American Journal of Pathology, Vol. 153, No. 3, September 1998

Correlation of Reduction in *MRP-1/CD9* and *KAI1/CD82* Expression with Recurrences in Breast Cancer Patients

Cheng-long Huang,* Nobuoki Kohno,† Eiji Ogawa,* Masashi Adachi,* Toshihiko Taki,* and Masayuki Miyake*

From the Department of Thoracic Surgery and Department V of Oncology, Kitano Hospital, Tazuke Kofukai Medical Research Institute, Osaka, and the Second Department of Internal Medicine,† Ehime University School of Medicine, Ehime, Japan*

MRP-1/CD9*, *KAI1/CD82*, and *ME491/CD63*, have been reported to be associated with the metastatic potential of solid tumors. The aim of this study was to determine whether their expression in tumor tissues is a useful indicator for prognosis in breast cancer patients. We studied 109 breast cancer patients who underwent surgery. Quantitative reverse transcription-polymerase chain reaction analysis was performed to evaluate the expression of these genes. The results were confirmed with immunohistochemistry. All of the carcinomas were *ME491/CD63* positive. Thirty-six tumors were *MRP-1/CD9* negative. The disease-free survival rate and the 5-year survival rate of patients with *MRP-1/CD9*-negative tumors were both significantly lower than that in patients with *MRP-1/CD9*-positive tumors ($P = 0.0005$ and $P = 0.0380$, respectively). Sixty-five tumors were *KAI1/CD82* negative. The disease-free survival rate of patients with *KAI1/CD82*-negative tumors was significantly lower than that of patients with *KAI1/CD82*-positive tumors ($P = 0.0065$). Cox regression analysis demonstrated that *MRP-1/CD9* status ($P = 0.0016$) and *KAI1/CD82* status ($P = 0.0234$) were useful indicators for the disease-free survival of breast cancer patients. The disease-free survival rate and 5-year survival rate of patients with either *MRP-1/CD9*-negative or *KAI1/CD82*-negative tumors were both significantly lower than patients who were positive for both genes ($P = 0.0003$ and $P = 0.0292$, respectively). The expression of *MRP-1/CD9* and *KAI1/CD82* genes are useful indicators of a poor prognosis in breast cancer patients. (Am J Pathol 1998, 153:973–983)

When compared with other types of solid human cancers, breast cancer is interesting because of its high sensitivity to hormonal therapy and chemotherapy.[1] However, the prognosis varies according to the extent of the disease and its biological behavior. Although the histopathological presence of axillary lymph node metastases is considered to be the most informative parameter for predicting the occurrence of relapses and the prognosis in breast cancer patients, there is a possibility of recurrence after resection even in patients with an early stage of node-negative breast cancer.[2] Therefore, it is important to understand the biological behavior of each individual tumor and to determine which types of tumor will have a more malignant course and thus need more intensive adjuvant therapy. It is widely accepted that several kinds of cancers are caused by the accumulation of genetic alterations.[3–6] Recent investigations have revealed that some of these genetic changes can be used as prognostic factors for predicting a poor prognosis. For example, the amplification of some oncogenes such as c-*erbB-2*,[3,4] *myc*,[5] *ras*,[6] and c-*fos*[6] have been found to be associated with a poor prognosis in patients with breast cancers. Mutations of *p53*, a well known tumor suppressor gene, also may be important for the prognosis of breast cancer patients.[5,7] In addition, assessing a combination of these genetic alterations may enable the more precise prediction of the outcomes of patients with breast cancers.[6]

Recently, three members of the transmembrane 4 superfamily *MRP-1/CD9*,[8,9] *KAI1/CD82*,[10] and *ME491/CD63*[11] have been reported to be associated with the biological behavior of solid tumors, especially with their metastatic potential. Initially, we found that MRP-1/CD9 was recognized by the murine MAb M31–15, which inhibited cell motility.[12] After the transfection of *MRP-1/CD9* into human lung cancer cell lines, we demonstrated that cell motility was suppressed in the *MRP-1/CD9*-expressing cells.[8] In addition, we showed that reduced MRP-1/CD9 protein expression was associated with metastasis and a poor prognosis in breast cancer patients[13] and that reduced *MRP-1/CD9* gene expression was also correlated with a poor prognosis in non-small cell lung cancer patients.[14] *KAI1/CD82* gene is located on human chromosome 11p11.2–13, and encodes a protein of 267 amino acids.[10] Initially, it was identified by cDNA cloning

Supported in part by Grants-in-Aid from the Ministry of Education, Science and Culture of Japan (No 07557253 and 08407040) to M. Miyake.

Accepted for publication June 3, 1998.

Address reprint requests to Dr. Masayuki Miyake, Department of Thoracic Surgery and Department V of Oncology, Kitano Hospital, Tazuke Kofukai Medical Research Institute, 13-3, Kamiyama-cho, Kita-ku, Osaka 530-0026, Japan.

as the R2 antigen, which was strongly up-regulated in mitogen-activated human T cells.[15] *KAI1/CD82* gene was also found to suppress tumor metastasis in a prostate cancer cell line[10] and a breast cancer cell line,[16,17] and thus may function as a metastatic suppressor gene.[10] A clinical analysis of patients with non-small cell lung cancers also revealed that reduced *KAI1/CD82* gene expression was associated with the metastasis of these tumors.[18] In addition, it has been reported that ME491/CD63 is strongly expressed on the cell surface during the early stage of malignant melanoma but becomes weaker in the advanced stages.[19] These findings are similar to those observed for MRP-1/CD9 and KAI1/CD82. Thus, of the many genetic markers available for evaluating the prognosis in breast cancers, *MRP-1/CD9*, *KAI1/CD82*, and *ME491/CD63* gene expression may have significant value as prognostic indicators in breast cancer patients. In the present study, we investigated whether the levels of *MRP-1/CD9*, *KAI1/CD82*, and *ME491/CD63* gene expression in tumor tissues are of value as prognostic factors in predicting the clinical behavior of breast cancer. Therefore, we performed a reverse transcription-polymerase chain reaction (RT-PCR) analysis to quantify the expression of these genes in tumor tissues from 109 patients with breast cancer. Immunohistochemical assays were also performed to confirm the results of the RT-PCR.

Materials and Methods

Clinical Characteristics of the Patients

From February 1987 to December 1995, 109 patients who underwent surgery at the Department of Thoracic Surgery of Kitano Hospital, Medical Research Institute of Osaka in Japan, were studied. The complete clinical records of all patients were available, and their histopathological diagnoses were fully documented. The postsurgical stage of each tumor was classified according to the Union International Contre Cancer TNM system.[20] In total, 109 patients with breast cancer up to stage IIIB were investigated.

Ninety-five patients had undergone a mastectomy, and 14 patients had undergone a quadrantectomy followed by immediate radiotherapy. Adjuvant systemic chemotherapy was given according to the patients' estrogen receptor (ER) status. Patients who were node positive or premenopausal ($n = 73$) underwent chemotherapy with oral 5-fluorouracil (200 mg/day) for 2 years, and eight patients with N2 disease were also treated with six cycles of cyclophosphamide/Adriamycin. Fifty-two ER-positive patients were treated with tamoxifen (20 mg/day) for 2 years or before recurrence. Sixteen postmenopausal patients of node-negative and receptor-negative status did not have any further adjuvant treatment. Thirty-three patients had recurrences during the observation period. After the recurrence, the locoregional tumor or lymph nodes were principally resected, followed by radiotherapy. Patients with distant metastases were treated with more effective adjuvant chemotherapies, including cisplatin and pirarubicin. This report includes follow-up data as of May 1, 1997. The median follow-up period was 48.5 months.

