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# Serum Levels of Laminin, Type IV Collagen and Type III Procollagen Peptide as Markers for Detection of Metastasis

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**We investigated the clinical usefulness of serum laminin, type IV collagen and type III procollagen peptide (PIIIP) as markers for detection of metastasis in patients with primary or metastatic bone and soft part tumors. The subjects consisted of 28 patients with metastatic bone tumors, 18 with primary bone tumors (benign; 10, malignant; 8), 22 with primary soft part tumors (benign; 12, malignant; 10), 18 with cancer without metastasis (as controls to metastatic bone tumor) and 60 healthy controls. Elevated levels of serum laminin, type IV collagen and PIIIP were not associated with any specific histological subtype, tumor size or location, and were clearly related to evidence of metastasis. Mean serum concentrations of laminin, type IV collagen and PIIIP were significantly higher in patients with metastasis than in patients without metastasis. Positive correlations were observed among serum laminin, type IV collagen and PIIIP levels in tumor patients. The sensitivity values for laminin, type IV collagen and PIIIP in detecting metastasis were 83.7%, 83.3% and 80.5%, respectively, with specificity of 90.0%, 86.1% and 86.1%. When two of the three markers were evaluated in identical blood samples, combined sensitivity and specificity values exhibited further increases as compared to the sensitivity and specificity of each marker. The use of all three markers led to the best combined sensitivity and specificity. These findings suggest that the combination of these markers would be a valuable screening test in predicting metastasis.**

Key words: Laminin — Type IV collagen — Type III procollagen peptide — Tumor marker — Metastasis

In malignant tumor patients, therapeutic approaches and prognoses are quite different if a patient has metastases. It is often difficult to distinguish metastases from primary tumors, especially in patients with no evidence of primary tumor and in a solitary lesion, although newer diagnostic approaches, such as magnetic resonance imaging are of some help.[1,2] Thus, a sensitive method for detection of metastases is still required.

During metastatic cascades, tumor cells must pass through the extracellular matrix (basement membrane) prior to invasion into and out of the lymphatic or blood vessels.[3–5] The extracellular matrix, which consists of laminin, type IV collagen and other glycoproteins, acts as a tissue barrier in the process of metastasis formation.[4] Type IV collagen composes the polygonal network forming the main structure of the basement membrane. Laminin, which has a cell-adhesive function, binds the polygonal network of type IV collagen in the basement membrane. It has been reported that metastatic potential of tumor cells was correlated with enzymatic degradation of type IV collagen by proteolytic enzymes (metalloprotease).[6,7] Thus, in the process of tumor cell invasion into the extracellular matrix, it is considered that serum levels of matrix components such as laminin and type IV collagen might be elevated by the degradation of the extracellular matrix.

Type III collagen is found in most tissues, such as skin, arterial wall, muscle and bone marrow and periosteum.[8] Type III procollagen peptide (PIIIP) is produced in the synthetic and degradative processes of type III collagen metabolism.[9] It is suggested that the serum concentration of PIIIP is correlated with clinical behavior such as the stage of tumor, histological malignancy and metastasis and so would be useful as a prognostic indicator in various cancers.[10–12]

In the present study, we investigated the serum concentrations of laminin, type IV collagen and PIIIP in patients with primary or metastatic bone and soft part tumors and examined their usefulness as diagnostic markers for metastasis.

## PATIENTS AND METHODS

**Patients** The subjects consisted of patients receiving follow-up care at the Toyama Medical and Pharmaceutical University Hospital and Chiba Cancer Center for verified bone and soft part tumors between March 1992 and December 1993. Blood samples were taken before surgery of chemotherapy. We excluded patients operated on within 1 year before sampling (with the exception of

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patients underwent minor incisional biopsies), and patients who had received chemotherapy within 1 year before sampling. Patients with other diseases which might affect the connective tissue metabolism, such as hepatitis, liver metastasis and rheumatoid arthritis, were also excluded.

