**Hepatoma-derived Growth Factor Is Associated with Reduced Sensitivity to Irradiation in Esophageal Cancer**

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**Abstract**

Radiotherapy is a useful component of treatment for esophageal cancer. Identification of the genes that are differentially expressed between radiosensitive and radioresistant cancer cells is important for predicting clinical effectiveness of radiotherapy. We established human esophageal cancer cell lines resistant to X-ray. Using differential display, we obtained one gene that was expressed in radiosensitive cells but was rarely expressed in radioresistant cells, and that gene was identical with hepatoma-derived growth factor (HDGF), a acidic polypeptide with mitogenic activity for fibroblasts. The semi-quantitative reverse transcription-PCR assay confirmed that HDGF mRNA expression was reduced in established radioresistant cells, and its reduction was associated with reduced sensitivity to irradiation. Radiotherapy was more effective in clinical cases with high HDGF mRNA expression compared with cases with low expression (P < 0.05). The findings demonstrate that HDGF may play an important role in radiosensitivity, and it could be a novel marker predicting effectiveness of radiotherapy in clinical cases.

**Introduction**

The prognosis of patients with esophageal cancer is poor. A combined surgical and chemo-radiotherapy treatment is needed in most cases, except for those with early disease. Radiotherapy is effective in selected patients, but there exist patients who show no response and suffer from side effects such as immunosuppression. Thus, it is very important to clarify markers that are useful to estimate the response to radiotherapy before treatment. Recently, the relationship between radiosensitivity and the function or expression of several genes has been reported, including p53 (1–3), jun (4, 5), ras (6, 7) raf-1 (8), src (4, 9), erbB (4), bcl-2 (10), and FGF**2** (11, 12). However, thus far, little is known about the clinical significance of these genes to estimate radiotherapy effectiveness. We have tried to identify differentially expressed genes between radiosensitive and radioresistant esophageal cancer cell lines. Radioresistant cell lines were established by a continuous challenge of the parent cell line with X-ray irradiation. Next, we applied the differential display technique, which is a PCR-based method of differential expression and subsequent cloning, to the parent (radiosensitive) and subsequent radioresistant cell lines. Consequently, one interesting gene, that of HDGF, was identified; its expression was suppressed in radioresistant cell lines. HDGF had been purified from the conditioned medium of the human hepatoma-derived cell line, HuH-7, and is an acidic polypeptide with growth-stimulating activity for fibroblasts (13–16). Finally, for the purpose of the clinical application of this gene, we studied the expression status of HDGF mRNA in biopsy specimens obtained from “radio-effective” and “non-effective” cases of esophageal cancer. Here, we report the identification of the HDGF gene and the relationship of its expression status to radiosensitivity in clinical cases of esophageal cancer.

**Materials and Methods**

**Cell Lines and Culture.** The human esophageal squamous cancer cell lines TE-2, TE-3, TE-9, TE-11, and TE-13 were provided from the Cell Resource Center for Biomedical Research Institute of Development, Aging and Cancer (Tohoku University, Japan). Cell lines KYSE110 and KYSE410 were a kind gift from Dr. Shimada (Kyoto University, Kyoto, Japan). They were cultured in RPMI-410 (Life Technologies, Inc., Rockville, MD) with antibiotics, 100 units/ml of penicillin and 100 μg/ml of streptomycin, and 10% fetal bovine serum.

**Establishment of Radioresistant Cell Lines.** The method for establishing resistant cell lines to anticancer drug (17) was applied to irradiation. On day 1, cells were counted and passed. On day 2, cells were treated with 2 Gy of X-ray irradiation (100 kV and 3.5 mA for 5 min) using an X-ray generator (MAB-1505R; Hitachi Medical Co., Tokyo, Japan); they were cultured in conditioned medium before the next passage on day 15. This challenge was repeated every 2 weeks until the radioresistant cell lines were established.

**Detection of Apoptosis after X-ray Irradiation.** The sensitivity to irradiation was confirmed by apoptosis after bolus 10 Gy of X-ray irradiation. Apoptotic cells were stained with APO 2.7 (Immunootech, Marseille, France), an antibody to a mitochondrial membrane protein, and examined with the FACScan (Becton Dickinson, Sunnyvale, CA; Ref. 18).