Tumor Specimens

To ascertain the presence of cancer cells, one-half of each fresh tumor tissue specimen was immediately embedded in optimum cutting temperature compound (Miles, Kankakee, IL), and frozen sections were then cut on the cryostat to a thickness of 6 μm and immediately stained with hematoxylin and eosin. After the connective tissues were trimmed off, the other half of the tumor specimen containing greater than 80% cancer cells of all tissue cells was selected for the RT-PCR analysis.

Quantitative RT-PCR Analysis

Total cellular RNA was extracted from the frozen tumor tissues by the acid guanidinium thiocyanate procedure.[21] First-strand cDNA synthesis was performed with 5 μg of total RNA using a cDNA synthesis kit (Pharmacia, Piscataway, NJ) according to the manufacturer's protocol. All of the subsequent assays were then carried out using the same procedures as described previously.[14,18] The generated cDNAs were amplified using primers for *MRP-1/CD9* (5′-TGCATCTGTATCCAGCGCCA-3′ and 5′-CTCAGGGATGTAAGCTGACT-3′), *KAI1/CD82* (5′-AGTCCTCCCTGCTGCTGTGTG-3′ and 5′-TCAGTCAGGGTGGGCAAGAGG-3′) and *ME491/CD63* (5′-CCCGAAAAACAACCACACTGC-3′ and 5′-GATGAGGAGGCTGAGGAGACC-3′). The internal control was *β-actin* (5′-GAGAAGATGACCCAGATCATGT-3′ and 5′-ACTCCATGCCCAGGAAGGAAGG-3′).[22] All of the subsequent assays were then carried out under conditions that yielded amplifications of *MRP-1/CD9*, *KAI1/CD82*, *ME491/CD63*, and *β-actin* within a linear range. Twenty-six cycles of PCR amplification were performed as follows: denaturation at 94°C for 40 seconds, annealing at 60°C for 40 seconds, and extension at 72°C for 90 seconds, followed by the final extension at 72°C for 7 minutes. The same PCR conditions were used to amplify the *β-actin* DNA. Tubes containing all of the ingredients except templates were included in all runs and served as negative controls. The human endothelial cell line ECV304 was used as a positive control, which has positive expression of *MRP-1/CD9*, *KAI1/CD82*, and *ME491/CD63*.[23] The amplified PCR products were electrophoresed on a 1% agarose gel containing ethidium bromide, and the bands were visualized under ultraviolet light followed by densitometric analysis (Figure 1).

Because it has been difficult to quantitate the absolute amount of specific mRNA without an internal standard of known concentration, the adjustment with a housekeeping gene has been used for the precise quantitation of mRNA of a specific gene in Northern blotting. Recently, quantitative RT-PCR has been developed using this method.[24] The densitometric values obtained for *MRP-1/CD9*, *KAI1/CD82*, and *ME491/CD63* bands in a given tumor tissue sample were divided by the corresponding value of *β-actin* for normalization, and the ratio was referred to as the gene expression ratio for each gene. The

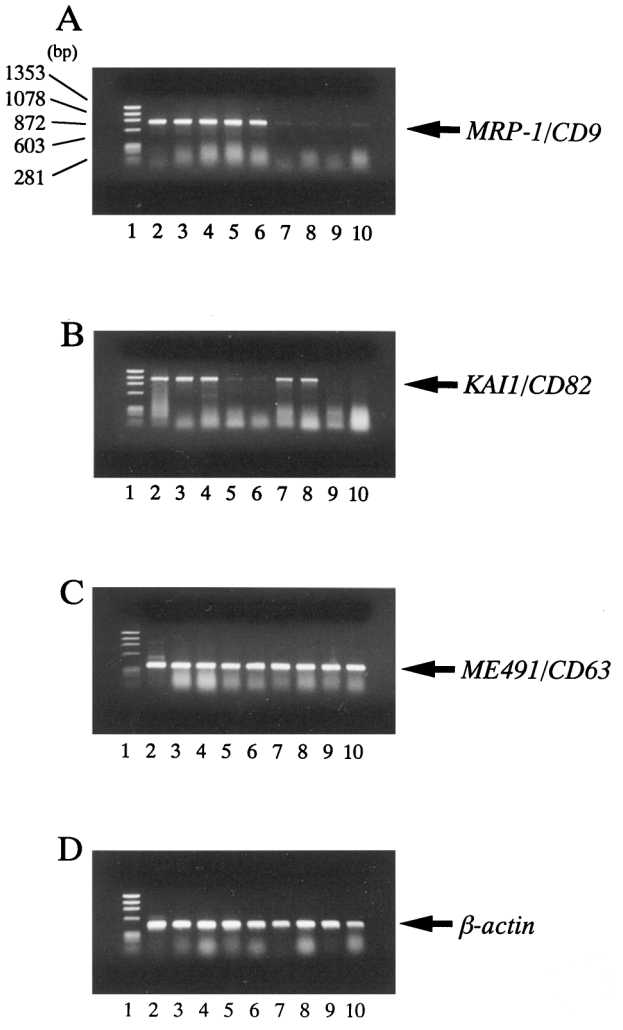

Figure 1. Agarose gel electrophoresis of RT-PCR-amplified *MRP-1/CD9 KAI1/CD82* and *ME491/CD63* cDNA. A: *MRP-1/CD9* cDNA. B: *KAI1/CD82* cDNA. C: *ME491/CD63* cDNA. D: *β-actin* cDNA (internal PCR control). Lane 1, size marker; lane 2, human endothelial cell line ECV304 (positive control); lanes 3 and 4, breast cancers with both *MRP-1/CD9*- and *KAI1/CD82*-positive expression; lanes 5 and 6, breast cancers with *MRP-1/CD9*-positive but *KAI1/CD82*-reduced expression; lanes 7 and 8, breast cancers with *MRP-1/CD9*-reduced but *KAI1/CD82*-positive expression; lanes 9 and 10, breast cancers with both *MRP-1/CD9*- and *KAI1/CD82*-reduced expression.

expression ratio of the tumor was divided by the expression ratio of the human endothelial cell line ECV304 to obtain the gene conservation rates. When the conservation rate of a given specimen was ≥1.0, it was considered to indicate conserved (positive) gene expression. If the value was <1.0, this denoted nonconserved (reduced) gene expression.

Immunohistochemical Assays

To confirm the results of *MRP-1/CD9* and *KAI1/CD82* gene expression on RT-PCR, immunohistochemical studies were performed as described previously.[25] Because MRP-1/CD9 and KAI1/CD82 are not well preserved in formalin-fixed, paraffin-embedded tissues, frozen sections were used instead. After quenching the endogenous peroxidase activity with 0.3% H_2O_2 (in absolute methanol) for 30 minutes, the sections were blocked for 2 hours at room temperature with 5% bovine serum albumin. Subsequently, duplicate sections were incubated for 2 hours with the anti-MRP-1/CD9 monoclonal antibody M31-15[12] and the anti-KAI1/CD82 monoclonal antibody C33,[26] respectively, and were then incubated for 1 hour with biotinylated horse anti-mouse immunoglobulin G (Vector Laboratories Inc., Burlingame, CA). The sections were incubated with the avidin-biotin-peroxidase complex (Vector) for 1 hour, and the antibody binding was visualized with 3,3′-diaminobenzidine tetrahydrochloride. Finally, the sections were lightly counterstained with Mayer's hematoxylin (Figure 2). Specimens of fibroadenoma of the breast were used as positive controls.

