N-Cadherin Is Regulated by Activin A and Associated with Tumor Aggressiveness in Esophageal Carcinoma

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ABSTRACT

Purpose: Activin A is a member of the transforming growth factor β superfamily and plays an important role in the differentiation of embryonic stem cells. We have reported previously that the expression of activin A is associated with lymph node metastasis in esophageal cancer, and our purpose in the current work is to clarify the molecular mechanism of the aggressive behavior of tumors that have high activin A expression.

Experimental Design: We have compared the gene expression profiles of human esophageal carcinoma cell lines that were stably transfected with activin βA, which is a subunit of activin A, with those of control human esophageal carcinoma cell lines, using a cDNA microarray.

Results: We found that the expression level of neuronal cadherin (N-cadherin) was higher in the transfectants than in the control cells. N-cadherin was located on the cell surface of the transfectants, irrespective of the expression of epithelial cadherin (E-cadherin), and the expression of N-cadherin mRNA was significantly associated with that of neuronal cadherin (n = 51; r = 0.855). A clinicopathologic analysis suggested that expression of N-cadherin mRNA was associated with the depth of tumor wall invasion, and a group of patients with high expression of N-cadherin mRNA showed a significantly poorer prognosis than a group of patients with low N-cadherin expression (P = 0.046).

Conclusions: These results indicate that activin A might mediate the expression of N-cadherin and that this may be associated with depth of invasion and poor prognosis.

INTRODUCTION

We have reported previously that overexpression of activin A in esophageal carcinoma tissues is associated with lymph node metastasis (1), and there have also been studies that have shown elevated activin A expression in pancreatic (2), prostate (3), ovarian (4), and colon (5) carcinomas. Moreover, patients with endometrial and cervical carcinomas have high serum levels of activin A (6), and activin A also stimulates the growth of BALB/c 3T3 fibroblasts, granulosa cells, and ovarian carcinoma cell lines (7–9). Interestingly, mice deficient in inhibin A, which is a specific inhibitor of activin A, exhibit high levels of circulating activin and develop gonadal stromal tumors, raising the possibility that increased activin expression may be tumorigenic under certain circumstances (10).

In the current study, we have used two esophageal carcinoma cell lines stably transfected with activin βA, a subunit of activin A, and investigated their gene expression profiles using a cDNA microarray analysis. We show that N-cadherin expression levels increased significantly in the two activin βA transfectants and that N-cadherin was expressed on the cell surface of the transfectants. As far as we are aware, there are no reports of the expression of N-cadherin in esophageal carcinoma cell lines and/or tissues. The expression of N-cadherin is associated with carcinoma cell invasion in breast carcinoma cell lines (11, 12), and N-cadherin induced by transforming growth factor β in carcinoma cells has been shown to contribute to carcinoma cell migration in a mouse fibroblast cell line (13). Hence, it is of interest to understand whether activin βA mediates N-cadherin expression in esophageal carcinoma tissues and to study the clinical significance of N-cadherin. To this end, we investigated the expression of activin βA and N-cadherin mRNA expression using real-time polymerase chain reaction (PCR), and we studied the association of clinicopathologic factors and prognosis with mRNA expression levels.

MATERIALS AND METHODS

Patients and Sample Collection

Activin A and related genes were investigated in a series of 65 esophageal carcinoma specimens from patients who underwent surgery at the Medical Institute of Bioregulation Hospital, Kyushu University and at the Saitama Cancer Center. All 65 patients were clearly diagnosed with esophageal carcinoma based on clinicopathologic findings. All patients underwent a...
reseion of the primary tumor, and no patients received chemotherapy or radiotherapy. The specimens were collected from the tumor edge, thus avoiding the necrotic center, immediately after resection and were quick-frozen in liquid nitrogen and stored at −80°C until processing. Corresponding normal mucosa specimens were obtained by sharply dissecting the mucosa from the muscularis propria.

Cell Lines and Activin βA Transfection

Esophageal carcinoma cell lines (KYSE110 and KYSE140) were obtained from the Department of Surgery and Surgical Basic Science, Kyoto University (Kyoto, Japan). KYSE110 and KYSE140 stably transfected with activin βA were then prepared as follows. The PCR product of full-length activin βA cDNA was ligated into pCDNA 3.1 expression vector (Invitrogen, Carlsbad, CA), and it was confirmed that no mutation had occurred. After transient transfection with activin βA using LipofectAMINE (Life Technologies, Inc., Rockville, MD), stable activin βA transfectants were selected using Geneticin (G418; Life Technologies, Inc.). Expression of activin βA mRNA was confirmed by both Northern blot and Western blot (Fig. 1). All cell lines were maintained in RPMI 1640 (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% fetal bovine serum (Equitech-Bio, Inc., Kerrville, TX).

