 1: reactive band (arrows) with MAb 12C3; Lane 2: no reactive band with MAb CA72-4.

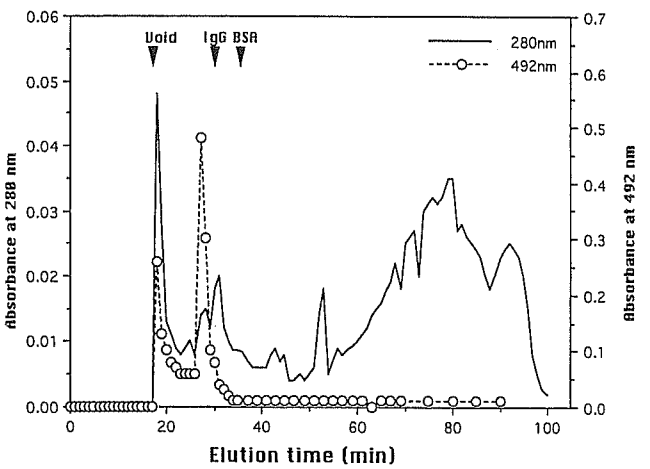

FIG. 5. FPLC-gel filtration elution profile. The MAb 12C3 antigen content in each fraction was determined by Western blot followed by densitometric analysis as described in Materials and Methods section.

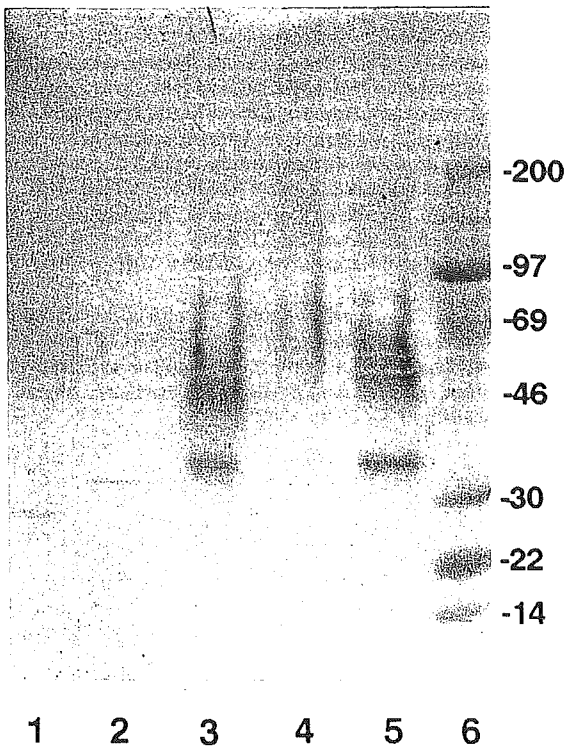

FIG. 6. Treatment of MAb 12C3 antigen with either enzymes or chemical reagents. The treated antigen was analyzed using SDSPAGE-Western blot analysis. Lane 1: trypsin; Lane 2: proteinase K; Lane 3: neuraminidase; Lane 4: $NaIO_4$; Lane 5: PBS as control; Lane 6: size marker in kDa.

cells protruding into the lumen that exhibited moderate nuclear atypia. The follow-up clinical data as well as the prognosis of these cases will be investigated further to determine whether the MAb 12C3-reactive cases have greater malignant potential.

In comparative immunohistologic examinations of serial tissue sections, there was a slightly lower frequency of reactivity of MAb 12C3 with the suspected malignant lesions. There was a higher accuracy and specificity than that of MAb OC125. Unlike the shed antigens, such as CA125,[5] CA72-4,[10-13] or CEA,[7] the antigen defined by MAb 12C3 was only cell-associated because it has not yet been found in spent culture medium from JOHYC-2 nor in the serum of ovarian carcinoma patients. The antigen recognized by MAb 12C3 was a glycoprotein with a molecular mass of 200 kDa consisting of subunit components. Competitive inhibition ELISA, Western blot analysis, and immunohistochemical distribution of the recognized antigen ruled out the possibility of cross-reactivity of MAb 12C3 with CA125,[5] CA72-4,[10-13] CA19-9,[8] and CEA[7] as well as with the ABH antigens.

These results indicate that MAb 12C3 detects a novel antigen whose distribution in normal tissue is quite restricted. In ovarian epithelial tumors, the expression of the epitope defined by MAb 12C3 increases with the level of morphologic atypia. MAb 12C3 will allow the biologic significance of the expression of this antigen to be investigated during malignant transformation, and will serve as a powerful new tool for the histologic detection of early malignant changes in borderline epithelial neoplasms. MAb 12C3 may also be useful as a targeting agent for cancer chemotherapy because the MAb 12C3-defined antigen is not a shed moiety. Further investigations are necessary to elucidate the true relation between the survival rate of the patients and the frequency of MAb 12C3 antigen expression in borderline malignant ovarian tumors.

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CD147 and Matrix Metalloproteinase-2 Protein Expression as Significant Prognostic Factors in Esophageal Squamous Cell Carcinoma

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BACKGROUND. The authors investigated whether the presence of matrix metalloproteinase-2 (MMP-2) and its inducer, CD147, in cancerous esophageal lesions and surrounding tissue might help to predict patient prognosis.

METHODS. Tissue samples from 101 patients with esophageal squamous cell carcinoma were stained with anti-CD147 and anti–MMP-2 antibodies for immunohistochemical analysis.

RESULTS. CD147 was expressed in cancerous and dysplastic lesions, but not in normal tissue. In contrast, MMP-2 was detected mainly in normal interstitial tissue adjacent to cancerous lesions, but it was detected also in cancerous lesions in some patients. Pathologic findings demonstrated that the intensity of MMP-2 staining in normal tissue was associated positively with the depth of tumor infiltration and the stage of disease, whereas MMP-2 staining in cancerous tissue was associated positively with vascular and lymphatic vessel invasion as well as with immature differentiation of cancer cells. Using a proportional hazard model, including information on CD147 staining patterns within cancerous lesions along with clinical cancer staging, improved the accuracy of predicting patient prognosis.