All of the immunostained sections were reviewed by two pathologists who had no knowledge of the patients' clinical status. Slides were examined under low power (×4 objective) to identify regions containing low-staining invasive tumors cells. In cases of multiple areas of low intensity, five areas selected at random were scored, and in sections where all of the staining appeared intense, one random field was selected. The proportion of high- and low-staining tumor cells in each selected field was determined by counting individual tumor cells at high magnification. At least 200 tumor cells were scored per ×40 field. Positive tumor cells were stained equivalent to normal breast glands and benign fibroadenoma tumor cells. All sections were scored in a semiquantitative fashion according to the method described previously,[27] which considers both the intensity and percentage of cells staining at each intensity. Intensities were classified as 0 (no staining), +1 (weak staining), +2 (distinct staining), and +3 (very strong staining), whereas 10% groupings were used for the percentage of cells that stained positive. For each slide, a value designated HSCORE was obtained by application of the following algorithm: HSCORE = $\Sigma(I \times PC)$, where I and PC represent intensity and percentage cells that stain at each intensity, respectively, and corresponding HSCOREs were calculated separately. Specimens with an HSCORE of ≥50 were classified as MRP-1/CD9 or KAI1/CD82-positive (+), and when HSCORE was <50, specimens were classified as reduced (−).

Statistical Analyses

The statistical significance of differences between *MRP-1/CD9* or *KAI1/CD82* gene expression and several other clinical pathological parameters was assessed by the χ^2 test. The disease-free survival and the overall survival curves were constructed according to the Kaplan-Meier method,[28] and differences in the survival of subgroups of patients were compared using Mantel's log-rank test.[29] Multivariate analyses were performed using the Cox regression model to study the effects of different variables on survival,[30] and six factors (*MRP-1/CD9* status, *KAI1/CD82* status, ER status, age at surgery, T status, and N status) were studied. Scores were assigned to each variable for the regression analysis. All P values were based on two-tailed statistical analyses, and a P value < 0.05 was considered to indicate statistical significance.

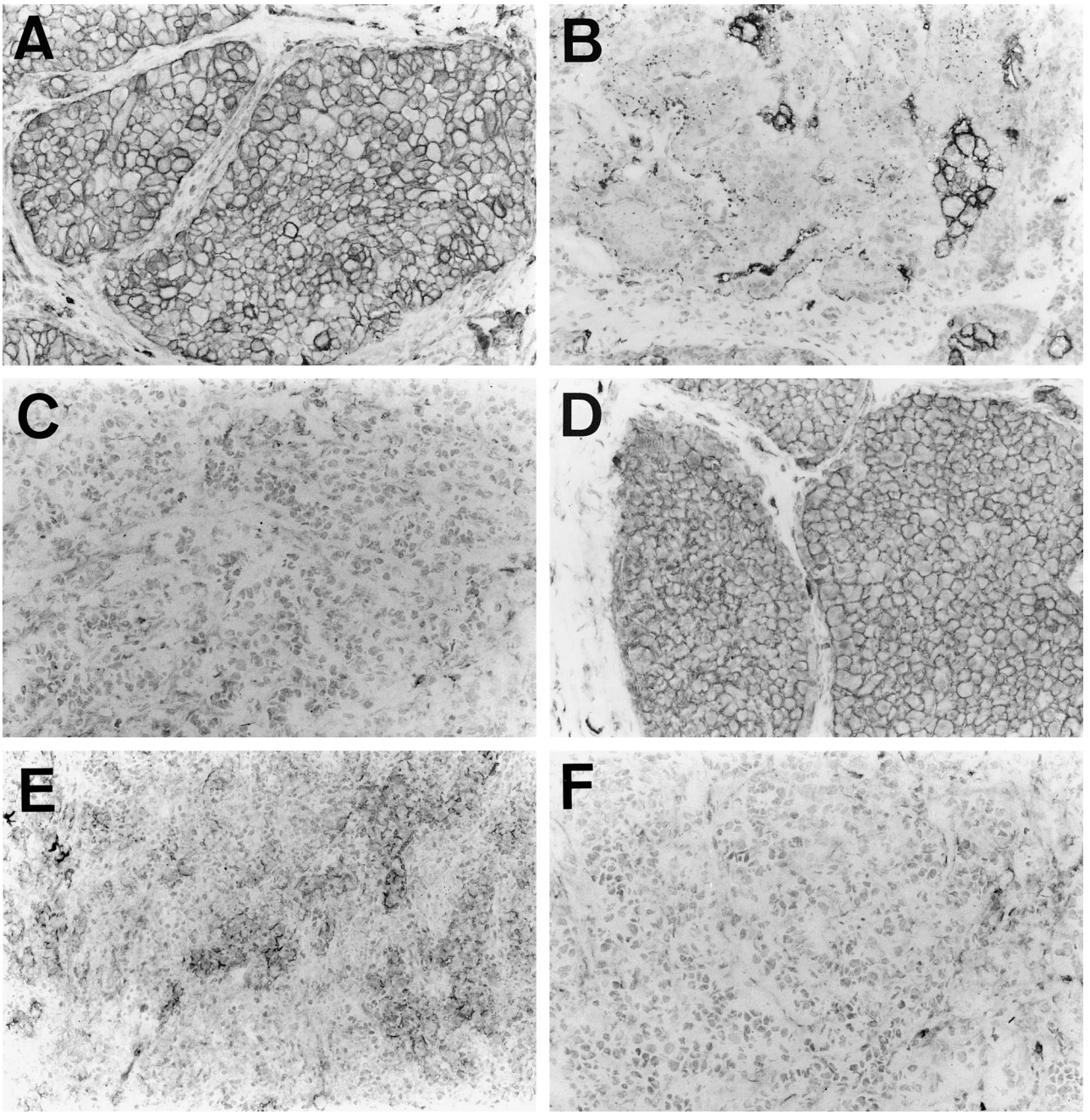

Figure 2. Immunohistochemical staining of human breast cancer tissues using the avidin-biotin-peroxidase complex procedure (original magnification, ×100). A: Invasive ductal carcinoma with positive MRP-1/CD9 expression. B: Invasive ductal carcinoma with lower MRP-1/CD9 expression classified as negative MRP-1/CD9 expression. C: Invasive ductal carcinoma with negative MRP-1/CD9 expression. D: Invasive ductal carcinoma with positive KAI1/CD82 expression. E: Invasive ductal carcinoma with lower KAI1/CD82 expression classified as negative KAI1/CD82 expression. F: Invasive ductal carcinoma with negative KAI1/CD82 expression.