RNA Extraction and Northern Blot

Total RNA Isolation. Frozen tissue specimens or cultured cell lines in a state of subconfluence were homogenized in 0.85 mol/L guanidine thiocyanate, and total RNA was obtained by ultracentrifugation through a cesium chloride cushion, as described previously (14).

RNA Analysis. An equal amount (15 μg) of total cellular RNA was loaded onto each lane of a 1.2% agarose gel and electrophoresed for 7 hours. The RNA samples were then transferred to nylon membranes (Hybond-N+; Amersham Pharmacia Biotech United Kingdom, Inc., Little Chalfont, United Kingdom). The membranes were cross-linked with UV Stratalinker 1800 (Stratagene, La Jolla, CA). After overnight hybridization at 42°C, the blots were washed at a final stringency of 0.1× saline-sodium phosphate-EDTA and 0.1% SDS at 65°C. Autoradiography was performed at room temperature with an identifying screen using a BioImage analyzer BAS 2500 (Fuji Photo Film Co., Tokyo, Japan).

Probe Preparation. The DNA probes for the open reading frame of activin βA were subcloned into pCR II cloning vector (Invitrogen), digested with EcoRI, loaded onto 1.0% agarose gels, and electrophoresed. The inserts were extracted using a gel extraction kit (TaKaRa Inc., Otsu, Japan) and labeled with [32P]dCTP using a random-primed DNA labeling kit (TaKaRa Inc.). A DNA probe for gliceraldehyde-3-phosphate dehydrogenase (GAPDH) was glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was made as described previously (15).

Reverse Transcription-Polymerase Chain Reaction (LightCycler Technique). Complementary DNAs were synthesized from 8 μg of total RNA in a 30-μL reaction mixture, as described previously (15). The products were loaded onto 1.2% agarose gels with 400 ng/mL ethidium bromide and electrophoresed. Real-time, no-terminated PCR for activin βA and N-cadherin cDNA was performed using a LightCycler thermal cycler system (Roche Diagnostics, Basel, Switzerland), according to the manufacturer’s instructions. The primers used were as follows: activin βA sense, 5’-TGGCAGAAGAAAGAAAGAAAAAG-3’; activin βA antisense, 5’-CCGAGATTTGGCGATGATACG-3’; N-cadherin sense, 5’-GGCCCTACTAGGTTA-CCTCAA-3’; N-cadherin antisense, 5’-AGCGGAGTGGATG-GTCCAAATTT-3’; E-cadherin sense, 5’-TGCTGATCTGCTG-TTTCTTCG-3’; E-cadherin antisense, 5’-CCGCTCTCTCTCTC-GTTATC-3’; and, as described previously (16), GAPDH sense, 5’-TTGGATTCGGAAAGGACTCA-3’; and GAPDH antisense, 5’-TGCATCATATTTGGCAGGTTT-3’. For PCR, 1 μL of RNA was placed into a 19-μL reaction volume containing 0.67 μL of primer, 1.2 μL of 25 mmol/L MgCl2, and 2 μL of LightCycler-FastStart DNA Master SYBR Green I mix (Roche Diagnostics). The protocol included a denaturation step at 95°C for 600 seconds followed by 40 cycles of 95°C denaturation for 20 seconds, 55°C annealing for 20 seconds, and 72°C extension for 20 seconds. Negative controls were run concomitantly to confirm that the samples were not cross-contaminated. A sample with 1 μL of diethylpyrocarbonate-treated water instead of RNA was concomitantly examined for each of the reactions described above. To confirm the amplification specificity, the PCR products were subjected to a melting curve analysis. A positive control using RNA obtained from the KYSE70 cell line was also performed for each LightCycler run.