**RNA Extraction.** Total RNA was prepared by a modification of the guanidinium thiocyanate method as described previously (19). In addition, 50 μg of total RNA were treated with 1 unit of DNase I (Message Clean kit; Gene Hunter, Nashville, TN). The treated RNAs were dissolved in diethyl pyrocarbonate-treated water to a concentration of 1.0 μg/μl and then stored at −90°C until use.

**Differential Display.** The mRNA expression in three pairs of esophageal cancer cell lines (radiosensitive and radioresistant) were analyzed by differential display. Because there have been several reports regarding false-positive results of differential displays (20), we used the modified protocol of fluorescent differential displays (21). The cDNAs were synthesized from 2.5 μg of total RNA using anchor primers, gT15X (where X represents G, C, or A). The anchor primers were end-labeled with [γ-32P]adenosine triphosphate (Amerham, Tokyo, Japan) using T4 polynucleotide kinase (New England Biolabs, Beverly, MA). A differential display PCR amplification was performed with 25 cycles for 1 min at 95°C, 1 min at 40°C, and 1 min at 72°C in a thermal cycler ASTEC PC800 (ASTEC, Fukuoka, Japan). The amplified cDNA was electrophoresed on 6% polyacrylamide sequence gels. The gels were dried and analyzed using a BAS1000 Bio Image Analyzer (Fuji, Kanagawa, Japan). Any interesting bands were cut out and cloned by the TA cloning method (Invitrogen, San Diego, CA). These clones were sequenced by an ABI PRISM 310 DNA sequencing System (Applied Biosystems, Foster City, CA) and analyzed by the GenBank using BLAST, FAST, and EST homology search programs.

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**The abbreviations used are:** FGF, fibroblast growth factor; bFGF, basic FGF; HDGF, hepatoma-derived growth factor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RT-PCR, reverse transcription-PCR; RSG, radiosensitive gene.
RNA Analyses: Northern Blotting. An equal amount (15 μg) of total cellular RNA was loaded onto each lane of 1.2% agarose-formaldehyde gels and electrophoresed for 7 h. The RNAs were transferred to nylon membranes (Hybond-N+; Amersham Pharmacia Biotech UK, Inc., Little Chalfont, United Kingdom). The membranes were UV cross-linked with 120,000 mJ/cm² using an UV Stratalinker 1800 (Stratagene, Inc., La Jolla, CA). After overnight hybridization at 42°C, the blots were washed to a final stringency of 65°C in 0.1× SSPE and 0.1% SDS. Autoradiography was performed at room temperature with an intensifying screen. To ensure that comparable amounts of mRNA from both tumor and adjacent normal tissue had been transferred, blots were rehybridized with GAPDH probe.

RT-PCR. The cDNAs for RT-PCR analysis were synthesized from 8 μg of total RNA extracted from the esophageal cancer cell lines in a 30-μl reaction mixture as described previously (22). The mixture was incubated at 37°C for 60 min, heated to 80°C for 10 min, and chilled on ice. The PCR amplification of 3’-untranslated region segments for RSG1 and HDGF which encompasses RSG1 was carried out with 28 cycles with 94°C for 1 min, 53°C for 1 min, and 72°C for 1 min (Fig. 1B). For pairs of primers specific to HDGF within its open reading frame, 94°C for 1 min, 59°C for 1 min, and 72°C for 1 min in 24 cycles (Fig. 1C) and to bFGF, 94°C for 1 min, 54°C for 1 min, and 72°C for 1 min in 26 cycles were used (Fig. 2A). To ensure that the RNA was of sufficient purity for RT-PCR, a PCR assay with primers specific for the gene GAPDH cDNA was carried out in each case, except that 22 cycles were performed under previously described PCR conditions (22).

Clinical Specimens. Esophageal tumors were biopsied at endoscopy for histopathological diagnosis before treatment in 25 patients who were treated at the Medical Institute of Bioregulation Hospital and the Saitama Prefectural Cancer Center Hospital between 1999 and 2000. The mean age was 61.6 years (range, 38–80 years), and all 25 patients were male. Two or three biopsy specimens were obtained and examined after obtaining informed consent from the patients. All 25 tumors were diagnosed as squamous cell carcinoma, and the patients underwent radiotherapy, with a daily dose of 2 Gy, 5 days/week for 4 weeks, prior to surgical resection. The effects of radiotherapy in the resected specimens were determined using the histopathological criteria of the Japanese Society for Esophageal Diseases. For the expression analysis of RSG1 mRNA, 10–40 μg of total RNA were extracted from the pretreatment biopsy specimens in each case, and cDNA was synthesized from 2.5 μg of total RNA as described.