Results

MRP-1/CD9, KAI1/CD82 and ME491/CD63 Gene Expression in Breast Cancer Tissues Analyzed by RT-PCR

Of all 109 breast cancers studied, *ME491/CD63* gene expression was preserved and no reduced levels of *ME491/CD63* DNA were detected (Figure 1). All of the carcinomas were evaluated to be *ME491/CD63* positive, and no statistically significant relationships were found between *ME491/CD63* gene expression and other known prognostic factors (Figure 1C). Thus, *ME491/CD63* might play a different role from the other two transmembrane 4 superfamily members in breast cancer. On the other hand, of the 109 breast cancer patients, 73 tumors (67.0%) were evaluated as *MRP-1/CD9* positive, and 36 tumors (33.0%) were *MRP-1/CD9* negative (Figure 1A). Forty-four tumors (40.4%) were evaluated as *KAI1/CD82* positive, and 65 tumors (59.6%) were *KAI1/CD82* nega-

tive (Figure 1B). However, no relationship was found between *MRP-1/CD9* expression and *KAI1/CD82* expression ($r = 0.138$, $P = 0.3526$, data not shown), and the expression of these genes were independent of each other.

MRP-1/CD9 and KAI1/CD82 Protein Expression Analyzed by Immunohistochemistry

Of the 109 breast cancers studied using the immunohistochemical method, 72 (66.0%) were classified as MRP-1/CD9 positive. In these cases, the MRP-1/CD9 expression resembled that of benign fibroadenomas, and the immunostaining was intense and uniform on the cell surface membrane (Figure 2A). There were 37 cases (34.0%) with reduced MRP-1/CD9 expression (Figure 2, B and C), and the immunostaining from most of these tumors was heterogeneous. The *MRP-1/CD9* gene expression was readily evident in those primary tumors that were classified as positive in the immunohistochemical assays. In contrast, the *MRP-1/CD9* gene expression was weak or entirely absent in those breast cancers that had reduced immunohistochemically detectable MRP-1/CD9. The *MRP-1/CD9* gene expression evaluated by RT-PCR was highly associated with MRP-1/CD9 protein expression, as determined by immunohistochemical staining ($r = 0.755$, $P < 0.0001$) (Figure 3A). Overall, the immunohistochemical results agreed well with those from the RT-PCR assays, and 89.9% of the samples coincided exactly.

On the other hand, there were 44 cases (40.4%) with positive KAI1/CD82 expression and 65 cases (59.6%) with reduced KAI1/CD82 expression (Figure 2, D to F). The *KAI1/CD82* gene expression evaluated by RT-PCR was also associated with KAI1/CD82 protein expression, as determined by immunohistochemical staining ($r = 0.803$, $P < 0.0001$) (Figure 3B). These results also agreed well with those from the RT-PCR assays, and 90.8% of the samples coincided exactly. In cases of discrepancy, the results from the RT-PCR analysis were used in the specimen classification.

Association of Tumor MRP-1/CD9 Status with Disease-Free and 5-Year Survival of Breast Cancer Patients

As described in our results from the Western blotting analysis, comparing survival of 109 patients with breast cancer demonstrated that the disease-free survival rate of patients with *MRP-1/CD9*-negative tumors was significantly lower than that of patients with *MRP-1/CD9*-positive tumors (37.7% *versus* 65.7%, $P = 0.0005$) (Table 1 and Figure 4A). In particular, *MRP-1/CD9* was an effective indicator of patients with early-stage tumors such as T1, N0, stage I, and stage II ($P = 0.0005$, $P = 0.0007$, $P = 0.0168$, and $P = 0.0249$, respectively). In addition, the 5-year survival rate of patients with *MRP-1/CD9*-negative tumors was significantly lower than that of patients with

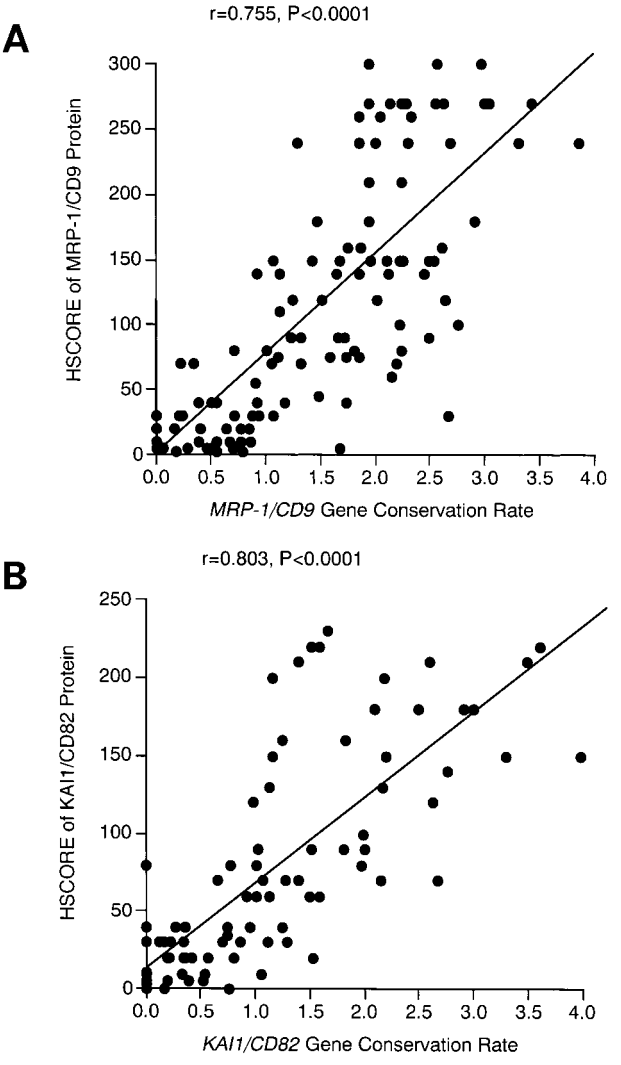

Figure 3. Pearson's correlation coefficient between gene conservation rate by RT-PCR and HSCORE by immunohistochemistry. A: MRP-1/CD9. B: KAI1/CD82.

MRP-1/CD9-positive tumors (80.8% *versus* 94.0%, $P = 0.0380$) (Table 1 and Figure 4B).

Association of Tumor KAI1/CD82 Status with Disease-Free and 5-Year Survival of Breast Cancer Patients

The disease-free survival rate of patients with *KAI1/CD82*-negative tumors was significantly lower than that of patients with *KAI1/CD82*-positive tumors (38.2% *versus* 80.2%, $P = 0.0065$) (Table 2 and Figure 4C). In particular, the disease-free survival rate of patients with *KAI1/CD82*-negative, early-stage tumors (ie, T1, T2, N0, stage I, and stage II) was significantly lower than that of patients with *KAI1/CD82*-positive, early-stage tumors ($P = 0.0226$, $P = 0.0105$, $P = 0.0243$, $P = 0.0429$, and $P = 0.0108$, respectively). However, there was no significant difference between the 5-year survival rates of patients with *KAI1/CD82*-negative tumors and patients with *KAI1/CD82*-positive tumors (Table 2 and Figure 4D).