Fig. 1 Expression of activin βA by Northern blot (A) and Western blot (B). A. Whereas the expression level of 28S is almost the same in the two transfectants (KYSE110A and KYSE140A) and control cells (KYSE110M and KYSE140M), expression of activin βA is not detected in KYSE110M and KYSE140M cells but is observed in KYSE110A and KYSE140A cells. B. Expression of activin A is not detected in KYSE110M and KYSE140M cells. However, expression of pro-activin A and activin A is detected in KYSE110A and KYSE140A cells. The Western blot shows that activin A is produced in the transfectants (KYSE110A and KYSE140A) via cleavage of pro-activin A.
Western Blot

Total protein was extracted from the esophageal carcinoma cell lines using 1× sample buffer, as described elsewhere (17). Identical aliquots (30 μg) of total protein were applied to several 10% acrylamide gradient gels. After electrophoresis, the samples were electroblotted onto a polyvinylidene difluoride membrane (Immobilon; Millipore, Bedford, MA) at 0.5 A for 0.5 hour at 4°C. Activin βA and N-cadherin were detected using mouse monoclonal primary antibodies to activin βA (R&D Systems, Minneapolis, MN) and N-cadherin (Transduction Laboratories, Lexington, KY) in an immunoblot analysis at a dilution of 1:1,000. The blots were developed using horseradish peroxidase-linked antimouse immunoglobulin whole antibody (Promega, Madison, WI). Signals for activin βA and N-cadherin were detected using Supersignal (Pierce, Rockford, IL). Pre-stained high molecular weight markers were also run on the gels (Amersham Pharmacia Biotech United Kingdom, Inc.).

Complementary DNA Microarray

A cDNA microarray (human cancer chip version 2.1; TaKaRa Biochemicals, Tokyo, Japan) was used, and the analysis was performed as follows. For competitive hybridization, cDNAs carrying a Cy5- or Cy3-dUTP (Amersham Pharmacia, Piscataway, NJ) label were constructed from 15 μg of total cellular RNA of control (mock-transfected) cells (KYSE110M and KYSE140M) and activin βA transfectants (KYSE110A and KYSE140A) using a RNA labeling kit (TaKaRa Biochemicals). The microarray was then hybridized to the probes. After overnight hybridization at 65°C, the slide was washed twice in 2× SSC and 0.5% SDS for 30 minutes at 55°C, washed in 2× SSC and 0.5% SDS for 5 minutes at 65°C, and then washed in 0.05× SSC and 0.5% SDS for 5 minutes at room temperature. The slides were then scanned using a GMS418 (Molecular Dynamics, Sunnyvale, CA) with separate measurement of the sample intensities for Cy3 and Cy5. The intensity of each hybridization signal was evaluated photometrically using Imagene software (BioDiscovery, Marina Del Rey, CA) and normalized to the average signal of a housekeeping gene, GAPDH. The Cy3/Cy5 ratio for each sample was calculated by averaging the spots, and the cutoff value for each expression level was calculated according to the background noise. For this cutoff, we used an expression level of above 100 where the fluctuation was less than a critical value (1.0) because other genes (those with low expression) are embedded in the background noise. Up-regulated and down-regulated genes in the activin βA transfectants were defined as those having a Cy3/Cy5 signal ratio of >2.0 and <0.50, respectively.

Immunofluorescence Detection

Cells that were subsequently to be stained for N-cadherin and E-cadherin (Transduction Laboratories) were grown on coverslips and fixed in 4% formaldehyde at 4°C. They were subsequently permeabilized in PBS containing 0.1% Triton X-100 for 10 minutes. Nonspecific sites were blocked with 3% bovine serum albumin, and the cells were then incubated with diluted primary antibody (1:100) for 1 hour and visualized by fluorescence with a Zeiss Axiovert fluorescence microscope, using a secondary antibody conjugated to Alexa Fluor (Molecular Probes, Eugene, OR).

Quantitation and Statistical Methods

The association between the variables was tested by Student’s t test or Fisher’s exact probability test. A prognosis analysis was performed by the Kaplan-Meier method (log rank; Mantel-Cox method). Correlation between the variables was tested using StatView J-4.5 software (SAS Institute, Cary, NC).

RESULTS

Activin A Expression in Activin βA Transfectants and Identification of Elevated N-Cadherin Expression by cDNA Microarray Analysis. The open reading frame of activin βA cDNA subcloned in pcDNA 3.1 was transfected into two esophageal carcinoma cell lines (KYSE110 and KYSE140). Expression of activin βA mRNA was shown by a Northern blot (Fig. 1A), and expression of activin A itself was shown by a Western blot (Fig. 1B). These results show that the transfection of activin βA was successful and that the full-length form of activin A was produced by the activin βA transfectants.

A cDNA microarray analysis was then performed on the transfectants and control cells. The number of up-regulated and down-regulated genes is two and six, respectively. Among those are two genes confirmed reproducibly using reverse transcription-polymerase chain reaction (RT-PCR): N-cadherin and keratin 18. The expression of N-cadherin in the two transfectants (KYSE110A and KYSE140A) was higher than that in the control cells (KYSE110M and KYSE140M). The expression ratios of N-cadherin (transfectant versus control) for KYSE110 and KYSE140 were 6.50 and 2.50, respectively.

