Motility-related Protein-1 (MRP-1/CD9) Reduction as a Factor of Poor Prognosis in Breast Cancer

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Abstract

The application of reliable markers is of major importance for predicting the prognosis of and instituting the appropriate post-surgical treatment of patients with breast cancer. Previously, we showed that motility-related protein-1 (MRP-1), which is identical to CD9, regulates cell motility, and that cultured tumor cells transfected with MRP-1/CD9 cDNA have low motility and low metastatic potential. In addition, MRP-1/CD9 immunoblotting and immunohistochemical study with breast cancer revealed that MRP-1/CD9 expression diminished as the clinical stage of a given breast cancer advanced, and that the MRP-1/CD9 gene and protein expression in the metastatic lymph nodes was strikingly lower than in the primary breast cancers. In this study, we also investigated the expression of MRP-1/CD9 by immunoblotting and immunohistochemical analysis in 143 freshly resected invasive ductal carcinomas of the breast: 52 tumors were stage I, 61 were stage II, and 30 were stage III. Tumors were classified as MRP-1/CD9 positive when a band intensity of >30% compared with positive control cells, ZR-75-30 were evaluated with the antibody M31-15, and those classified as MRP-1/CD9 reduced had <50% of the cancer cells immunostained with M31-15. There were 97 patients with MRP-1/CD9 positive tumors and 46 patients whose tumors had reduced MRP-1/CD9 levels. The disease-free rate of the former group of patients was strikingly higher than that of the latter (84.7% versus 51.4%, P < 0.001). Similarly, the overall survival rate was also significantly different between the two groups (93.6% versus 69.6%, P = 0.004). Multivariate analysis with the Cox regression model indicated that MRP-1/CD9 positivity correlated better with disease-free survival (P < 0.001) than estrogen receptor, tumor, and lymph node status. Our data suggest that low MRP-1/CD9 expression by tumors of the breast may be associated with poor prognosis. It is conceivable that testing for MRP-1/CD9 may identify node-negative breast cancer patients who are at high risk for early disease recurrence.

Introduction

Several randomized clinical trials have shown that adjuvant chemotherapy improves the survival of patients with breast cancer (1–3). However, because it is not clear which clinical characteristics determine the need for adjuvant chemotherapy, it is very difficult to distinguish patients for whom surgery alone is adequate from those who may benefit from both surgery and additional treatment. Hence, the search for sensitive and reliable prognostic tests is of cardinal importance since they would be valuable for identifying patients for whom intensive adjuvant therapy is warranted (3). The prognostic factors that may help predict recurrences of breast cancer include the number of involved lymph nodes, the size of the tumor, its histological grade, and estrogen receptor status. Recently, the focus pertaining to factors of potential prognostic utility has turned to the expression of molecular markers. A few of these have been reported to be associated with poor prognosis, and their presence or levels may be an indication for adjuvant therapy. For example, the c-erbB-2 oncogene overexpression is found to be associated with shorter survival, particularly in breast cancer patients with involved lymph nodes (3–5). Mutations of the tumor suppressor gene p53 also may be of prognostic importance, especially for patients with node-negative disease (6, 7). On the other hand, it has been reported that nm23 protein expression in breast cancer was associated with good prognosis and absence of lymph node metastases, but that low nm23 mRNA levels correlated with the presence of lymph node metastases at surgery (8, 9).

We have previously generated a murine MAb1, M31-15, that inhibits cell motility and established that the antibody recognizes the MRP-1,2 a transmembrane glycoprotein (10). The amino acid sequence of MRP-1 is identical to that of CD9, a WBC differentiation antigen, reported in the same year (11). MRP-1/CD9 is expressed not only by hematopoietic cells, but also by most established cell lines derived from solid human tumors. However, its role in nonhematopoietic cells remains unknown. In efforts to elucidate the functions of MRP-1/CD9 in such cells, we transfected various types of cultured cells with plasmid constructs containing human MRP-1/CD9 cDNA (12). These experiments revealed that cell motility and growth were suppressed in the MRP-1/CD9-expressing cells. In addition, investigations with mouse melanoma BL6 cells and the BALB/c-nu/nu mouse system disclosed that the metastatic potential of all transformants expressing MRP-1/CD9 was lower than that of the parent BL6 cells (12). This set of observations indicated that MRP-1/CD9 regulates cell motility, and that it is a receptor for negative signal ligands.