Table 1. Disease-Free Survival Rate and 5-Year Survival Rate of 109 Patients with Breast Cancer According to Their Clinicopathological Characteristics and *MRP-1/CD9* Gene Status

Characteristics	Disease-free survival rate (%)			5-year survival rate (%)		
	MRP-1/CD9 +	*MRP-1/CD9* −	*P* value	*MRP-1/CD9* +	*MRP-1/CD9* −	*P* value
Age at surgery (years)						
≤50	68.8	45.9	0.0409	96.2	83.7	0.1855
>50	63.9	28.6	0.0013	92.8	76.2	0.0815
ER status						
+	66.2	45.0	0.0244	100.0	90.0	0.4000
−	64.4	34.3	0.0081	88.7	70.3	0.0587
Tumor status						
T1	95.2	44.4	0.0005	100.0	100.0	>0.9999
T2	52.0	52.9	0.4539	90.3	83.7	0.4179
T3	80.0	25.0	0.0570	100.0	75.0	>0.9999
T4	100.0	0.0	>0.9999	100.0	33.3	>0.9999
Nodal status						
N0	93.0	45.0	0.0007	100.0	85.6	0.2000
N1	52.5	33.9	0.0438	90.1	85.1	0.6659
N2	40.0	0.0	0.2536	66.7	0.0	0.0445
Pathological stage						
I	93.8	37.5	0.0168	100.0	100.0	>0.9999
II	69.5	46.3	0.0249	93.2	81.5	0.1302
III	55.6	14.3	0.0887	85.7	68.6	0.3759
Total number of patients	65.7	37.7	0.0005	94.0	80.8	0.0380

Prognostic Value of MRP-1/CD9 and KAI1/CD82 Status

The Cox regression model was used to evaluate the disease-free survival and overall survival as shown in Table 3. The *MRP-1/CD9* status (hazard ratio, 3.332; $P = 0.0016$), *KAI1/CD82* status (hazard ratio, 2.778; $P = 0.0234$), ER status (hazard ratio, 2.242; $P = 0.0336$), and nodal status (hazard ratio, 3.089; $P = 0.0003$) were found to be useful indicators for the disease-free survival of breast cancer patients. On the other hand, only two variables, ER status (hazard ratio, 6.358; $P = 0.0230$) and nodal status (hazard ratio, 3.376; $P = 0.0221$), were significant factors for predicting the overall survival of breast cancer patients.

Classification of Breast Cancer According to MRP-1/CD9 and KAI1/CD82 Gene Expression

Initially, the 109 breast cancer patients were divided into four groups according to their *MRP-1/CD9* and *KAI1/CD82* gene status; 34 patients had both *MRP-1/CD9*- and *KAI1/CD82*-positive tumors, 10 patients had *MRP-1/CD9*-negative but *KAI1/CD82*-positive tumors, 39 patients had *MRP-1/CD9*-positive but *KAI1/CD82*-negative tumors, and 26 patients had both *MRP-1/CD9*- and *KAI1/CD82*-negative tumors. The disease-free survival rates of these patients were 94.1%, 40.0%, 26.9%, and 37.6%, respectively. The disease-free survival rate of patients with tumors positive for both genes was significantly higher than that of patients with the other three types of tumors. On the other hand, there were no significant differences among the latter three groups. Therefore, the 109 breast cancer patients were reclassified into two subgroups: one subgroup with both *MRP-1/CD9*- and *KAI1/CD82*-positive tumors and the other subgroup with either or both negative tumors. The disease-free survival rate of the former double-positive subgroup was significantly higher than the latter subgroup (94.1% *versus* 38.4%, $P = 0.0003$) (Table 4 and Figure 4E). In addition, the former subgroup also had a higher 5-year survival rate than the latter subgroup (100.0% *versus* 84.4%, $P = 0.0292$) (Table 4 and Figure 4F). The Cox multivariate regression analysis of disease-free survival is shown in Table 5. This simultaneous evaluation for both *MRP-1/CD9* and *KAI1/CD82* expression was found to be a significant indicator of a poor prognosis ($P = 0.0006$), and nodal status is also a significant indicator of a poor prognosis. The other variables (age at surgery, ER status, and T status) did not correlate with the *MRP-1/CD9* and *KAI1/CD82* gene status or its prognostic value. However, because no patients in the double-positive subgroup have died, it is impossible to perform a Cox multivariate regression analysis for their 5-year survival.

Discussion

Previously, we have shown that MRP-1/CD9 protein expression was a good predictive factor for poor prognosis in breast cancer patients.[13] As part of our evaluation of the members of the transmembrane 4 superfamily as possible prognostic predictors for breast cancer, we have extended our study to other genes, such as *ME491/CD63* and *KAI1/CD82*. It has been reported that ME491/CD63 is strongly expressed on the cell surface during the early stage of malignant melanoma, but is weaker or absent in the more malignant stages and in the normal melanocyte.[19] However, the *ME491/CD63* mRNA levels in almost all of the breast cancers were well preserved, and no cases with a reduction in *ME491/CD63* mRNA levels were detected. This suggests that *ME491/CD63* may limit the progression only in malignant melanomas