CONCLUSIONS. These results suggested that measurement of CD147 and MMP-2 expression with simple immunohistochemical staining may enhance further the understanding of the pathophysiology of invading tumor cells and, when used in combination with cancer staging, may increase the ability of investigators to predict prognosis in patients with esophageal squamous cell carcinoma. *Cancer* **2004;101:1994–2000.** © *2004 American Cancer Society.*

KEYWORDS: CD147, metalloproteinase, esophagus, cancer, prognosis.

Interactions between cancer cells and the surrounding microenvironment may allow tumor invasion into adjacent organs and trigger metastasis via vascular/lymphatic vessels.[1] Results of investigations into tumor biology can contribute to the development of novel promising therapies, such as protease inhibitors for myeloma treatment.[2] CD147, also known as extracellular matrix (ECM) metalloproteinase inducer (EMMPRIN) or basigin, is highly expressed on the outer surface of carcinoma cells, but not on normal mucosal cells. CD147 stimulates adjacent interstitial normal cells to produce matrix metalloproteinases (MMPs).[3] MMPs are proteases known to degrade the ECM.[4] Thus, carcinoma cells can interact with adjacent normal cells to produce MMPs via CD147 on their surface, and, in turn, invade lymphatic tissue and blood vessels and penetrate through the ECM to adjacent organs with the help of MMPs.[5]

The roles of CD147 and MMPs in tumor invasiveness have been confirmed immunohistochemically in several types of cancer cells

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and surrounding tissue, including astrocytomas[6] and melanomas.[7] Moreover, the expression of MMPs is reported to correlate with the clinical prognosis of patients with breast carcinoma[8] and other types of cancers.[9–11]

Esophageal squamous cell carcinoma is an aggressive cancer with a poor prognosis. The median survival ranges from 1 to 2 years even with chemoradiotherapy before surgery,[12,13] although survival has continued to improve.[14] Currently, clinical TNM staging before treatment is the best way to predict the prognosis of patients with esophageal carcinoma.[15] However, the combination of immunohistochemical examinations with ordinal TNM staging has been reported to improve diagnostic accuracy in terms of predicting prognosis.[16,17] In addition, recent progress in molecular diagnostics using gene expression profiles may help to predict the prognosis of patients with esophageal carcinoma[18] and other types of cancer.[19,20]

In the current study, CD147 and MMP protein expression patterns within esophageal cancer cells and surrounding tissue were examined immunohistochemically to determine their relation to clinicopathologic findings and disease recurrence-free survival of patients.

MATERIALS AND METHODS

Patients

Between February 1994 and December 2001, 117 patients with esophageal tumors were treated at Jikei University Hospital (Tokyo, Japan). Of the 117 patients, 16 were excluded (4 patients did not have paraffin-embedded specimens available, 6 patients displayed adenocarcinoma, and 6 patients had carcinoma in situ [CIS]). Therefore, 101 patients for whom specimens and clinical information could be obtained were included in the current study.

Clinical information was abstracted from surgical and clinical charts. Some patients received radiotherapy to improve quality of life. Tumor stages were classified according to the 5th edition of the TNM classification system.[21] Patients were periodically (every 1–3 months) examined on an outpatient basis to make sure they did not have disease recurrence. Examinations consisted of standard tests, including endoscopy and computed tomography scans of the chest and abdomen.

Pathologic Specimens

Specimens were obtained by endoscopic mucosal resection or surgery. Formalin-fixed, paraffin-embedded specimens of esophageal tumors were processed for conventional histologic assessment by hematoxylin and eosin (H & E) staining. Only patients with squamous cell carcinoma of the esophagus confirmed by two or more board-certified pathologists were included. Patients with all other forms of carcinoma, including partly adenomatous lesions, were excluded. All specimens were free of cancer invasion of the tumor margin and patients with CIS were excluded. Histologic features of the extent of the lesions, invasion into lymphatic or blood vessels, intramural metastasis, and lymph node metastasis were evaluated. Pathologic diagnosis and classification of specimens were accomplished using the guidelines of the Japanese Society for Esophageal Diseases.[22] Lymphatic vessel invasion was determined to be definite when cancer cells were detected in thin-walled, endothelium-lined spaces containing no red blood cells (RBC) and occasional lymph fluid. Similarly, blood vessel invasion was defined by the presence of cancer cells and RBC within round or ovoid endothelium-lined spaces surrounded by a layer of smooth muscle.[23–25] Intramural metastasis was defined as evidence of tumor within the esophageal wall, not directly related to the primary tumor.[26,27] Histologic grades of differentiation were assigned according to whether a tumor was well, moderately, or poorly differentiated.[28]

Establishment of Murine Anti-CD147 Antibody

A murine monoclonal antibody (MoAb 12C3) that is specific to human ovarian carcinoma was generated by immunizing mice with a human ovarian germinoma cell line (JOHYC-2).[29] To recognize the antigen epitope defined by MoAb 12C3, a cDNA library was constructed from the SKOV3 human ovarian carcinoma cell line using a T7Select 10-3 vector (Novagen, Madison, WI). The phages were bound to microplate wells (Immulon-2, Dynatec Laboratory, Chantilly, VA), which were immobilized with MoAb 12C3, and were selected from either the SKOV3 cDNA or the colon carcinoma cDNA library (Novagen) by the panning method. Furthermore, a single-phage clone was purified by immunostaining with MoAb 12C3 from the SKOV3 and colon carcinoma cDNA libraries. These single-phase clones were termed SKOV3-1 and Colon-1, respectively. Then, the DNA sequences of the phage selected were determined by dideoxynucleotide chain termination. A homology search using amino acid sequence with the BLAST program was performed and revealed that SKOV3-1 and Colon-1 matched with the 17th to 174th and the 17th to 149th amino acid residues, respectively, which were on the extracellular region of CD147 (Fig. 1).