Sixty-eight samples from 68 patients met our criteria; 28 patients with metastatic bone tumors, 18 patients with primary bone tumors (benign; 10, malignant; 8 including 4 lung metastases), 22 patients with primary soft part tumors (benign; 12, malignant; 10 including 3 lung metastases and 1 brain metastases.). The primary lesion of 28 metastatic bone tumors consisted of 8 breast cancers, 6 gastric cancers, 6 lung cancers, 5 prostatic cancers, and 3 ovarian cancers. As the control for metastatic bone tumors, we investigated 18 patients with cancer without metastasis who consisted of 6 breast cancers, 4 gastric cancers, 4 prostatic cancers and 4 lung cancers (before surgery and chemotherapy). All tumors were histologically confirmed. The cancers, primary malignant bone and soft part tumors were graded as high or low malignancy using the respective histological classification. The characteristics of tumor patients are summarized in Table I.

**Analysis of serum laminin, type IV collagen and PIIIP levels** Serum concentrations of laminin and type IV collagen were measured by a sandwich enzyme-linked immunosorbent assay. In brief, each well of a 96-well

Table I. Characteristics of Tumor Patients with or without Metastasis

| Histological type | Number of patients | Counts: Histological grade malignancy low/high | Counts: Size <5 cm/>5 cm | Site of primary lesion or metastases |
|---|---|---|---|---|
| Metastasis (+) | | | | |
| Metastatic bone tumor | | | | |
| Breast cancer | 8 | 4/4 | 4/4 | bone metastases |
| Gastric cancer | 6 | 2/4 | 3/3 | bone metastases |
| Lung cancer | 6 | 3/3 | 2/4 | bone metastases |
| Prostatic cancer | 5 | 2/3 | 3/2 | bone metastases |
| Ovarian cancer | 3 | 1/2 | 1/2 | bone metastases |
| Primary bone tumor | | | | |
| Osteosarcoma | 2 | 1/1 | 1/1 | primary: extremity, metastasis: lung |
| Ewing's sarcoma | 1 | 0/1 | 1/0 | primary: extremity, metastasis: lung |
| Primary soft part tumor | | | | |
| Liposarcoma | 3 | 2/1 | 2/1 | primary: extremity, metastasis: lung |
| Leiomyosarcoma | 1 | 0/1 | 1/0 | primary: extremity, metastasis: brain |
| Metastasis (−) | | | | |
| Breast cancer | 6 | 3/3 | 4/2 | |
| Gastric cancer | 4 | 2/2 | 2/2 | |
| Lung cancer | 4 | 1/3 | 2/2 | |
| Prostatic cancer | 4 | 2/2 | 1/3 | |
| Primary bone tumor | | | | |
| Osteosarcoma | 2 | 1/1 | 1/1 | extremity |
| Chondrosarcoma | 3 | 1/2 | 2/1 | trunk: 1, extremity: 2 |
| Enchondroma | 3 | Benign | 1/2 | extremity |
| Osteochondroma | 3 | Benign | 1/2 | extremity |
| Osteoid osteoma | 2 | Benign | 1/1 | extremity |
| Giant cell tumor | 2 | Benign | 1/1 | trunk: 1, extremity: 1 |
| Primary soft part tumor | | | | |
| Leiomyosarcoma | 2 | 1/1 | 1/1 | trunk |
| Synovial sarcoma | 2 | 1/1 | 1/1 | extremity |
| MFH[a)] | 2 | 1/1 | 1/1 | trunk |
| Lipoma | 4 | Benign | 2/2 | trunk: 3, extremity: 1 |
| Hemangioma | 3 | Benign | 2/1 | trunk: 1, extremity: 2 |
| Neurofibroma | 3 | Benign | 1/2 | trunk |
| Neurilemmoma | 2 | Benign | 1/1 | extremity |

*a*) MFH: Malignant fibrous histiocytoma.

microtiter plate was sensitized with 100 $\mu$l of 10 $\mu$g/ml of anti-human laminin (Calbiochem Corp., USA) or type IV collagen (Shiseido, Tokyo) monoclonal antibody at room temperature for 1 h. The plate was washed with phosphate-buffered saline (PBS) containing 0.05% Tween 20 and 0.1% bovine serum albumin (BSA; Sigma Chemical Co., USA), then 100 $\mu$l of purified human laminin (0–100 ng/ml; Chemicon International, Inc., USA), type IV collagen (0–100 ng/ml; Collaborative Research, Inc., USA) or serum sample was placed in each well of a microtiter plate, and incubated for 2 h at 37°C. After washing three times, 100 $\mu$l of anti-human laminin diluted 1,000-fold (Fuji Yakuhin, Tokyo) or type IV collagen diluted 5,000-fold (Chemicon International, Inc., USA) monoclonal antibody was allowed to react in each well at 37°C for 2 h. The wells were washed with PBS, and then peroxidase-conjugated anti-IgG antibody (Cappel Products, USA) was added to each well and the plate was incubated for 2 h at 37°C. *o*-Phenylenediamine (40 mg/ml; Tokyo Kasei, Tokyo) was added to 100 ml of citrate buffer, and 100 $\mu$l of this solution was added to each well and allowed to react for 30 min. The reaction was halted by adding 50 $\mu$l of 0.4 *M* $H_2SO_4$, and the optical density was measured with an automatic plate reader (Model 450, Bio-Rad, USA) at absorbance 490–655 nm.