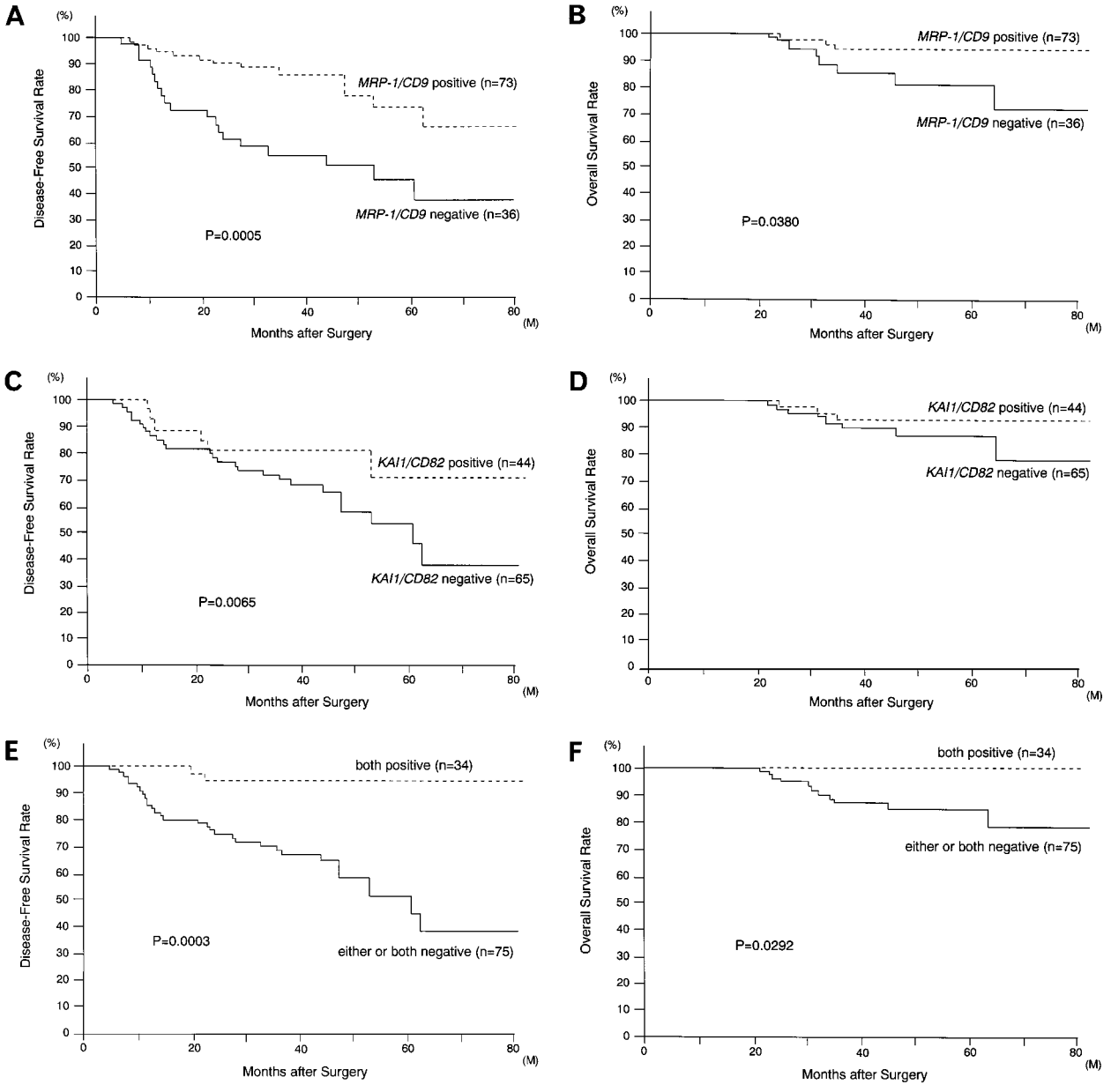

Figure 4. A: Disease-free survival of 109 breast cancer patients according to their tumor *MRP-1/CD9* gene status. B: Overall survival of 109 breast cancer patients according to their tumor *MRP-1/CD9* gene status. C: Disease-free survival of 109 breast cancer patients according to their tumor *KAI1/CD82* gene status. D: Overall survival of 109 breast cancer patients according to their tumor *KAI1/CD82* gene status. E: Disease-free survival of 109 breast cancer patients in relation to their classification (both *MRP-1/CD9*-positive and *KAI1/CD82*-positive subgroup *versus* either *MRP-1/CD9*-negative or *KAI1/CD82*-negative subgroup). F: Overall survival of 109 breast cancer patients in relation to their classification (both *MRP-1/CD9*-positive and *KAI1/CD82*-positive subgroup *versus* either *MRP-1/CD9*-negative or *KAI1/CD82*-negative subgroup).

and that its expression might have no effect on the characteristics of breast cancers. On the other hand, our present study demonstrated that a reduction in not only *MRP-1/CD9* gene expression but also *KAI1/CD82* gene expression in breast tumors correlated with a poor prognosis. In addition, a simultaneous classification according to both *MRP-1/CD9* and *KAI1/CD82* gene expression was a very useful indicator for predicting the recurrence of breast cancer. Of patients with N0 status or stage I diseases, those with both *MRP-1/CD9*- and *KAI1/CD82*-positive tumors had no recurrences. However, among patients with either *MRP-1/CD9*- or *KAI1/CD82*-negative tumors, the disease-free survival rates were only 55.1% for patients with N0 status and 72.9% for patients with stage I. Therefore, when breast cancer patients have either *MRP-1/CD9*- or *KAI1/CD82*-negative tumors, they should be carefully observed after surgery even in early pathological stages. Furthermore, these patients might need additional adjuvant chemotherapy and/or hormonal therapy. In contrast, of the 34 patients with both *MRP-1/CD9*- and *KAI1/CD82*-positive tumors, only 2 patients (T2N2 and T3N1) had recurrences, and all of the patients were alive for 5 years after their surgery. These results suggested that the tumors positive for both *MRP-1/CD9*

Table 2. Disease-Free Survival Rate and 5-Year Survival Rate of 109 Patients with Breast Cancer According to Their Clinicopathological Characteristics and *KAI1/CD82* Gene Status

Characteristics	Disease-free survival rate (%)			5-year survival rate (%)		
	KAI1/CD82 +	*KAI1/CD82* −	*P* value	*KAI1/CD82* +	*KAI1/CD82* −	*P* value
Age at surgery (years)						
≤50	64.3	54.6	0.8587	87.1	93.2	0.5449
>50	92.9	25.7	0.0005	96.2	81.4	0.0860
ER status						
+	94.4	39.6	0.0207	100.0	94.4	>0.9999
−	70.7	40.8	0.0719	87.8	78.2	0.2174
Tumor status						
T1	75.0	67.3	0.0226	100.0	100.0	>0.9999
T2	90.9	35.7	0.0105	95.0	84.4	0.2012
T3	57.1	50.0	0.6415	83.3	66.7	0.5637
T4	0.0	33.3	0.5151	0.0	66.7	0.0833
Nodal status						
N0	95.7	54.1	0.0243	95.2	93.3	0.8570
N1	72.9	27.5	0.0729	94.4	85.5	0.3488
N2	33.3	20.0	0.3727	66.7	33.3	0.9883
Pathological stage						
I	100.0	70.1	0.0429	100.0	100.0	>0.9999
II	83.6	36.6	0.0108	92.3	86.8	0.3911
III	42.9	33.3	0.5373	85.7	71.4	0.6945
Total number of patients	80.2	38.2	0.0065	92.8	87.0	0.3080

and *KAI1/CD82* may have a lower-grade malignancy and that adjuvant chemotherapy might not be necessary before recurrence.

Both MRP-1/CD9 and KAI1/CD82 are cell surface membrane glycoproteins that are widely expressed in various normal epithelia.[25] Members of the transmembrane 4 superfamily include MRP-1/CD9,[8] KAI1/CD82,[10] ME491/CD63,[19] TAPA-1/CD81,[31] CD53,[32] CD37,[33] and others. The members of this family have four hydrophobic transmembrane domains divided by two extracellular loops, with cytoplasmic N and C termini.[34] Their extracellular loops usually have some *N*-glycosylation sites. This type of structure suggests that these cell surface glycoproteins might play an important role in signal transduc-

Table 3. Multivariate Regression Analysis in Predicting the Disease-Free Survival and Overall Survival of 109 Patients with Breast Cancer

Variables	Assigned score	Disease-free survival		Overall survival	
		Hazard ratio (95% CI)	*P* value	Hazard ratio (95% CI)	*P* value
MRP-1/CD9 status					
+	0	3.332 (1.579–7.032)	0.0016	3.092 (0.732–13.052)	0.1245
−	1				
KAI1/CD82 status					
+	0	2.778 (1.149–6.721)	0.0234	2.260 (0.559–9.142)	0.2528
−	1				
Age at Surgery (years)					
≤50	0	1.996 (0.972–4.101)	0.0598	1.983 (0.549–7.158)	0.2959
>50	1				
ER status					
+	0	2.242 (1.065–4.723)	0.0336	6.358 (1.291–31.316)	0.0230
−	1				
Tumor status					
T1	1	1.239 (0.721–2.128)	0.4374	1.353 (0.552–3.316)	0.5080
T2	2				
T3	3				
T4	4				
Nodal status					
N0	0	3.089 (1.688–5.654)	0.0003	3.376 (1.190–9.573)	0.0221
N1	1				
N2	2				

CI, confidence interval.