Immunohistochemical Staining for CD147 and MMP-2

Using paraffin-embedded specimens from patients with esophageal squamous cell carcinoma, CD147

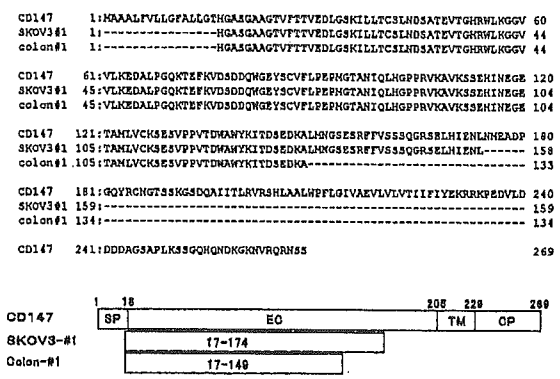

FIGURE 1. Single-phage clone purified by immunostaining with monoclonal antibody 12C3 from the SKOV3 and colon carcinoma cDNA libraries, termed SKOV3-1 and Colon-1, respectively. A homology search using an amino acid sequence with the BLAST program was performed and revealed that SKOV3-1 and Colon-1 matched the 17th to the 174th and the 17th to the 149th amino acid residues, respectively, which were on the extracellular region of CD147. SP: signal peptide region; EC: extracellular region; TM: transmembrane region; CP: cytoplasmic region. The numbers within the rectangles indicate amino acid residues encoded by isolated clones.

protein was detected using the anti-CD147 murine MoAb using a dextran polymer conjugate two-step visualization system.[30] Using different slices from the same paraffin-embedded specimens, MMP-2 protein was stained with anti-human MMP-2 murine MoAb and with purified immunoglbulin G (F68; Daiichi Fine Chemical Co., Ltd., Toyama, Japan).[31] Results of staining for CD147 in both cancerous and dysplastic lesions were classified into the following three patterns: no staining, partial staining, and diffuse and strong staining. Results of staining for MMP-2 both in normal tissue adjacent to cancerous lesions and in cancerous lesions were classified into just negative or positive. The examiners were blinded to patients' clinicohistologic (H & E staining) information when assigning staining patterns as positive/negative. Two investigators (Y.I. and M.U.) evaluated the staining levels independently, after which discordant evaluations were adjusted by connected microscopes.

Statistical Analysis

Kappa statistical analyses were applied to measure agreements of CD147 immunohistochemical staining between dysplastic and cancerous lesions. Chi-square tests and simple linear regression analysis were used to evaluate the relation between immunohistochemical staining patterns and several clinicopathologic parameters. Survival curves of the patients were compared using the Kaplan–Meier method and analyzed by the log-rank test. Cox proportional hazards models were fitted for multivariate analysis.

RESULTS

Patients' age ranged from 43 to 83 years (mean ± standard deviation: 61 ± 9 years) and there were more men ($n = 69$) than women ($n = 32$). All patients were followed from Day 16 after surgery to a maximum of 3175 days (median, 981 days). Forty-seven patients died of esophageal carcinoma and six of other causes. Thus, 48 patients were alive and 53 had died. Three patients were lost to follow-up and included in the current study as censored on the final day in the outpatient clinic.

Immunohistochemistry for CD147 and MMP-2 Protein Expression Patterns in Patients with Esophageal Lesions

Typical histologic pictures of CD147 staining of cancerous and dysplastic tissue are shown in Figure 2A–C. CD147 was enriched on the surface of cancer cells. Forty-six cancerous lesions stained with the CD147 antibody showed a diffuse and strong pattern, whereas 24 showed a partial pattern and 31 showed no staining. Conversely, in dysplastic tissue, staining with the CD147 antibody was negative in 45 lesions, partial in 38 lesions, and diffuse and strong in 18 lesions. In 45 specimens, CD147 expression patterns were matched between cancerous and dysplastic lesions (chi-square test: $P < 0.001$). The other 56 specimens did not exhibit any matching between cancerous and dysplastic lesions (kappa = 0.20, 95% confidence interval, 0.08–0.32). Neither normal esophageal mucosal tissue nor submucosal interstitial cells were stained with the anti-CD147 antibody.

Submucosal interstitial cells around cancerous lesions expressed MMP-2 protein (Fig. 2D). The frequency for each staining was as follows: negative, 24 specimens; positive, 77 specimens. In contrast, submucosal interstitial cells distant from cancerous tissue were not stained with the anti–MMP-2 antibody. Moreover, cancerous tissue was stained with the anti–MMP-2 antibody in 25 patients (Fig. 2E), which showed also positive MMP-2 staining in interstitial cells. However, the expression of CD147 in cancerous or dysplastic lesions had no association with the expression of MMP-2 on submucosal or cancerous lesions.

CD147 and MMP-2 Expression Patterns and Clinicopathologic Variables

CD147 protein expression patterns within cancerous and dysplastic lesions were not associated with any of the following variables: TNM classification, stage, extent of the lesions, invasion into lymphatic or blood vessels, intramural metastasis, or histologic grades of differentiation.