More recently, to investigate whether the levels of MRP-1/CD9 in tumor tissues are valuable for predicting the clinical behavior of actual human cancer, we tested MRP-1/CD9 expression in invasive ductal carcinomas of the breast with immunohistochemical study and immunoblotting. Thus, we found that MRP-1/CD9 expression diminished as the clinical stage of a given breast cancer advanced (13). Besides, primary tumors in almost half of the cases had higher MRP-1/CD9 protein levels than their respective metastatic lymph nodes. These findings, obtained with immunohistochemical and immunoblotting methods, were corroborated by determining MRP-1/CD9 gene expression with a quantitative reverse transcription-PCR-based assay. Gene overexpression was not observed in any of the samples studied (13). Moreover, we have applied quantitative reverse transcription-PCR analysis to determine MRP-1/CD9 gene expression in non-small cell lung cancer and found that low MRP-1/CD9 gene expression by tumors of the lung may be associated with poor prognosis (14). Based on these results, in this report we present the findings of the prospective evaluation of MRP-1/CD9 protein expression in tumor tissues

1 The abbreviations used are: MAb, monoclonal antibody; MRP-1, motility-related protein-1; TM4SF, transmembrane 4 superfamily.
from 143 breast cancer patients for whom adequate clinicopathological data were available.

Materials and Methods

Clinical Characteristics of the Patients. This study was carried out on 143 patients with invasive ductal carcinoma of the breast. All of them underwent mastectomy or quadrantectomy at the Department of Thoracic Surgery of the Kitano Hospital Medical Research Institute (Osaka, Japan) between May 1990 and December 1992. Complete clinical records of all of them were available, and the histopathological diagnoses were fully documented. The postsurgical pathological stage of each tumor was classified according to the TNM system (15). The salient clinical features of the patients are presented in Table 1. The median postoperative follow-up for the patients was 45.7 (range, 30–61) months. This report includes follow-up data as of July 1, 1995. Ten patients with intraductal carcinoma and nine with two or more forms of cancer were not included in the study. In addition, four women with stage IV disease were also excluded because even if their primary tumors are resected, such patients usually have cancer cells at the metastatic sites. This would make the disease-free survival analysis of these patients meaningless in the context of the present study. Eleven patients had quadrantectomy followed by immediate radiotherapy, and the other patients had no radiotherapy before recurrence. Adjuvant systemic chemotherapy was given according to estrogen receptor status, menopausal status, and lymph node involvement. Node-negative or premenopausal patients (n = 108) underwent treatment by oral 5-fluorouracil (200 mg/day) for 2 years, and, furthermore, 19 patients with N1 disease were treated with six cycles of cyclophosphamide/Adriamycin. Sixty-two estrogen receptor-positive patients were treated with tamoxifen (20 mg/day) for 2 years or before recurrence. Twenty-two patients (postmenopausal node negative and receptor positive) did not have any further adjuvant treatment. We detected distant metastases in 25 patients during the observation period, and among these 25 patients, 3 patients also had local recurrences. Twelve patients had locoregional recurrences. After recurrence, locoregional tumors or lymph nodes were principally resected and then these patients were treated with radiotherapy. Patients with distant metastases were treated with more effective adjuvant chemotherapy, including cisplatin and pirarubicin.

Surgical Specimens. One-half of each freshly resected tumor tissue was immediately embedded in optimum cutting temperature compound (Miles, Kankakee, IL) and frozen at -80°C. Frozen sections were cut on the cryostat to a thickness of 6 µm, mounted on poly-l-lysine-coated slides, and either used immediately or stored at -80°C for no more than 2 weeks. To verify the presence of cancer cells, a frozen section from each specimen was stained immediately with H&E. One-half of a given tumor sample containing only cancer cells was used for Western blotting.

Immunoblot Analysis. Tissue samples containing only cancer cells were solubilized with 1% CHAPS. An aliquot of the soluble fraction was subjected to slab gel electrophoresis, followed by Western blotting and probing with the anti-MRP-1/CD9 MAb, rabbit anti-mouse IgG (γ chain specific; Cappel, Malvern, PA) and 125I-labeled protein A (DuPont, Boston, MA); Refs. 16 and 17). The total protein content applied to the lanes was adjusted to the same concentration. Soluble extracts of the established human breast cancer cell line ZR-75-30 were used as positive controls. Band intensity was evaluated with densitometry.