Table 1 Comparative analysis of induced apoptosis after bolus 10 Gy irradiation in three treated cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>10 h</th>
<th>24 h</th>
<th>48 h</th>
</tr>
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<tbody>
<tr>
<td>TE-11</td>
<td>C</td>
<td>17.6 ± 2.6</td>
<td>3.4 ± 1.0</td>
</tr>
<tr>
<td>R15</td>
<td>0.7 ± 0.2</td>
<td>7.5 ± 1.7</td>
<td>4.2 ± 1.5</td>
</tr>
<tr>
<td>TE-13</td>
<td>C</td>
<td>6.7 ± 1.9</td>
<td>1.7 ± 1.1</td>
</tr>
<tr>
<td>R15</td>
<td>5.8 ± 2.8</td>
<td>2.0 ± 0.6</td>
<td>1.2 ± 0.4</td>
</tr>
<tr>
<td>KYSSE410</td>
<td>C</td>
<td>3.7 ± 1.3</td>
<td>1.8 ± 0.8</td>
</tr>
<tr>
<td>R15</td>
<td>5.4 ± 1.6</td>
<td>1.9 ± 1.0</td>
<td>2.4 ± 1.6</td>
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</table>

a Average ± SD.

b P < 0.005.

c P < 0.001.

d C, control parent cells; R15, cells with 15 treatments of 2 Gy irradiation (total 30 Gy).

Results

Establishment of Cell Lines Resistant to Irradiation. The human esophageal cancer cell lines were treated repetitively with 2 Gy of X-ray irradiation. Three cell lines, TE-11, TE-13, and KYSSE410, survived this challenge; the others did not. After 15 treatments (total dose of 30 Gy), the apoptosis induced by bolus 10 Gy irradiation was detected with APO 2.7 in cell lines TE-11, TE-13, and KYSSE410. A significant difference was recognized in the early phase in TE-11 and in the late phase in TE-13 and KYSSE410. As shown in Table 1, the rate of apoptosis in TE-11 cells—parent cells compared with treated cells—was 17.60 ± 2.61% compared with 0.73 ± 0.21% at 10 h after treatment in TE-13 and KYSSE410 cell lines, 17.90 ± 1.47% to 1.20 ± 0.36% and 17.40 ± 1.95% to 2.43 ± 1.64% at 48 h (P < 0.05). Apoptosis after irradiation was examined also with terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling assays and with Annexin V, and similar results were obtained (data not shown). Thus, the acquirement of radioresistance was re-
Differentially Expressed Genes. The established three radioresistant cell lines (R15; treated with 15 repetitive irradiation cycles) were compared with their parent control cell lines (C). The PCR products obtained by the differential displays were electrophoresed, and each lane displayed ~50 bands. Most bands showed the same pattern between C and R15 in the three cell lines. However, several bands appeared in all parent lines but either disappeared or were substantially decreased in all resistant lines (Fig. 1A). We named those cDNA bands as RSG. The cDNA reamplified from the band of RSG1 showed ~300-bp size DNA fragment, and RT-PCR confirmed the C>R15 differential expression of RSG1. The reamplified cDNA of RSG1 was cloned by the TA cloning method. RSG2 and RSG3 have not yet been identified.

Identification of HDGF. The sequence of RSG1 was homologous to one gene entered in the BLAST program. The sequence of RSG1, nucleotides 1–229, showed complete homology to HDGF, 1983–2124 (Fig. 1B). HDGF expression in the C and R15 samples was examined with RT-PCR using a primer pair for HDGF that includes the RSG1 sequence. The expression of HDGF showed the same pattern as RSG1, i.e., decreased expression in the R15 samples (Fig. 1C). Therefore, the expression of HDGF was decreased in radioresistant cells.

Status of HDGF Expression, bFGF Expression, and Radioresistivity. The relationship of HDGF expression and radioresistivity was analyzed by Northern blotting and RT-PCR using primers specific to HDGF and RNA isolated from cells treated with varying times and doses of radiation treatment. The expression of HDGF mRNA was reduced with repetitive treatments in all three treated cell lines (Fig. 2). Two untreated cell lines, TE-9 and KYSE110, without or with reduced HDGF expression (Fig. 2B), were resistant to irradiation (Table 2). In other words, the expression of HDGF was reduced as the sensitivity to irradiation decreased. On the other hand, bFGF expression was not related to radioresistance in the three treated cell lines (Fig. 2A).