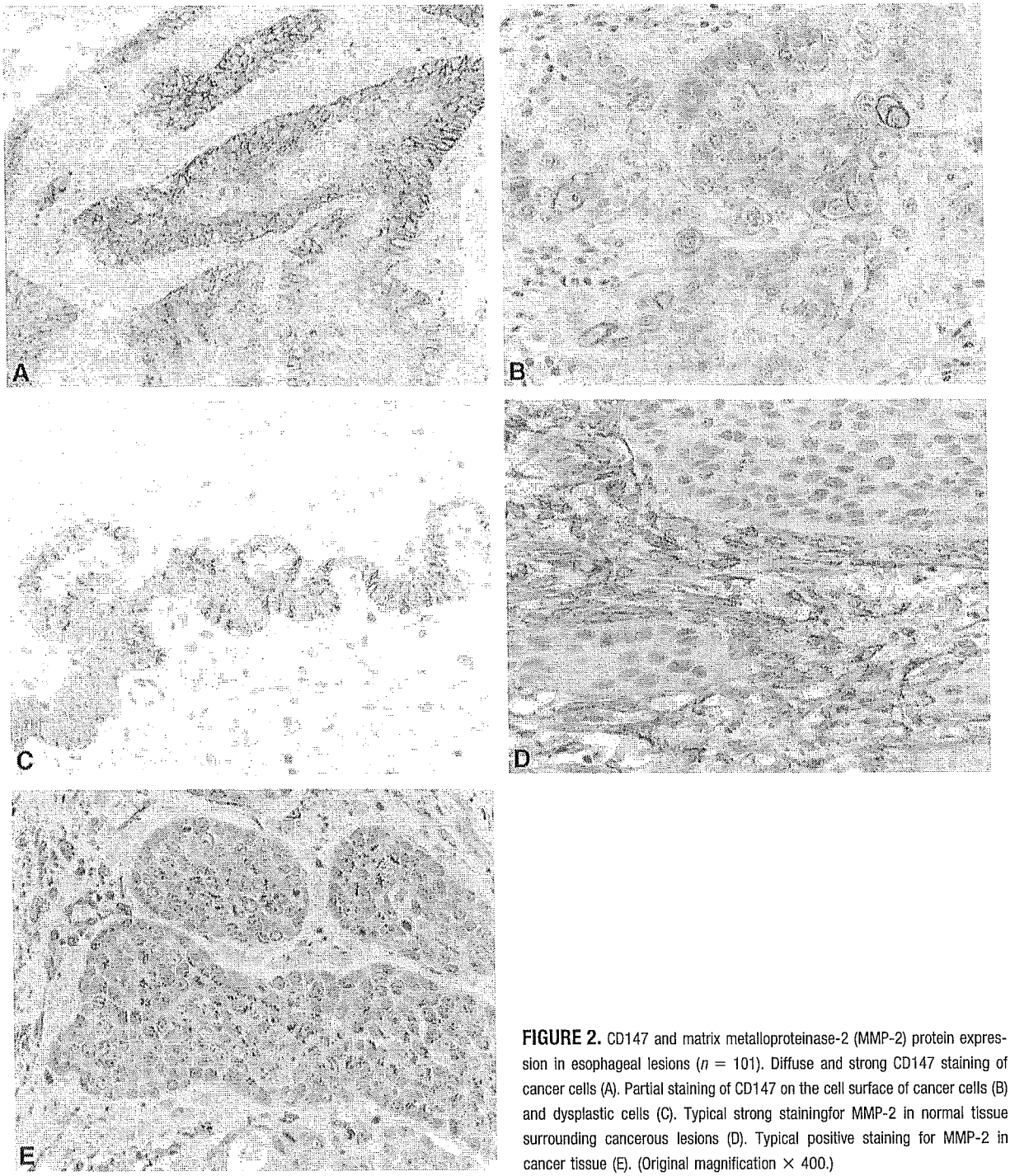

FIGURE 2. CD147 and matrix metalloproteinase-2 (MMP-2) protein expression in esophageal lesions (n = 101). Diffuse and strong CD147 staining of cancer cells (A). Partial staining of CD147 on the cell surface of cancer cells (B) and dysplastic cells (C). Typical strong stainingfor MMP-2 in normal tissue surrounding cancerous lesions (D). Typical positive staining for MMP-2 in cancer tissue (E). (Original magnification × 400.)

The presence of MMP-2 protein within the normal tissue around cancerous lesions was associated positively with tumor infiltration in the TNM classification system (chi-square test: P = 0.018) as well as with stage of cancer (P = 0.04), but not with lymph node metastasis, distant metastasis, extent of the lesions, invasion into lymphatic or blood vessels, intramural metastasis, or histologic grades of differentiation. In contrast, staining of MMP-2 protein within cancerous lesions was associated positively with invasion into

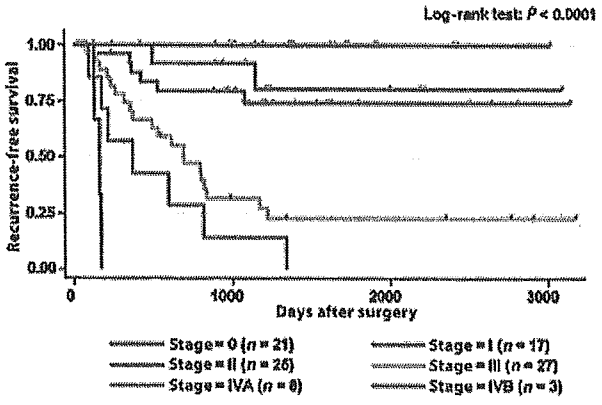

FIGURE 3. Stage (0, I, II, III, IVA, IVB) and disease recurrence-free survival using the Kaplan–Meier method (n = 101).

lymphatic tissue (P = 0.005), as well as with blood vessels (P = 0.023) and immature differentiation (P = 0.003) of histopathology.

CD147 and MMP-2 Expression Patterns and Disease Recurrence-Free Survival

Kaplan–Meier survival curves were first created based on tumor stages (0, n = 21; I, n = 17; II, n = 25; III, n = 27; IVA, n = 8; and IVB, n = 3) for the 101 patients (Fig. 3). As expected, patients with more advanced stages of cancer had shorter median disease recurrence-free survival periods: Stage III, 697 days; Stage IVA, 373 days; Stage IVB, 162 days (log-rank test: P < 0.0001).

According to the pattern of CD147 expression noted among cancerous and dysplastic cells, Kaplan–Meier survival curves were compared using the log-rank test. Neither cancerous nor dysplastic tissue expression of CD147 was associated with disease recurrence-free survival without adjusting for stages.

MMP-2 protein expression within normal tissue reduced disease recurrence-free survival significantly (P = 0.022) in patients with Stage IVA and IVB disease (Fig. 4A), but not in patients with Stage III and lower stages of disease (Fig. 4B). The presence of MMP-2 protein within cancerous tissue had no significant effect on disease recurrence-free survival, regardless of cancer stage.