Table 4. Disease-Free Survival Rate and 5-Year Survival Rate of 109 Patients with Breast Cancer according to Their *MRP-1/CD9* and *KAI-1/CD82* Gene Status

Characteristics	Disease-free survival rate (%)			5-year survival rate (%)		
	Both +	Either − or both −	*P* value	Both +	Either − or both −	*P* value
Age at surgery (years)						
≤50	80.0	51.9	0.3928	100.0	88.4	0.5242
>50	100.0	25.4	0.0001	100.0	80.4	0.0609
ER status						
+	92.9	46.1	0.0782	100.0	94.7	>0.9999
−	95.0	35.6	0.0010	100.0	73.0	0.0571
Tumor status						
T1	100.0	72.0	0.0360	100.0	100.0	>0.9999
T2	94.1	40.3	0.0178	100.0	83.7	0.2782
T3	80.0	25.0	0.0570	100.0	75.0	>0.9999
T4		25.0			50.0	
Nodal status						
N0	100.0	55.1	0.0147	100.0	90.9	0.5238
N1	92.9	28.3	0.0090	100.0	83.9	0.2621
N2	50.0	16.7	0.2125	100.0	26.7	>0.9999
Pathological stage						
I	100.0	72.9	0.2157	100.0	100.0	>0.9999
II	100.0	38.3	0.0080	100.0	84.4	0.1725
III	60.0	27.3	0.1465	100.0	67.5	0.4965
Total number of patients	94.1	38.4	0.0003	100.0	84.4	0.0292

tion and may regulate cell growth, cell differentiation, cell adhesion, and cell motility.[34,35] Although the precise functions of this family still remain unclear, many studies using immunoprecipitation have demonstrated the possible existence of a "tetraspan network."[36] By connecting with other molecules such as integrins[35,37,38] and human lymphocyte antigens,[39–41] this family may organize the positioning of cell surface proteins and thus play a role in signal transduction. In addition, MRP-1/CD9, KAI1/CD82, CD63, and CD81 are considered to form complexes with each other.[36] These results suggest that *MRP-1/CD9* or *KAI1/CD82* gene expression might be declining as the malignant tumors advance and could disrupt the tetraspan network and enable the malignant cells to acquire their metastatic potential. In fact, *MRP-1/CD9* gene has been reported to be more highly expressed in a primary colon cancer as compared with its corresponding metastatic tumor using differential display cloning.[42] These studies also indicated that *MRP-1/CD9* and *KAI1/CD82* might function as metastatic suppressor genes and are useful indicators of a poor outcome in patients with some solid tumors.[13,14,18]

On the other hand, the down-regulation of *KAI1/CD82* gene during the progression of human prostate cancer infrequently involves a gene mutation or allelic loss.[43] We also have found no mutations of *MRP-1/CD9* gene in 143 resected lung tumor specimens (data not shown). To date, we believe that the mechanism underlying the reduction in the expression of these genes in tumor tissues might be an abnormal gene promoter or an abnormal down-regulation somewhere upstream in their signal pathway. Various oncogenes and oncosuppressor genes have their actions relatively upstream of the signal pathway. During the progression of these tumors, the accu-

Table 5. Multivariate Regression Analysis in Predicting the Disease-Free Survival of 109 Patients with Breast Cancer

Variables	Assigned score	Hazard ratio (95% CI)	*P* value
MRP-1/CD9 and *KAI1/CD82* status			
Both +	0	13.375 (3.068–58.312)	0.0006
Either − or both −	1		
Age at surgery (years)			
≤50	0	2.468 (1.175–5.183)	0.0170
>50	1		
ER status			
+	0	2.299 (1.112–4.752)	0.0247
−	1		
Tumor status			
T1	1	1.347 (0.791–2.293)	0.2727
T2	2		
T3	3		
T4	4		
Nodal status			
N0	0	2.870 (1.572–5.241)	0.0006
N1	1		
N2	2		

CI, confidence interval.

mulations of abnormalities might act as triggers inducing the tumors to become more aggressive.

Another interesting aspect of the current research is that our study showed that the results of *MRP-1/CD9* and *KAI1/CD82* gene expression as evaluated by RT-PCR agreed well quantitatively with their protein expression as evaluated by immunohistochemistry. In addition, because the results in larger-scale studies might also depend on the quality of the mRNA, it may be relatively easy to quantitate MRP-1/CD9 and KAI1/CD82 protein expression in an actual clinical setting. Our present study demonstrated that *MRP-1/CD9* and *KAI1/CD82* gene expression in breast cancers are important factors for predicting recurrence. The classification of breast cancers according to both *MRP-1/CD9* and *KAI-1/CD82* gene expression will prove to be useful for the clinical treatment of breast cancers patients.

Acknowledgments

We thank Tatsuya Hirai for his technical assistance and Drs. Tadashi Obayashi and Sadayuki Matsumoto for the pathological examination.