Serum PIIIP concentration was determined with the use of a commercial radioimmunoassay kit (Hoechst Japan, Tokyo).

Serum was stored at −20°C until analyses. Cut-off values of serum laminin, type IV collagen and PIIIP levels were set at 1.6 ng/ml, 5.0 ng/ml and 0.8 unit/ml (values are means+2 standard deviation (SD) in 60 age- and sex-matched healthy controls), respectively. The frequency of above cut-off value in laminin, type IV collagen and PIIIP was computed as a positive % ratio. Sensitivity was calculated as the percentage of individuals among patients with metastasis who showed serum concentrations of these markers above the respective cut-off limits. Specificity was calculated as the percentage of individuals in patients without metastasis who had concentrations of these markers within the normal range.

**Statistical analysis** Results were expressed as means± SD. Variances were analyzed by F test. Student's *t* test (equal variance groups) and the Wilcoxon U test (unequal variance group) were used for statistical comparisons between the two groups. A *P* value of less than 0.05 was considered to indicate a significant difference.

## RESULTS

**Serum concentrations of laminin, type IV collagen and PIIIP in patients with primary or metastatic bone and soft part tumors** The mean serum concentration and positive ratio of laminin in patients with metastatic bone tumor were 2.3±0.5 ng/ml and 82.1%, whereas those in cancer patients without metastasis were 1.4±0.5 ng/ml and 22.2% (Fig. 1A). Mean serum laminin level was significantly higher in patients with metastatic bone tumor as compared with the nonmetastasized cancer patients ($P<0.05$). The mean serum level of type IV collagen was significantly higher in patients with metastatic bone tumor (8.0±3.6 ng/ml, positive ratio: 78.6%) than in cancer patients without metastasis (3.4± 1.8 ng/ml, positive ratio: 27.8%) ($P<0.05$) (Fig. 1B). The mean serum level of PIIIP was also significantly elevated in patients with metastatic bone tumors (1.6± 0.8 unit/ml, positive ratio: 75.0%) as compared with that in cancer patients without metastasis (0.5±0.3 unit/ml, positive ratio: 27.8%) ($P<0.05$) (Fig. 1C).

In the primary bone and soft part tumor groups, serum levels of all three markers showed no significant difference between benign and malignant tumor groups. However, since primary malignant bone and soft part tumor patients with metastasis all showed pathologic high levels above the cut-off values for all three markers, the mean concentrations and positive ratios of the three markers were higher in malignant tumor patients than in benign tumor patients (Fig. 1).

The serum concentrations of the three markers in primary malignant bone tumor patients with metastasis (n=4) and without metastasis (n=4) were 1.8±0.3 ng/ml and 1.1±0.4 ng/ml for laminin, 5.5±0.8 ng/ml and 3.8±1.2 ng/ml for type IV collagen, and 1.0±0.4 unit/ml and 0.6±0.2 unit/ml for PIIIP, respectively, whereas those in malignant soft part tumor patients with metastasis (n=4) and without metastasis (n=6) were 1.9±0.4 ng/ml and 1.2±0.6 ng/ml for laminin, 5.6±0.9 ng/ml and 3.6±1.4 ng/ml for type IV collagen, and 0.9±0.3 unit/ml and 0.4±0.2 unit/ml for PIIIP, respectively. In the primary malignant bone and soft part tumor patients, serum levels of the three markers were significantly higher in patients with metastasis than in patients without metastasis ($P<0.05$).

**Serum concentrations of laminin, type IV collagen and PIIIP in patients with or without metastasis** The elevated levels of serum laminin, type IV collagen and PIIIP were not associated with any specific histological type, tumor size and location, and were related to evidence of metastasis. In all tumor patients, the mean serum concentrations and positive ratios of laminin, type IV collagen and PIIIP in patients with metastasis showed significantly higher levels than in patients without metastasis (Table II).