N-and E-Cadherin Expression and Localization in Cell Lines. Based on analysis of the Western blot, N-cadherin expression was observed in both control cell lines, but the level of expression of N-cadherin was higher in both transfectants. In contrast, the expression level of E-cadherin was unrelated to activin βA transfection. Hence, E-cadherin was expressed in KYSE110M cells, but KYSE110A cells showed little E-cadherin expression. In contrast, E-cadherin was only weakly expressed in KYSE140M cells but was strongly expressed in KYSE140A cells.

Immunofluorescent staining showed a slight background stain for KYSE110M cells (Fig. 2B) but a honeycomb-like stain for KYSE110A cells (Fig. 2C). This suggests that N-cadherin was not localized on the cell surface of KYSE110M cells but was present on the surface of KYSE110A cells. The same results were observed for KYSE140M and KYSE140A cells (Fig. 2D and E). E-cadherin was weakly expressed on the cell surface of KYSE110A cells but was present on the surface of KYSE110M cells (Fig. 2F and G). However, E-cadherin was only weakly expressed on the cell surface of KYSE140M cells (Fig. 2H) but was strongly expressed on the surface of KYSE140A cells (Fig. 2I). The expression of N-cadherin on the cell surface of both transfectants might have a function in cell-to-cell adhesion and may be mediated by activin A.

Correlated Expression of Activin βA and N-Cadherin in Esophageal Carcinoma Tissues. A quantitative RT-PCR was performed on mRNA derived from 65 esophageal carcinoma tissues using real-time PCR. The expression levels of activin βA, N-cadherin, E-cadherin, and GAPDH were calculated relative to their expression in a positive control cell line,
KYSE70. To adjust the expression levels of activin A, N-cadherin, and E-cadherin in each sample, we used the expression of GAPDH in the sample and the control, using the following equations: the value for activin A expression = log(activin A/GAPDH); the value for N-cadherin expression = log(N-cadherin/GAPDH); and the value for E-cadherin expression = log(E-cadherin/GAPDH). We then examined the correlation among the values for activin A, N-cadherin, and E-cadherin. The correlation coefficient between activin A and N-cadherin was 0.882 (Fig. 3A), and that between activin A and E-cadherin was 0.302 (Fig. 3B).

Clinical Significance of the Expression of N-Cadherin. The expression levels of N-cadherin in tumor and normal tissues were calculated as described above. To adjust these data for the individuality of patients, we used the tumor to normal (T/N) ratio, as described previously (18), to analyze their significance in terms of clinicopathologic factors and prognosis. The T/N ratio ranged from 0.00 to 155, with an average of 9.47 and a median value of 1.84. Forty-four patients (61.2%) had a T/N ratio > 1.00, indicating that the expression of N-cadherin in carcinoma tissues was higher than that in normal tissues in more than half of the cases. We classified the patients into two groups based on a cutoff value of 2.00 for the T/N ratio, a high expression group (n = 33) and a low expression group (n = 32).

An analysis of clinicopathologic factors performed with reference to the high expression and low expression groups is shown in Table 1. A significant difference in the depth of wall invasion was observed between the two groups (P = 0.035). Because most clinical cases in this analysis were of an advanced stage (stage I, 2 cases; stage II, 1 case; stage III, 24 cases; stage IV, 16 cases), it was difficult to analyze the role of N-cadherin expression in the early clinical stage. Furthermore, there were no cases of carcinoma in situ, and the number of lymph node metastasis-negative cases was only 17. Hence, no significant difference was observed between the two groups for any other clinicopathologic factor. However, the high expression group showed a poorer prognosis than the low expression group (Fig. 4; P < 0.05).

DISCUSSION

Activin A, which is a member of the transforming growth factor β superfamily, has the ability to induce embryonic stem cell differentiation. Hence, it is possible to produce certain organs (heart, liver, kidney, and so forth) by exposing a Xenopus embryo to an appropriate concentration of activin A in culture medium (19).

As described above, several studies have examined the expression of activin A in pancreatic, prostate, ovarian, and colon cancers (2–5). Moreover, we have reported previously that activin A is overexpressed in esophageal squamous cell carcinoma tissues, that almost all esophageal squamous cell
carcinoma cell lines express activin A, and that its expression is associated with tumor aggressiveness, irrespective of the histologic differentiation of the carcinoma cells. To clarify the molecular mechanisms of the aggressive behavior of tumors with high expression of activin A, we established activin A transfectants of two esophageal cancer cell lines. A cDNA microarray analysis of these cells showed that they overexpressed N-cadherin.