References

1. Early Breast Cancer Trialists' Collaborative Group: Systemic treatment of early breast cancer by hormonal, cytotoxic, or immune therapy: 133 randomized trials involving 31 000 recurrences and 24 000 deaths among 75 000 women. Lancet 1992, 339:1–15,71–85
2. Allred DC, Clark GM, Elledge R, Fuqua SA, Brown RW, Chamness GC, Osborne CK, McGuire WL: Association of p53 protein expression with tumor cell proliferation rate and clinical outcome in node-negative breast cancer. J Natl Cancer Inst 1993, 85:200–206
3. Hartmann LC, Ingle JN, Wold LE, Farr GH, Grill JP, Su JQ, Maihle NJ, Krook JE, Witzig TE, Roche PC: Prognostic value of c-erbB2 overexpression in axillary lymph node positive breast cancer. Cancer 1994, 74:2956–2963
4. Giai M, Roagna R, Ponzone R, Bortoli MD, Dati C, Sismondi P: Prognostic and predictive relevance of c-erbB-2 and ras expression in node positive and negative breast cancer. Anticancer Res 1994, 14:1441–1450
5. Berns EMJJ, Klijn JGM, Smid M, Staveren IL, Look MP, Putten WLJ, Foekens JA: TP53, and MYC gene alterations independently predict poor prognosis in breast cancer patients. Genes Chromosomes Cancer 1996, 16:170–179
6. Bland KI, Konstadoulakis MM, Vezeridis MP, Wanebo HJ: Oncogene protein co-expression: value of Ha-ras, c-myc, c-fos, and p53 as prognostic discriminants for breast carcinoma. Ann Surg 1995, 221: 706–720
7. Kovach JS, Hartmann A, Blaszyk H, Cunningham J, Schaid D, Sommer SS: Mutation detection by highly sensitive methods indicates that p53 gene mutations in breast cancer can have important prognostic value. Proc Natl Acad Sci USA 1996, 93:1093–1096
8. Ikeyama S, Koyama M, Yamaoko M, Sasada R, Miyake M: Suppression of cell motility and metastasis by transfection with human motility-related protein (MRP-1/CD9) DNA. J Exp Med 1993, 177:1231–1237
9. Miyake M, Nakano K, Ieki Y, Adachi M, Huang C, Itoi S, Koh T, Taki T: Motility related protein 1 (MRP-1/CD9) expression: inverse correlation with metastases in breast cancer. Cancer Res 1995, 55:4127–4131
10. Dong JT, Lamb PW, Rinker-Schaeffer CW, Vukanovic J, Ichikawa T, Isaacs JT, Barrett JC: KAI1, a metastasis suppressor gene for prostate cancer on human chromosome 11p11.2. Science 1995, 268: 884–886
11. Radford KJ, Mallesch J, Hersey P: Suppression of human melanoma cell growth and metastasis by the melanoma-associated antigen CD63 (ME491). Int J Cancer 1995, 62:631–635
12. Miyake M, Koyama M, Seno M, Ikeyama S: Identification of the motility-related protein (MRP-1), recognized by monoclonal antibody M31–15, which inhibits cell motility. J Exp Med 1991, 174:1347–1354
13. Miyake M, Nakano K, Itoi S, Koh T, Taki T: Motility-related protein-1 (MRP-1/CD9) reduction as a factor of poor prognosis in breast cancer. Cancer Res 1996, 56:1244–1249
14. Higashiyama M, Taki T, Ieki Y, Adachi M, Huang C, Koh T, Kodama K, Doi O, Miyake M: Reduced motility related protein-1 (MRP-1/CD9) gene expression as a factor of poor prognosis in non-small cell lung cancer. Cancer Res 1995, 55:6040–6044
15. Gaugitsch HW, Hofer E, Huber NE, Schnabl E, Baumruker T: A new superfamily of lymphoid and melanoma cell proteins with extensive homology to Schistosoma mansoni antigen Sm23. Eur J Immunol 1991, 21:377–383
16. Yang X, Welch DR, Phillips KK, Weissman BE, Wei LL: KAI1, a putative marker for metastatic potential in human breast cancer. Cancer Lett 1997, 119:149–155
17. Phillips KK, White AE, Hicks DJ, Welch DR, Barrett JC, Wei LL, Weissman BE: Correlation between reduction of metastasis in the MDA-MB-435 model system and increased expression of the Kai-1 protein. Mol Carcinog 1998, 21:111–120
18. Adachi M, Taki T, Ieki Y, Huang C, Higashiyama M, Miyake M: Correlation of KAI1/CD82 gene expression with good prognosis in patients with non-small cell lung cancer. Cancer Res 1996, 56:1751–1755
19. Hotta H, Ross AH, Huebner K, Isobe M, Wendeborn S, Chao MV, Ricciardi RP, Tsujimoto Y, Croce CM, Koprowski H: Molecular cloning and characterization of an antigen associated with early stages of melanoma tumor progression. Cancer Res 1988, 48:2955–2962
20. Hermanek P, Sobin LH: TNM Classification of Malignant Tumors, ed 4. Edited by P Hermanek, LH Sobin. Berlin, Springer-Verlag, 1987, pp 103–109
21. Chomczynski P, Sacchi N: Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 1987, 162:156–159
22. Nakajima-Iijima S, Hamada H, Reddy P, Kakunaga T: Molecular structure of the human cytoplasmic β-actin gene: interspecies homology of sequences in the introns. Proc Natl Acad Sci USA 1985, 82:6133–6137
23. Takahasi K, Sawasaki Y: Rare spontaneously transformed human endothelial cell line provides useful research tool. In Vitro Cell Dev Biol 1992, 28A:380–382
24. Coen DM: Quantitation of rare DNAs by PCR. Current Protocols in Molecular Biology. Edited by FM Ausubel, R Brent, RE Kingston, DD Moore, JG Seidman, JA Smith, K Struhl. New York, John Wiley & Sons, 1995, pp 15.3.1–15.3.8
25. Huang C, Taki T, Adachi M, Yagita M, Sawada S, Takabayashi A, Inufusa H, Yoshie O, Miyake M: MRP-1/CD9 and KAI1/CD82 expression in normal and various cancer tissues. Int J Oncol 1997, 11:1045–1051
26. Imai T, Fukudome K, Takagi S, Nagira M, Furuse M, Fukuhara N, Nishimura M, Hinuma Y, Yoshie O: C33 antigen recognized by monoclonal antibodies inhibitory to human T cell leukemia virus type 1-induced syncytium formation is a member of a new family of transmembrane proteins including CD9, CD37, CD53, and CD63. J Immunol 1992, 149:2879–2886
27. McCarty KS Jr, Szabo E, Flowers JL, Cox EB, Leight GS, Miller L, Konrath J, Soper JT, Budwit DA, Creasman WT, Seigler HF, McCarty KS Sr: Use of a monoclonal anti-estrogen receptor antibody in the immunohistochemical evaluation of human tumors. Cancer Res 1986, 46:4244s–4248s
28. Kaplan EL, Meier P: Nonparametric estimation from incomplete observations. J Am Stat Assoc 1958, 53:457–481
29. Mantel N: Evaluation of survival data and two new rank order statistics arising in its consideration. Cancer Chemother Rep 1966, 50:163–170
30. Cox DR: Regression models and life-tables. J R Stat Soc B 1972, 34:187–220
31. Oren R, Takahashi S, Doss C, Levy R, Levy S: TAPA-1, the target of an antiproliferative antibody, defines a new family of transmembrane proteins. Mol Cell Biol 1990, 10:4007–4015

32. Amiot M: Identification and analysis of cDNA clones encoding CD53: a pan-leukocyte antigen related to membrane transport proteins. J Immunol 1990, 145:4322–4325
33. Classon BJ, Williams AF, Willis AC, Seed B, Stamenkovic I: The primary structure of the human leukocyte antigen CD37, a species homologue of the rat MRC OX-44 antigen. J Exp Med 1989, 169: 1497–1502
34. Wright MD, Tomlinson MG: The ins and outs of the transmembrane 4 superfamily. Immunol Today 1994, 15:588–594
35. Hemler ME, Mannion BA, Berditchevski F: Association of TM4SF proteins with integrins: relevance to cancer. Biochim Biophys Acta 1996, 1287:67–71
36. Rubinstein E, Naour FL, Lagaudriere-Gesbert C, Billard M, Conjeaud H, Boucheix C: CD9, CD63, CD81, and CD82 are components of a surface tetraspan network connected to HLA-DR, and VLA integrins. Eur J Immunol 1996, 26:2657–2665
37. Berditchevski F, Zutter MM, Hemler ME: Characterization of novel complexes on the cell surface between integrins and proteins with 4 transmembrane domains (TM4 proteins). Mol Biol Cell 1996, 7:193–207
38. Mannion BA, Berditchevski F, Kraeft SK, Chen LB, Hemler ME: Transmembrane-4 superfamily proteins CD81 (TAPA-1), CD82, CD63, and CD53 specifically associate with integrin $\alpha 4\beta 1$ (CD49d/CD29). J Immunol 1996, 157:2039–2047
39. Angelisova P, Hilgert I, Horejsi V: Association of four antigens of the tetraspans family (CD37, CD53, TAPA-1, and R2/C33) with MHC class II glycoproteins. Immunogenetics 1994, 39:249–256
40. Szollosi J, Horejsi V, Bene L, Angelisova P, Damjanovich S: Supramolecular complexes of MHC class I, MHC class II, CD20, and tetraspan molecules (CD53, CD81, and CD82) at the surface of a B cell line JY. J Immunol 1996, 157:2939–2946
41. Lagaudriere-Gesbert C, Lebel-Binay S, Wiertz E, Ploegh HL, Fradelizi D, Conjeaud H: The tetraspanin protein CD82 associates with both free HLA class I heavy chain and heterodimeric β_2-microglobulin complexes. J Immunol 1997, 158:2790–2797
42. Cajot JF, Sordat I, Silvestre T, Sordat B: Differential display cloning identifies motility-related protein (MRP1/CD9) as highly expressed in primary compared to metastatic human colon carcinoma cells. Cancer Res 1997, 57:2593–2597
43. Dong JT, Suzuki H, Pin SS, Bova GS, Schalken JA, Isaacs WB, Barrett JC, Isaacs JT: Down-regulation of the KAI1 metastasis suppressor gene during the progression of human prostatic cancer infrequently involves gene mutation or allelic loss. Cancer Res 1996, 56:4387–4390