Degradation of Tenascin-C and Activity of Matrix Metalloproteinase-2 Are Associated with Tumor Recurrence in Early Stage Non-Small Cell Lung Cancer

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ABSTRACT

Purpose: To find out an effective prognostic factor for early stage non-small cell lung cancer (NSCLC), we examined the relationship of the degree of tenascin-C (TN-C) degradation in relapsed NSCLC tumors with the prognosis of the patients. The molecular mechanism of TN-C degradation was also evaluated.

Experimental Design: In 63 stage-1 NSCLC patients, TN-C protein was analyzed by Western blotting, and the activity of matrix metalloproteinase (MMP)-2 was examined by gelatin zymography in 23 stage-1 NSCLC patients.

Results: Degradation of TN-C was detected in 12 of 63 patients. TN-C degradation was detected in 9 of 17 patients (52.9%) that showed local and distant cancer recurrences. In short, in 9 of 12 patients (75%) showing TN-C degradation, lung cancer recurrence was recognized. The actual frequency of free-from-recurrence at 4 years was 28.1% in patients with tumors showing TN-C degradation, and actual frequency of free-from-recurrence at 4 years and 10 years was 82.1% and 76.6% in patients without TN-C degradation (P < 0.001). In 23 stage-1 NSCLC patients, in tumors with or without degraded TN-C, the mean ratio of tumor:normal tissue of activated MMP-2 was 3.5 ± 0.4 or 1.54 ± 0.4, respectively. Significantly increased activity of MMP-2 was recognized in tumors showing TN-C degradation (P < 0.001).

Conclusions: These results suggest that TN-C degradation is a reliable marker for recurrence potential of stage-1 NSCLC and that MMP-2 may be a protease breaking down TN-C in lung cancer.

INTRODUCTION

Surgical treatment is the most efficient therapy for primary lung cancer in the early stage. However, the 5-year survival rate is only ~80%; the remaining (20%) patients dying of recurrence of lung cancer (1–3). In these patients, it is considered that cancer cells have already metastasized via the blood or lymph streams before operation. If there is a reliable marker that can predict the recurrence in early staged cancer, these patients may receive aggressive chemotherapy that may result in an improvement of survival rate.

TN-C (4) is a component of the ECM that has been shown to be involved in tissue interactions during fetal development and oncogenesis. It is a hexomeric glycoprotein consisting of different monomer variants generated by alternative splicing (5, 6), in the range of 190–250 kDa of molecular sizes. Expression of a high molecular weight variant of TN-C has been suggested to be of significance for tumor progression in cancers (7). This variant is known to be easily broken down into the fragments by MMPs (8). We have reported recently that degraded fragments of TN-C are frequently found in lung tumors with metastasis to lymph nodes (9). In the present study we hypothesized that degraded fragments of TN-C may be potential prognostic indicators of cancer recurrence after surgical treatment.

Therefore, to assess whether TN-C may be an indicator of cancer recurrence and patient prognosis, we examined the relation of TN-C degradation with clinical characteristics in 63 cases with NSCLC at stage 1 without lymph node metastasis. Furthermore, we investigated an association of TN-C degradation with activity of MMPs, especially MMP-2.

MATERIALS AND METHODS

Patients and Methods. Between August 1989 and November 1998, tumor tissues were obtained from 63 consecutive patients with primary lung cancer in stage 1, who received curative operation with routine systematic nodal dissection of both hilar and mediastinal lymph nodes, at Mie University Hospital, Mie, Japan. The stage of the disease was classified according to the International Staging System for Lung Cancer (10). There were 51 male and 12 female patients with a mean age of 65.3 ± 8.2 years (range, 48–84 years). The pathological types were 35 squamous cell carcinomas and 28 adenocarcinomas. Twenty-eight patients had stage T1N0M0 disease and 35...
had T2N0M0 disease. The basic clinical features of the patients are summarized in Table 1.

In addition, in 23 consecutive stage-1 NSCLCs the association of TN-C degradation with the activity of MMP was evaluated.