**Sensitivity and specificity of laminin, type IV collagen and PIIIP for detection of metastasis** The accuracy of positive test results to predict metastasis was 86.1% for laminin, 81.1% for type IV collagen and 80.6% for

PIIIP, and that of negative test results in predicting no evidence of metastasis was 90.0% for laminin, 86.0% for type IV collagen and 86.0% for PIIIP. The sensitivity in predicting metastasis was thus more than 80% for each marker. The specificity in predicting metastasis was also about 90% for all three markers. When two markers were determined in identical blood samples, combined sensitivity and specificity were superior to those afforded

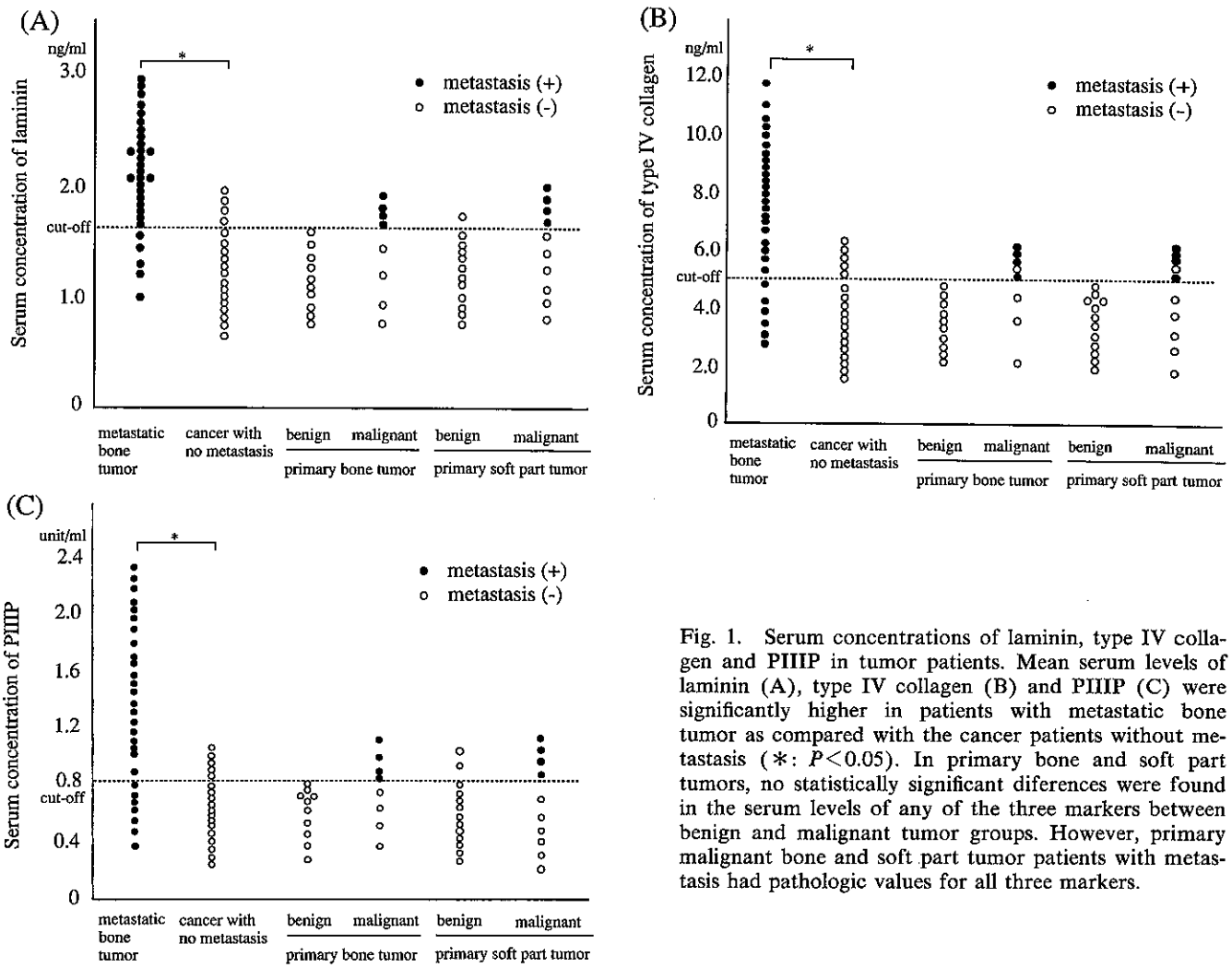


Fig. 1. Serum concentrations of laminin, type IV collagen and PIIIP in tumor patients. Mean serum levels of laminin (A), type IV collagen (B) and PIIIP (C) were significantly higher in patients with metastatic bone tumor as compared with the cancer patients without metastasis (*: $P<0.05$). In primary bone and soft part tumors, no statistically significant diferences were found in the serum levels of any of the three markers between benign and malignant tumor groups. However, primary malignant bone and soft part tumor patients with metastasis had pathologic values for all three markers.

Table II. Serum Concentrations of Laminin, Type IV Collagen and PIIIP in Patients with or without Metastasis

| Patient | Laminin (ng/ml) (positive ratio) | | Type IV collagen (ng/ml) (positive ratio) | | PIIIP[a] (unit/ml) (positive ratio) | |
|---|---|---|---|---|---|---|
| Metastasis (+) (n=36) | 2.6±0.6 (86.1%) | * | 8.6±3.8 (83.3%) | * | 1.7±0.8 (80.5%) | * |