The Cox Hazard Model

Cox regression analysis was performed to determine if the studied prognostic significance of CD147 and/or MMP-2 expression improved the accuracy of staging in predicting disease recurrence-free survival. Including data on CD147 expression in cancerous lesions significantly improved the model of ordinal staging

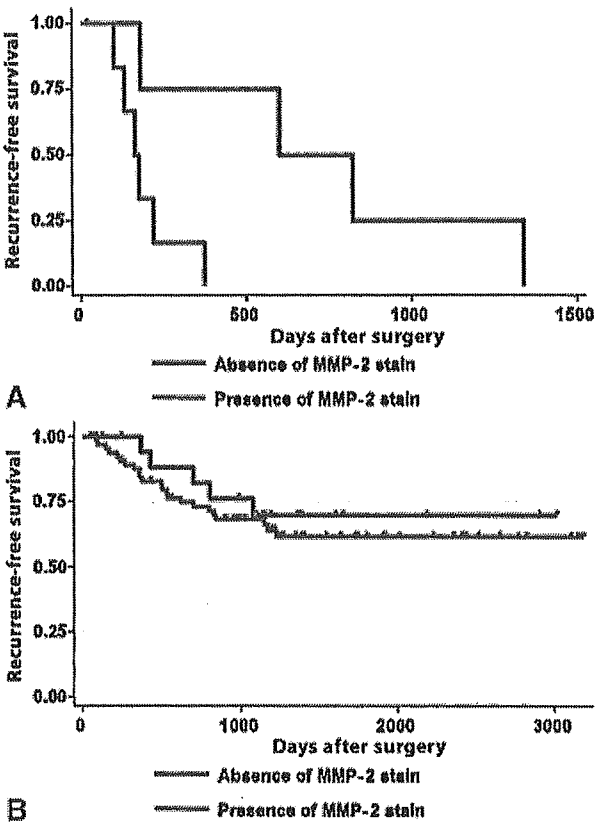

FIGURE 4. Matrix metalloproteinase-2 expression (presence/absence) in normal tissue adjacent to cancerous lesions and disease recurrence-free survival using the Kaplan–Meier method (n = 101). (A) Patients were restricted to Stage IVA and IVB disease. (B) Patients were restricted to Stage 0, I, II, and III disease.

TABLE 1
Cox Regression Analysis of CD147 Expression Levels in Cancerous Tissue Specimens Adjusted for Various Histologic Findings

Variables	Hazard ratio	95% Confidence interval	P value
Stage 0 and Stage I	1		
Stage II	5.2	1.0–25.9	0.041
Stage III	30.0	6.5–138	< 0.001
Stage IVA and Stage IVB	109.5	20.3–590	< 0.001
CD147 no staining	1		
CD147 partial staining	4.6	1.55–13.4	0.006
CD147 diffuse and strong staining	2.2	0.96–5.09	0.063

alone (log-likelihood test: P = 0.019; Table 1). However, including data on MMP-2 expression in normal and/or cancerous tissue with disease stage, or including cancer stage plus CD147 expression data, did not alter the accuracy of the models.

DISCUSSION

In the current study, we determined protein expression of CD147 and MMP-2 in cancerous and surrounding lesions with immunohistochemical staining, and explored the association of these protein expressions with clinicopathologic findings and disease recurrence-free survival in 101 patients with esophageal squamous cell carcinoma. CD147 protein was detected mainly in cancerous lesions and in some dysplastic lesions, but not in normal tissue. In contrast, MMP-2 protein was found in normal interstitial cells adjacent to cancer lesions but not in normal tissue far from cancer lesions. These findings are consistent with previous reports that CD147 expression on cancer cells stimulates surrounding normal tissue to produce MMP-2.[5–7] However, the intensity of the expression of CD147 in cancer lesions was not associated with that of MMP-2 in surrounding normal tissue, suggesting that reactivity of normal tissue to CD147 on cancer cells may be different among individuals.

Importantly, advanced T stage in the TNM classification system, which represents the depth of tumor infiltration, was associated positively with expression levels of MMP-2 in normal tissue. Moreover, greater expression of MMP-2 in normal tissue in patients with advanced-stage cancer was significantly associated with poor prognosis. These results suggest that MMP-2 expressed in normal cells may facilitate infiltration of cancer cells, resulting in advancement of T stage and reduced disease recurrence-free survival in patients with Stage IV disease.

In the current study, MMP-2 was expressed not only in normal tissue but also in cancer cells in some tissue specimens. Moreover, MMP-2 expression in cancer cells was associated with lymphatic and vascular invasion and cellular immaturity. In advanced gastric carcinoma, MMP-2 immunostaining has been observed exclusively and correlated with vascular invasion by tumor cells.[32,33] Furthermore, CD147 was shown to interact not only with adjacent stromal cells but also with MMP-1 on tumor cells.[34] These findings suggest that cancer cells may invade lymphatic and blood vessels by secreting MMP-2 themselves.

CD147 and MMP-2 expression have been demonstrated to be associated with tumor infiltration and invasion into vessels, suggesting that blockade of these molecules may prolong disease recurrence-free survival by interfering with tumor infiltration and invasion. In clinical trials, MMP inhibitors may enhance the effect of antitumor agents,[35,36] although the usage of an MMP inhibitor as a single agent has not been shown to be effective.[37,38]

In conclusion, measurement of CD147 and MMP-2 expression with simple immunohistochemical staining may further enhance understanding of the pathophysiology of invading tumor cells and, when used in combination with cancer staging, may increase our ability to predict prognosis in patients with esophageal squamous cell carcinoma.

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Profiling Gene Expression Ratios of Paired Cancerous and Normal Tissue Predicts Relapse of Esophageal Squamous Cell Carcinoma

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ABSTRACT

Esophageal squamous cell carcinoma has heterogeneous clinical outcomes that cannot be predicted well using any existing clinical or molecular prognostic factors. Gene expression profiling may enable more precise prediction of the clinical outcome of these patients. We developed a new approach using gene expression ratios of paired cancerous and normal tissue specimens from the same patient to reduce the effects of variation among individuals. Using oligonucleotide microarrays, we analyzed total RNA expression levels corresponding to 12,600 transcript sequences in 24 paired cancerous and normal tissue operative specimens from 12 patients with esophageal squamous cell carcinoma. Hierarchical clustering analysis using gene expression ratios (cancer:normal) divided the 12 patients into two groups; all 7 patients in the first cluster survived without relapse (median follow-up, 483 days), whereas all 5 patients in the second cluster relapsed (median relapse-free survival time, 280 days; log-rank test, $P = 0.006$). In contrast, clustering either with cancerous tissue alone or with normal tissue alone did not show significant differences in the outcomes. The expressions of a variety of genes related with cell cycle, gene-repair, apoptosis and chemoradiotherpay resistance were up-regulated in the poor prognostic cluster. These results suggest that ratios of paired gene expression profiles may more efficiently predict relapse-free survival of esophageal squamous cell carcinoma than existing prognostic factors or than gene expression profiling with cancerous tissue alone.