Tumor and nontumor (normal) tissues of the resected lung were obtained during open thoracotomy, immediately frozen in liquid nitrogen, and stored at −80°C until use.

**Western Blotting.** TN-C protein in lung tumors and normal lung tissues were extracted using the method of Kusagawa et al. (9). Briefly, the urea extracts (100 μg of protein/lane) were subjected to electrophoresis in 6% SDS-PAGE, as described by Laemmli (11). Western blotting was carried out using the Amersham enhanced chemiluminescence system. Rat monoclonal antibody against human TN-C (RCB1) was used at 1.3 μg/ml, and horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was used at a 1:2500 dilution. The blots were exposed to X-ray film (Eastman Kodak Co., Rochester, NY) for different intervals (30 s to 2 min) and exposure within the linear range of resolution of the X-ray film were chosen.

**Gelatin Zymography.** The gelatinolytic activity was analyzed according to a method described previously (12). Briefly, resected tissues were homogenized in sample buffer (10 μl buffer/mg tissue) containing 10 mm Tris/HCl (pH 6.8), 20% glycerin, 2% SDS, and 0.1% bromphenol blue. The homogenates were clarified by centrifugation and then separated by electrophoresis on 10% polyacrylamide gel containing 0.1% SDS and 1 mg/ml gelatin as a substrate. Thereafter, gels were washed in the reaction buffer [50 mM Tris/HCl (pH 7.6), 0.15 M NaCl, 10 mM CaCl₂, and 0.02% NaN₃] containing 2.5% Triton X-100 for 1 h to remove SDS. The gels were then incubated for 24 h at 37°C in the reaction buffer and stained with 0.1% Coomassie Brilliant Blue R250. The location of gelatinolytic activity was detectable as a clear band in the background of uniform staining. Pro- and activated MMP-2 (gelatinase A) bands were detected at 66 and 62 kDa, respectively. The ratio of activated MMP-2 to total MMP-2 activities (62 kDa/66 kDa + 62 kDa) was calculated from their gelatinolytic activities measured by computer-assisted image analysis according to the method of Davies et al. (13). We then determined the T:N ratio of the active form of MMP-2 by comparing the percentage of the active form in tumor tissue with that in normal tissue in each case.

**Follow-up.** Patients were followed at 3-month intervals after operation. Follow-up evaluation included blood examination and chest roentgenogram at 3-month intervals, and chest computed tomography at 6-month intervals. The follow-up periods have ranged from 4 to 131 months, and the median follow-up period was 62 months.

**Statistics.** The association between different variables was analyzed by the X² test.

The duration of the free-from-recurrence period was measured from the date of operation until the first evidence of recurrence from primary NSCLC or the last date of follow-up for patients who remained alive and with no recurrence. Survival was calculated from the date of operation until death or the date of the last follow-up. The free-from-recurrence period and survival were analyzed according to the Kaplan-Meier method, and differences in their distribution were evaluated by the log-rank test. Cox proportional hazards models were applied for univariate analysis. A P < 0.05 was defined as being statistically significant.

**RESULTS**

**Detection of TN-C Degradation in Lung Tumors.** On immunoblotting, the TN-C reactive bands sized 250 kDa and 190 kDa were observed in all 10 cases of normal lung tissues, but no bands sized <190 kDa were found in normal lung tissues (Fig. 1). In neoplastic tissues, bands sized 190 kDa and sized 250 kDa were observed in all 63 of the cases; in addition, bands <190 kDa were detected in 12 of 63 (19.0%) cases (Fig. 2). These small bands were concluded to be generated by degradation of TN-C.