| Metastasis (−) (n=50) | 1.4±0.5 (12.5%) | | 3.6±1.7 (17.5%) | | 0.8±0.5 (17.5%) | |

*a*) PIIIP: type III procollagen peptide.
*: $P<0.05$. Values: mean±SD.

Table III. Sensitivity and Specificity in Predicting Metastasis

| | Sensitivity (%) | Specificity (%) |
|---|---|---|
| Laminin | 83.7 | 90.0 |
| Type IV collagen | 83.3 | 86.1 |
| PIIIP[a)] | 80.5 | 86.1 |
| Laminin+Type IV collagen | 88.9 | 92.0 |
| Laminin+PIIIP | 86.1 | 90.0 |
| Type IV collagen+PIIIP | 86.1 | 90.0 |
| Laminin+Type IV collagen+PIIIP | 91.7 | 94.0 |

*a*) PIIIP: type III procollagen peptide.

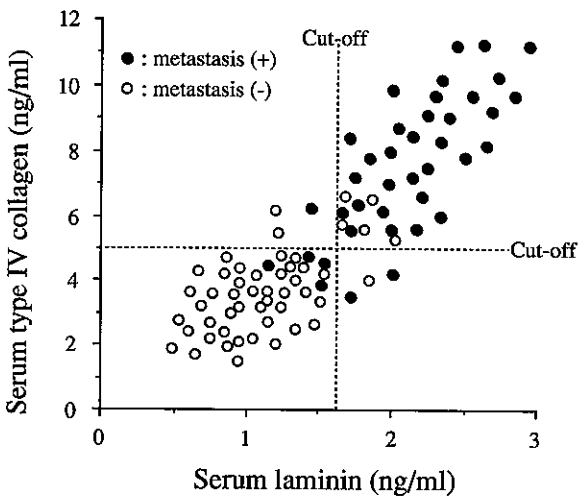


Fig. 2. Correlation of serum laminin and type IV collagen levels in tumor patients. There was a positive correlation between serum laminin and type IV collagen levels in tumor patients ($r$=0.89).

by the single use of each marker. The sensitivity and specificity disclosed the superiority of the combination of laminin with type IV collagen as compared with the combination of laminin with PIIIP or PIIIP with type IV collagen. Furthermore, the combined use of all three markers led to a further increase in sensitivity and specificity (Table III).

**Correlations among the three markers in tumor patients** There were significant positive correlations of serum concentrations between laminin and type IV collagen, laminin and PIIIP, and type IV collagen and PIIIP in tumor patients. The correlation coefficients between laminin and type IV collagen, laminin and PIIIP, and type IV collagen and PIIIP were 0.86, 0.77 and 0.75, respectively. The strongest positive correlation among the three markers was observed between laminin and type IV collagen (Fig. 2).