INTRODUCTION

In general, squamous cell cancer in the esophagus is an aggressive tumor with poor prognosis, with or without chemoradiotherapy (1). However, the clinical outcomes of esophageal cancer are heterogeneous, and some patients not only with early stage but also with advanced stage cancer may survive longer than clinically expected, and *vice versa*. Yet, only the stage based on TNM classification is widely accepted as a prognostic factor at present (2), which is still far from an accurate predictor. Molecular analyses of esophageal cancer have largely focused on individual candidate genes, with particular emphasis on $p16^{INK4A}$ and *p53* (3), which are no better prognostic factors than existing clinical staging and TNM classification.

The recent development of microarray analysis provides the opportunity to take a genome-wide approach to predict clinical outcomes in a variety of cancers based on the molecular classification of a similar pathological group of cancers with different prognoses (4). However, unlike classical clinical studies, data on thousands of gene expression patterns from a much smaller number of samples must be examined. In addition, whether differential gene expression originates from variation among individuals or from clinical heterogeneity of a given cancer remains to be clarified.

We hypothesized that using the gene expression ratio of paired cancerous and normal tissue samples from the same patient for a given gene could reduce the effects of individuality and, consequently, increase the accuracy of predicting clinical outcomes compared with analysis of cancerous tissue alone.

MATERIALS AND METHODS

Patients. Paired esophageal cancer and normal tissue samples (total 24) were obtained from 12 patients with esophageal cancer after provision of written informed consent, and a protocol approved by the Ethics Committee for Biomedical Research of the Jikei Institutional Review Board, Jikei University School of Medicine, Japan. Eligibility requirements for this study included squamous cell carcinoma of the esophagus that was confirmed by more than two board pathologists and that was free of cancer invasion in the tumor margin and was clinically limited to the locoregional area (no distant metastasis in TNM classification; stage I to III). Tumor stage and grade were classified according to the fifth edition of the TNM classification of the International Union against Cancer (2). In the present study, primary tumors T_1 and $T_{2/3}$ in TNM classification were defined as early (E) and advanced (A), respectively. All patients had adequate general health including hepatic, renal, and bone marrow reserve, and could tolerate the planned surgical procedure. Patients were ineligible if they had Barrett's esophageal adenocarcinoma, stage IV based on TNM classification, presence of other primary cancers, inability of total resection, or prior chemotherapy and/or radiotherapy. On an outpatient basis, the 12 patients were periodically (1–4 months) examined to exclude relapse of the disease using standard examinations including blood examination of tumor markers, chest X-ray, endoscopy, and computed tomography of the chest and abdomen.

Samples. Each cancer tissue sample was divided into two specimens; one for pathological confirmation that the sample was composed of more than 80% cancer cells and the other for RNA extraction. Paired normal tissue was obtained from an area that was at least 4 cm away from any cancerous tissue. Normal tissues were also divided into two specimens: one for pathological confirmation that the sample did not contain either cancer cells or premalignant tissue morphologically. Total RNA was extracted using the RNeasy kit (Qiagen, Chuoh-ku, Tokyo, Japan), and biotinylated cRNAs were then generated. Samples were hybridized onto Affymetrix U95A Array oligonucleotide arrays (Affymetrix, Santa Clara, CA). Arrays were subsequently developed with phycoerythrin-conjugated streptavidin and were scanned to obtain quantitative gene expression levels. Paired normal and cancerous tissue specimens from each patient were handled simultaneously during RNA extraction and hybridization.

Statistics. Genes with expression levels ≤ 0 units in at least one sample were deleted, which resulted in a reduction in the number of genes from 12,626 to 5,843. Gene expression ratios of paired cancerous and normal tissues were simply calculated as unit levels of cancerous tissue (>0 units) divided by unit levels of cancerous tissue (>0 units), which were then transformed with log2:log(cancer/normal) = log(cancer) − log(normal). Next, these were normalized with the z value. Hierarchical clustering analysis was then performed using Spotfire software version 7.0 (Spotfire, Somerville, MA). Associations between categorical parameters were examined using Fisher's exact and χ^2 tests. The Kaplan-Meier product-limit method was used to estimate relapse-free survival. For comparison purposes, the Mantel log-rank test was per-

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formed. Differences between groups were considered significant if the P was <0.05.

RESULTS

Patient characteristics are shown in Table 1. Age ranged from 52 to 82 (mean, 66 ± 10) years, and the majority were males. Patients 8 and 9 were brothers. All of the patients underwent surgery between November 2, 2000, and January, 29, 2002, and were followed for 121–644 days.

The summary of raw data were as follows; mean of normal, 1016; quartile of normal, 140 (25)/312(50)/777(75); mean of cancer, 1102; quartile of cancer, 156(25)/369(50)/937(75). Correlation-of-law data between normal and cancer were 0.86 in 70,116 observations. SDs among 12 normal samples and 12 cancer samples in each gene were compared with show distributions of heterogeneity in normal and cancer expression profiles (Fig. 1). Before attempting to predict the clinical outcome by gene expression profiling, we examined whether hierarchical clustering can distinguish cancerous tissue from normal counterparts. Twenty-four samples containing 12 normal and 12 cancerous tissue samples were analyzed using hierarchical clustering, resulting in two major clusters: (*a*) 12 normal samples and 1 cancer and (*b*) 11 cancer samples. The profiles of the most prominent genes expressed at high levels in cancerous and normal tissue samples are shown in Fig. 2*A* and *B*, respectively. Of the 29 kinds of genes expressed at a higher level in cancerous than in normal tissue (similarity at 2.74 among the gene cluster), 19 (66%) kinds had been previously reported to associate with cellular proliferation and gene repair (Fig. 2*A*). In contrast, one-half of the 38 kinds of genes expressed at higher levels in normal than in cancer tissue (similarity at 2.41 among the gene cluster) have been associated with enzymatic proteins, and only two genes associated with cellular proliferation and gene repair were present (Fig. 2*B*).