Statistical Analysis. Overall survival curves and disease-free survival curves were constructed according to the Kaplan–Meier method (18). Comparisons were made with the log rank test (19). The SAS statistical package (SAS Institute, Cary, NC) was used for the Cox proportional hazards model; five factors (age at surgery, T stage, N stage, estrogen receptor status; and tumor MRP-1/CD9 status) were studied, and scores were assigned to each variable for the regression analysis (20). The association between reduction in MRP-1/CD9 expression and T stage, N stage, and estrogen receptor status was investigated using a frequency table. All P values are based on two-tailed statistical analysis; a P value of <0.05 was considered to indicate statistical significance.

Results

Specimen Classification Based on Western Blotting Results. MRP-1/CD9 expression in breast tumor tissues was analyzed using Western blotting with MAb 31-15. The major Mr 25,000 MRP-1/CD9 band is usually accompanied by a minor Mr 28,000 band. The major band was readily evident in the immunoblots of the soluble fractions of the primary tumors that were classified as positive in the immunohistochemical assays, as described previously (Fig. 1; Ref. 13). By contrast, the band intensity was weak or entirely absent in the primary invasive ductal carcinomas that had reduced immunohistochemically detectable MRP-1/CD9. To classify the specimens on the basis of Western blotting, the band intensity was weak or entirely absent in the primary invasive ductal carcinomas that had reduced immunohistochemically detectable MRP-1/CD9. To classify the specimens on the basis of Western blotting, the Mr 25,000 band obtained with the positive control cells ZR-75-30 was set at 100%. The total protein content applied to the lanes was adjusted to the same concentration. Soluble extracts of the established human breast cancer cell line ZR-75-30 were used as positive controls. Band intensity was evaluated with densitometry.

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tumor cells. When >50% of the carcinoma cells in a given specimen

were counted per X40 field. Positive tumor cells were

stained equivalent to normal breast glands and benign

fibroadenoma (Fig. 3A) since immunostaining was intense and was

seen uniformly at the cell surface membrane (Fig. 3B). There were 46

cases (32.2%) with reduced MRP-1/CD9 expression (Fig. 3C). Im-

munostaining of most of these tumors was heterogeneous, and the

staining pattern along cell junctions was not linear, but granular. In

each instance, MRP-1/CD9 expression in metastatic lymph nodes was

lower than that in the corresponding primary tumor (Fig. 3D).

All immunostained sections were examined by two pathologists

who had no knowledge of the patients’ clinical status. At least 200

tumor cells were counted per ×40 field. Positive tumor cells were

stained equivalent to normal breast glands and benign fibroadenoma
tumor cells. When >50% of the carcinoma cells in a given specimen

were positively stained, the sample was classified as MRP-1/CD9

positive, and when <50% were stained, the sample was classified as

reduced. Overall, the immunoblotting results agreed well with

those of the immunohistochemical assays, and, in seven cases of

discrepancy, the results of Western blotting were used in specimen

classification.

Relationship between Tumor MRP-1/CD9 Immunoreactivity

and Known Prognostic Factors. Analysis of the results obtained

with the 143 breast carcinomas tested revealed no statistically signif-

icant relationship ($\chi^2$) between MRP-1/CD9 expression and the

patients’ age at surgery or estrogen and progesterone receptor status,
tumor size, or postoperative hormonal therapy and chemotherapy.
However, MRP-1/CD9 expression was associated with lymph node

status ($P = 0.045$). Thus, whereas 24.7% of the patients with N0 stage
disease had reduced expression, low MRP-1/CD9 was found in 37.2% of
the N1 stage patients and 52.6% of those with N2 stage.

Association of Tumor MRP-1/CD9 Status with Disease-free and

Overall Survival. Comparing survival among the 143 patients re-

classified the disease-free and overall survival rates for the 97

patients with MRP-1/CD9-positive tumors differed significantly from

those of the 46 individuals whose tumors had reduced MRP-1/CD9

levels (Figs. 4 and 5). The disease-free survival rates were 84.7% and

51.4% ($P < 0.001$), respectively (Table 1). Moreover, tumors with

reduced MRP-1/CD9 expression of N0 and N1 disease or with T1 and

T2 disease were associated with progressively reduced disease-free

survival (Table 1). The overall survival rate of patients with MRP-1/

CD9-positive tumors was 93.6% (Table 1) and that of the individuals

whose tumors had reduced MRP-1/CD9 was 69.6% ($P = 0.004$).