## DISCUSSION

In metastatic tumor cells, the penetration of cells through the extracellular matrix is indispensable for metastasis formation.[3,4)] From the results of *in vitro* invasion assay system, tumor cell invasiveness *in vitro* may be correlated with malignant potential *in vivo*.[13,14)] We have also demonstrated that the invasiveness through the extracellular matrix was higher in high-metastatic clone cells than in low-metastatic clone cells established from murine sarcoma.[15)]

In the present study, serum levels of laminin, type IV collagen and PIIIP were significantly higher in patients with metastatic bone tumor than in cancer patients without metastasis. In primary bone and soft part tumors, serum levels of all three markers showed no significant difference between benign and malignant tumor groups. However, primary malignant bone and soft part tumor patients with metastasis showed high values above the cut-off levels for all three markers. In patients with metastasis, serum levels of laminin, type IV collagen and PIIIP were significantly increased regardless of metastatic site. In all tumor patients, there was no correlation among serum levels of these markers, histological type, and size or location of tumors. Elevation of these markers was closely related to evidence of metastasis. Serum laminin, type IV collagen and PIIIP have been used as indicators of connective tissue disease, such as liver fibrosis and liver cirrhosis. In the present study, patients with liver dysfunction or liver metastases were excluded. Thus, the increases of these markers were suggested to be due to degradation of the extracellular matrix by tumor cell invasion.

We have found in the previous study that tumor cell attachment to laminin via cell surface laminin receptor might induce the production of type IV collagenase from tumor cells.[15)] Increased type IV collagenase production is associated with metastatic potential and is a good prognostic marker of malignant tumors.[7,16,17)] The triggering of extracellular matrix degradation might be mediated by the attachment of tumor cells to laminin in the extracellular matrix.[15,18)] In the process of tumor cell invasion into the matrix, it is considered that laminin is dissolved in the sera together with type IV collagen degraded by metalloproteases, because laminin binds the polygonal network composed of type IV collagen in the basement membrane. In the present study, there was a significant positive correlation between serum laminin and type IV collagen levels obtained from tumor patients. Using a murine sarcoma model of spontaneous pulmonary metastasis, we have investigated the correlation between serum levels of laminin and type IV collagen and pulmonary metastases. The results showed that serum laminin and type IV collagen levels were increased with

the advance of metastasis formation.[19] Our data provided evidence to suggest that elevation of serum laminin and type IV collagen might be caused by tumor cell invasion into the matrix during metastatic cascades.

PIIIP is a peptide produced in either synthetic or degradative processes of type III collagen metabolism. Serum PIIIP levels reflect active type III collagen turnover because the half-life of serum PIIIP is not more than a few minutes.[12] Elevation of PIIIP level might possess potential as a prognostic factor in prospective studies on various malignant tumors.[20, 21] Regarding the elevation of serum PIIIP level in the present study, the lack of correlation with histological type and the size of tumors, and the significant correlation with metastasis provide evidence to support the view that the rise in type III collagen metabolism might be due to the degradation of the extracellular matrix by tumor cells during the metastatic cascade.

In the present study, serum laminin, type IV collagen and PIIIP demonstrated high sensitivity and specificity values in cases of evident metastasis. The frequency of false-positive and false-negative rates in predicting metastasis was 13.9% and 10.0% for laminin, 16.7% and 14.0% for type IV collagen and 19.5% and 14.0% for PIIIP, respectively. These findings suggest that serum laminin, type IV collagen and PIIIP are useful markers for detection of metastasis. The simultaneous evaluation of these markers showed greater sensitivity and specificity compared with the single use of each marker for the detection of metastasis. The combined use of all three markers led to a better result in the detection of metastasis. Thus, it is suggested that combined determination of these markers is useful as a screening test for predicting metastasis, although these markers showed little organ specificity.

Tumor markers would be especially valuable for assessing prognosis, for early diagnosis of recurrence or metastasis and for early response evaluation during treatment of malignant tumors with surgery, chemotherapy or radiotherapy. We have found in murine sarcoma models that serum levels of laminin, type IV collagen and PIIIP were elevated before the microscopic appearance of metastasis in animal dissection.[19] This suggested that these markers might become positive at a very early stage of metastasis. In the metastatic process, tumor cell invasion into the extracellular matrix occurs before tumor cells penetrate and grow into the target organ. In the present retrospective study, the prognostic values for early detection of metastasis and tumor response to treatment could not be assessed, although these markers had high sensitivity and specificity values in predicting the evidence of metastasis as a screening test. The values of serum laminin, type IV collagen and PIIIP during the follow-up of nonmetastasized tumor patients are being studied to examine the possible clinical relevance of these markers for early detection of metastasis and early evaluation of response to treatment.

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