It is of interest to determine whether activin A mediates the expression of N-cadherin in vivo. Here, we have shown that the expression of mRNA for N-cadherin and activin βA in esophageal carcinoma tissues is significantly correlated (r = 0.882; P < 0.0001; Fig. 3A), suggesting that activin A might mediate the expression of N-cadherin in these tissues. On the other hand, the expression of E-cadherin mRNA was slightly correlated with that of activin βA (r = 0.302; P = 0.0126; Fig. 3B).

There are many reports suggesting that the expression of N-cadherin is strongly associated with invasive aspects of cancer cells in vitro. In breast carcinoma, N-cadherin promotes carcinoma cell migration regardless of the presence or absence of E-cadherin (11). In melanoma and breast and prostate adenocarcinoma, a switch in expression from E-cadherin to N-cadherin occurs during melanoma development, and the expression of N-cadherin promotes cancer cell migration (12, 20, 21). However, these reports also indicated that N-cadherin may promote carcinoma cell migration, regardless of the switch in expression levels of E-cadherin and N-cadherin.

There are no reports concerning the clinicopathologic significance of N-cadherin expression. Hence, our data obtained in clinical samples of esophageal carcinoma represent the first

<table>
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<tr>
<th>Table 1</th>
<th>Clinicopathologic characteristics of N-cadherin mRNA expression in esophageal carcinoma</th>
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<tbody>
<tr>
<td></td>
<td>N-cadherin ≤ 2.00</td>
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<tr>
<td>Age (y)</td>
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<tr>
<td>&lt;65</td>
<td>19</td>
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<tr>
<td>≥65</td>
<td>14</td>
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<tr>
<td>Sex</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>26</td>
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<tr>
<td>Female</td>
<td>2</td>
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<tr>
<td>Histological differentiation</td>
<td></td>
</tr>
<tr>
<td>Well/moderate</td>
<td>26</td>
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<tr>
<td>Poor</td>
<td>6</td>
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<tr>
<td>Other</td>
<td>4</td>
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<tr>
<td>Depth of invasion</td>
<td></td>
</tr>
<tr>
<td>Submucosa/muscularis propria</td>
<td>10</td>
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<tr>
<td>Adventitia</td>
<td>23</td>
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<tr>
<td>Lymph node metastasis</td>
<td></td>
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<tr>
<td>Absent</td>
<td>9</td>
</tr>
<tr>
<td>Present</td>
<td>24</td>
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<td>Vascular permeation</td>
<td></td>
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<td>Absent</td>
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<tr>
<td>Present</td>
<td>24</td>
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<tr>
<td>Lymph permeation</td>
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<tr>
<td>Present</td>
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<tr>
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<tr>
<td>III/IV</td>
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</table>

NOTE. This classification is based on that of the Japanese Esophageal Cancer Committee. This analysis includes 60 squamous cell carcinosmas, 3 basal cell carcinosmas, and 2 adenocarcinosmas.

Fig. 4 Kaplan-Meier overall survival curves in patients with esophageal carcinomas. Cases with N-cadherin expression of T/N ratio of ≥2.00 showed a poorer prognosis than those with T/N ratio of <2.00. Use of the Mantel-Cox method to compare the two groups showed a significant difference (P < 0.05).
evidence of such clinicopathologic significance. Our data suggest that the expression of N-cadherin mRNA is associated with the depth of wall invasion (Table 1), and this finding is consistent with previous reports regarding carcinoma cell migration in vitro. With regard to disease prognosis, the group of patients with high N-cadherin expression had a poorer prognosis than the group of patients with low N-cadherin expression group. Because the expression of N-cadherin in carcinoma cell lines shows an invasive phenotype, it is important to determine whether there is an influence of the depth of wall invasion on N-cadherin expression as a prognostic factor. Our findings showed such an association ($P = 0.035$). Moreover, univariate and multivariate analyses of 128 patients have shown that the depth of wall invasion and lymph node metastasis were significantly different prognostic factors (22). This report and our results suggest that high N-cadherin expression on the carcinoma cell surface is mediated by activin A and is associated with tumor aggressiveness and a poor prognosis based on the depth of wall invasion.

ACKNOWLEDGMENTS
We are grateful to M. Ikeda, J. Miyake, K. Ogata, and T. Shinooka for excellent technical assistance.

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