Hierarchical clustering analyses were then performed for the 12 paired cancer:normal gene expression ratios, 12 cancerous tissue samples alone, and 12 normal tissue samples alone (Fig. 3), and compared with clinical information. Two distinct clusters were formed when the cancer:normal expression ratio was used (Fig. 3*A*); all of the patients in the first cluster (cluster I) survived without relapse (median follow-up, 483 days), whereas all patients included in the second cluster (cluster II) relapsed (median relapse-free survival time, 280 days; $P = 0.0013$). In contrast, hierarchical clustering using either cancerous tissue alone (Fig. 3*B*) or normal tissue alone (Fig. 3*C*) from the 12 patients was not associated with clinical stages, TNM classification, or relapse. Other pathological prognostic factors (pathological grade of differentiation and lymphovascular invasion) did not show significant association with any clusters obtained by analysis of cancer:normal ratio, cancerous tissue alone, or normal tissue alone, using the two-sided Fisher's exact test.

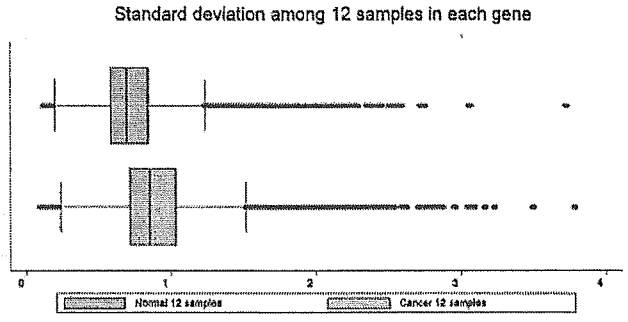

Fig. 1. SDs among 12 samples in each gene were compared between normal and cancer expression profiles. Raw data were transformed with log2, and then SDs in either normal or cancer profiling among 12 patients were calculated in each gene.

The two clusters based on gene expression ratios of cancer:normal tissue also showed a significant difference in relapse-free survival time (log-rank test, $P = 0.006$; Fig. 4*A*). There was also a significant difference in relapse-free survival time between presence (N_1) and absence (N_0) of lymph node metastasis ($P = 0.03$; Fig. 4*C*), but its difference was smaller than that of hierarchical clustering based on gene expression profiling. Other clinical prognostic factors, including stages based on TNM classification (Fig. 4*B*), invasiveness of primary tumor (early, T_1, or advanced, T2 and T3; Fig. 4*D*), grade of differentiation, and degree of lymphovascular invasion, did not make significant difference in relapse-free survival time, although the sample size was too small to conclude that these prognostic factors have no impact on predicting clinical outcomes.

The genes in cluster I with high paired cancer:normal expression ratios (Fig. 5*A*) were classified into several groups based on reported function: mucin and tumor antigen, chromosome (*ubiquitous Kruppel-like factor*, major centromere autoantigen: *CENP-B, histone H1, phosphoribosylpyrophosphate synthetase, CTF5*), signaling to direct cell fate (*Wnt-13, HOX2H, Bcl-2 interacting killer, DRAK2, SIM2, Smad3, RAIG1*), and others. In contrast, those in cluster II were mainly related to ubiquitin-related genes (*ubiquitin-conjugating enzyme, ubiquitin carrier protein, SUMO1*), viral oncogenes (*EBV small RNA-associated protein, 100 kDa EBNA coactivator, E6-AP, papillomavirus*; Fig. 5*B*), cell cycle, and gene repair [*Int-6, dynamin, CBP/p300-interacting transactivator* (*CITED2*), *c-myc binding protein* (*MBP-1*)], *serine/threonine protein kinase, SMARCA5, ckshs2, ku (p70/p80) subunit, MTPC1, hPMS1, RbAp48, PTTG1IP, CUTL1, replication protein 1,*

Table 1 *Characteristics and outcomes of patients*

No.	Age	Gender	Operation	TNM[a]	Stage[b]	Grade[c]	Inv[d]
1	69	Male	4/19/2001	$T_1N_0M_0$	I	II	0
2	70	Male	1/29/2002	$T_1N_1M_0$	IIB	I	1
3	55	Male	11/1/2001	$T_1N_0M_0$	I	I	0
4	66	Male	11/9/2000	$T_1N_0M_0$	I	II	2
5	76	Male	11/22/2001	$T_3N_0M_0$	IIA	II	2
6	58	Male	3/15/2001	$T_1N_1M_0$	IIB	II	0
7	75	Female	4/5/2001	$T_3N_1M_0$	III	I	1
8	79	Male	11/2/2000	$T_3N_1M_0$	III	III	2
9	82	Male	11/4/2000	$T_3N_1M_0$	III	III	2
10	52	Male	11/8/2001	$T_1N_1M_0$	IIB	I	2
11	59	Male	5/31/2001	$T_3N_1M_0$	III	I	2
12	56	Male	12/20/2001	$T_2N_1M_0$	IIB	II	2

[a] T, primary tumor; T_{is}, carcinoma *in situ*; T_1, invading the lamina propria or submucosa; T_2, invading the muscularis propria; T_3, invading the adventitia; T_4, invading adjacent structures; N_0, no regional lymph node metastasis; N_1, regional lymph node metastasis; M, metastasis; M_0, no metastasis; M_1, metastasis.

[b] Stage 0, $T_{is}N_0M_0$; stage I, $T_1N_0M_0$; stage IIA, $T_2N_0M_0$ or $T_3N_0M_0$; stage IIB, $T_1N_1M_0$ or $T_2N_1M_0$; stage III, $T_3N_1M_0$ or T_4 Any N M_0; stage IV, Any T Any N M_1.