Prognostic Value of MRP-1/CD9 Status. The variables used in

cox regression analysis are shown in Tables 2 and 3. The esti-

mated prognostic value of each variable in relation to disease-free

survival and overall survival among the 143 patients is expressed as a

P value. Four variables (MRP-1/CD9 status, estrogen receptor status,

N stage, and T stage) were found to be significant prognostic factors

of survival. MRP-1/CD9 had the most significant P value for disease-

free survival ($P < 0.001$), and the second with respect to overall

survival ($P = 0.0084$). Other variables (body weight, serum albumin,
carcinogenic antigen, progesterone receptor status, postoperative

hormonal therapy, and chemotherapy) had no relationship with

MRP-1/CD9 status or its prognostic value (data not shown).

Discussion

Prevention of metastasis is among the most important problems in

the treatment of patients with malignant tumors (21, 22). Despite

various types of antitumor defense mechanisms, the malignant tumor
cells that invade tissue boundaries and move through the host’s

cellular and extracellular matrix barriers resist the shear stresses
arising in the vascular bed, the frictional forces arising between their

peripheries, and the vessel walls. In addition, they traverse capillaries

that are generally rigid and whose diameter is smaller than the tumor

cells. It has been shown that motility of cancer cells is one of the

essential cellular functions closely related to the metastatic process

(21, 22).

We have previously demonstrated that motility of MAC10 cells,
derived from a human adenocarcinoma of the lung, is inhibited by

MAb M31-15, and that this antibody recognizes MRP-1, a transmem-

brane glycoprotein (10). We also determined that the sequence of

MRP-1 is identical to that of CD9, the sequence of which was reported

in the same year by another laboratory (11). Because disruption of cell
to cell contacts induces tumor cell invasion and metastases, it is of

interest in the present context that MAbs to CD9 elicit an enhance-

ment of Fc-independent heterotypic adhesion of pre-B cell lines to

bone marrow stromal fibroblasts, but not to bone marrow stroma (23).
Moreover, these antibodies specifically induce homotypic adhesion of

pre-B lymphocytes and augment neutrophil adherence to the endothe-
Fig. 3. Immunohistochemical staining of human breast tissues with the MRP-1/CD9-specific MAb M31-15. Avidin-biotin-peroxidase complex procedure. A, the cell surface in a benign fibroadenoma was strongly stained. B, positive immunostaining of an invasive ductal carcinoma of the breast. C, invasive ductal carcinoma with reduced MRP-1/CD9 expression. D, metastatic lymph node corresponding to the invasive carcinoma shown in B. Note that staining intensity is lower than in the primary tumor. A–D, ×150.

Fig. 4. Disease-free survival in 143 patients with invasive ductal carcinoma of the breast in relation to the tumor MRP-1/CD9 status (P < 0.001). P value was determined with the log rank test.

Fig. 5. Overall survival of the 143 patients with invasive ductal carcinoma of the breast in relation to the tumor MRP-1/CD9 status (P = 0.004).
product encoded by \textit{KAI1}, a recently described metastasis suppressor gene of prostate cancer, is identical to R2 and C33, which also belong to the TM4SF (28-30). It is thought that decreased \textit{KAI1} expression may be involved in the malignant progression of prostate cancer (28). These observations on other systems are very consistent with our data that the level of MRP-1/CD9 expression is inversely related to the clinical stage of a given cancer of the breast. Thus, MRP-1/CD9-positive tumors were evident in 73% of the patients whose tumors were in stages I or II, but only in 50% of patients with stage III tumors.

As the present results indicate, reduction in MRP-1/CD9 expression by primary invasive ductal carcinomas of the breast was associated with poor prognosis. The link between diminished MRP-1/CD9 and poor prognosis was independent of tumor stage, lymph node status, and estrogen receptor status. Reduction in tumor MRP-1/CD9 expression was strongly associated with an increased risk of recurrence among the patients with N0 and N1 stage disease and with T1 and T2 tumors. However, the interpretation of these findings has to be made with caution in view of the relatively short postoperative follow-up (30–61 months) of the patients.

Adjuvant endocrine therapy and chemotherapy have significantly improved the disease-free survival of patients with early stage breast cancer (1–3). However, the role of adjuvant chemotherapy after mastectomy, particularly in women whose disease is confined to the breast, has a poor prognosis, and may therefore benefit from adjuvant treatment. It is conceivable that testing tumors for MRP-1/CD9 status, in combination with other molecular and biochemical assays, may improve the prognostic evaluation of breast cancer patients and enhance the clinician’s ability to prospectively identify patients who will have early disease recurrence and who will require adjuvant chemotherapy.

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MRP-1/CD9 AS A PROGNOSTIC FACTOR OF BREAST CANCER


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