**Follow-up.** During the follow-up period, local and distant cancer recurrence was identified in 17 of 63 cases (26.9%) of stage-1 patients. The sites of recurrence include the bone in 2, the other lung lobe in 10, the brain in 3, the mediastinal lymph node in 1, and in the same lung in 1 patient. TN-C degraded fragments were observed in 12 patients (19%). No relationship was found between the histological type and TN-C degradation (Table 2). However, in recurrence tumors, small-sized bands were observed in 9 of 17 cases (52.9%); there was a significantly higher incidence of small-sized bands in patients with recurrence than in those without recurrence (6.5%; P < 0.001; Table 3).

The total 5-year survival rate was 73.9% for TN-C degra-
dation-negative patients and 45.8% for TN-C degradation-positive patients (P = 0.028; Fig. 3). The actual frequency of free-from-recurrence at 4 years was 28.1% for patients with TN-C degradation. The 4-year and 10-year free-from-recurrence for patients without TN-C degradation was 82.1% and 76.6%, respectively (P = 0.001; Fig. 4). Irrespective of sex, age, histological type, and operative procedure, the Cox regression analysis revealed that TN-C degradation was a predictor of lung cancer recurrence (hazard ratio, 9.353; 95% confidence interval, 3.447–25.376; P = 0.001).

MMP-2 Gelatin Zymography. The latent (inactive) and active MMP-2 migrated at positions of 66 and 62 kDa, respectively. Representative cases are shown in Fig. 5. Gelatin zymography demonstrated that the inactive form of MMP-2 is present in all 23 of the tumor and normal tissue samples examined. Although activated MMP-2 could be also seen in normal tissues, the activation ratio of MMP-2 in tumors (45.5 ± 8.4%) was significantly higher than that in the normal counterparts (25.9 ± 10.5%) as seen in Fig. 5.

Among 23 cases of lung cancer, TN-C degradation was observed in 5 cases. The MMP-2 T:N ratio was compared between tumors with and without TN-C degradation. The mean value for the tumor with TN-C degradation was 3.5 ± 0.4 (Fig. 5A). It was significantly higher than that without TN-C degradation (1.5 ± 0.4; Fig. 5B; P < 0.001).

DISCUSSION

TN-C, a component of the ECM, is produced by some carcinoma cells and mesenchymal cells surrounding the tumor (14–16). It includes two major TN-C variants with masses of ~250 and 190 kDa. These variants are generated by alternative splicing of TN-C RNAs. The larger variant was predominantly found in cancers of breast, prostate, and colon (7, 17, 18). We also reported that preferential expression of the high molecular weight variant is observed in lung cancer tissues (9). In regard to the biological activity of the alternative spliced domain of TN-C, previous in vitro studies show down-regulation of focal adhesion in cultured cells (19) and stimulation of cell migration in corneal epithelial cells (20). Using recombinant fragments of human TN-C, the alternative spliced domain was found to bind...
Clinicopathological studies have demonstrated the relationship between TN-C expression and metastasis (30, 31). Proteolytically digested TN-C can be also found in colon cancer (32), and degraded TN-C molecules were observed in lung cancer cases with lymph node metastasis at a high frequency (9). Furthermore, the present study demonstrates a significantly higher recurrence rate in early staged lung cancer patients with TN-C degradation than in those without TN-C degradation. The amount of degraded fragments of TN-C is considered to be related to MMP activities reflecting dynamic remodeling of cancer tissues. However, it is not clear whether TN-C degraded fragments are simply products of ECM degradation or whether the fragments per se have biological activities enhancing cancer invasion and metastasis.

In conclusion, in this study we analyzed the pattern of TN-C expression and its relation to clinic outcome in early staged lung cancer patients. Our data showed that significantly higher recurrence incidence is recognized in patients with TN-C degradation. These results suggest that TN-C degradation could be used as a prognostic indicator for recurrence potential of early stage lung cancer, and that active adjuvant therapy and careful follow-up was necessitated in the patients with TN-C degradation. In addition, we found that significantly higher activities of MMP-2 were detected in patients with TN-C degradation. These results suggest that MMP-2 may be a protease degrading TN-C in lung cancer. Additional in vitro studies necessitate elucidating the relationship between MMP-2 and TN-C in lung cancer.

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