[c] Grade I, well differentiated; grade II, moderately differentiated; grade III, poorly differentiated.

[d] Inv, lymphovascular invasion; 0, no invasion; 1, mild invasion; 2, more than moderate invasion.

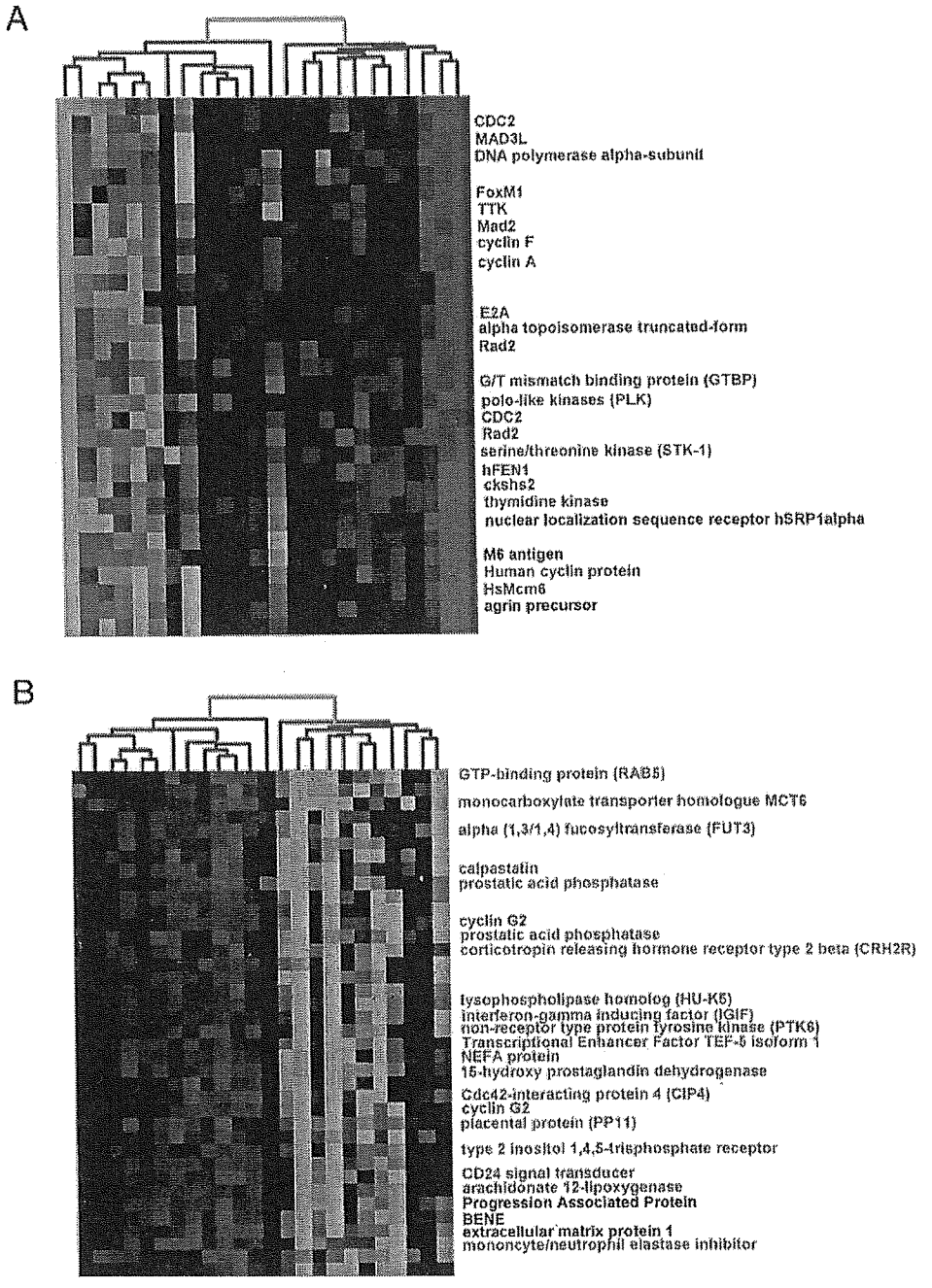

Fig. 2. Hierarchical clustering of 24 specimens composed of 12 pairs of cancer (*red lines in dendrogram at top*) and normal (*blue lines*) tissue samples. *Each column,* a single sample; *each row,* a single gene. *Red areas,* increased RNA expression; *green areas,* decreased RNA expression. *A,* a typical gene profile highly expressed in cancerous tissue (*red*); *B,* a typical gene profile highly expressed in normal esophageal tissue (*green*).

TTK, hMSH2, cAMP-dependent protein kinase, MZF1, TLE1, SPHAR, Centrin3, suppressin, CDK8, casein kinase II, CDC23; Fig. 5*C*). Genes related to resistance to chemoradiotherapy [*superoxide dismutase, MRP,*[3] *p53 binding protein, Rad6*] were also up-regulated in cluster II. Other gene sets [*NXP2, hnRNP A2, RNA polymerase II, Rho guanine nucleotide exchange factor 7, nucleotide binding protein, kinesin-related protein, arginine methyltransferase, histone-binding protein*] high in cluster II may contribute to nucleic acid metabolism. Metastasis-associated *mta1,* metalloproteinase-related collagenase, which was high in cluster II, may facilitate infiltration of tumor growth.

[3] The abbreviations used are: MRP, multidrug resistance-associated protein; HPV, human papillomavirus.

DISCUSSION

Profiling gene expression ratios of paired esophageal squamous cancerous and normal tissue specimens allowed us to predict relapse-free survival time more precisely than did any known clinical prognostic factors or the expression profiles of cancerous and normal tissues from each patient. Although the number of analyzed samples in this study was small and validation studies with larger numbers are required, the present findings do have statistically significant relationships (log-rank test in Kaplan-Meier survival curve, $P = 0.006$) that suggest less possibility of a type I error. Importantly, association with clinical outcomes was shown to be significant not by the gene expression profile of either cancerous tissue alone or normal tissue alone, but by the ratio of cancer to normal tissue specimens from the