Effects of Radiotherapy and Expression of HDGF in Clinical Cases. In 25 cases of squamous esophageal cancer, pretreatment HDGF mRNA expression was assayed by semiquantitative RT-PCR on biopsy samples (Fig. 3). The HDGF/GAPDH expression ratio was set at 1.0 using a control cell line, HuH-7. For KYSE410, the normalized HDGF/GAPDH ratios were 1.28 and 0.67 for C and R20 samples. Consequently, the association between HDGF expression and the sensitivity to irradiation was confirmed. In clinical cases, the average ratio was 1.63 ± 2.02 and the median was 1.14. There was a significant difference between high (ratio >1.14) and low (<1.14) HDGF expression groups with respect to the histopathological grade (P < 0.05; Table 3). Grade 0 represents with no radiotherapy effect; Grade 1, slightly effective, more than one-third of the cancer cells are viable; Grade 2, moderately effective, less than one-third of the cancer cells are viable; Grade 3, markedly effective, there are no viable cells of cancer.

Discussion

Radiation therapy is used frequently in the treatment of patients with esophageal cancer, and it occupies an important part in esophageal cancer treatments. When we analyzed the results of the National Cancer Database for patients treated with esophageal cancer in 1993, we found that radiation therapy was used as a component of treatment in 56% of patients (23). Recently, chemoradiotherapy, a combination of chemotherapy and radiotherapy, is the most frequent treatment for patients with esophageal cancer, and a complete histopathological response is achieved in 20–40% of cases (24, 25).

In this study, we aimed at two practical utilities. One was to look for genes related to radioresistivity. The other was to determine whether the expression levels of such genes would predict clinical radioresistivity in human esophageal cancer. To avoid the influence of factors other than radioresistivity, we established esophageal cancer cell lines resistant to X-ray irradiation by repeated challenge of the parent cell lines with irradiation.

Differential display is a useful tool to isolate differentially expressed genes (20). In this study, we applied the differential display strategy to identify irradiation-related genes. Consequently, we successfully obtained one gene, HDGF, the expression of which was drastically suppressed in radioresistant cell lines compared with radiosensitive parent cell lines (Fig. 1A). HDGF was initially purified from the conditioned medium of human hepatoma cell line HuH-7. It is an acidic polypeptide with mitogenic activity for fibroblasts and endothelial cells, and it is structurally related to bFGF (13, 26, 27). In addition, HDGF shows significant homology with the “high-mobility group protein,” and its expression has also been recognized in normal tissues (14, 16, 28). Lately, new members and functions of the HDGF

### Table 2. Comparative analysis of induced apoptosis after bolus 10 Gy irradiation in two cell lines without or with reduced HDGF expression.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>10 h</th>
<th>24 h</th>
<th>48 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>TE-9</td>
<td>1.6 ± 2.3</td>
<td>2.1 ± 2.5</td>
<td>2.1 ± 2.4</td>
</tr>
<tr>
<td>KYSE110</td>
<td>3.3 ± 3.1</td>
<td>5.6 ± 1.5</td>
<td>4.2 ± 3.3</td>
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</tbody>
</table>

* Average ± SD.
family have been reported (15, 29, 30). To our knowledge, there has been no report studying the expression of HDGF in clinical cases of human esophageal cancer or studying the relationship between HDGF expression and radiosensitivity. Our present study demonstrated that the expression of HDGF mRNA was down-regulated in radioresistant cases and the status of HDGF expression reflected the sensitivity to X-ray irradiation (Fig. 2A). We confirmed the relationship between HDGF expression and radiosensitivity in TE-9 and KYSE110 without repeated treatments. These two cell lines demonstrated no or reduced HDGF expression (Fig. 2B), indicating the resistance to the irradiation (Table 2). In clinical cases, radiotherapy was markedly effective in 10 of 13 patients with high pretreated HDGF expression and in only 4 of 12 patients with low HDGF expression. There was a significant difference between high and low HDGF expression groups with respect to radiosensitivity (Table 3). As the result of our analysis of clinical cases, it is possible that the radiotherapy might be predicted to be more effective in cases with high expression of HDGF.

There are many reports analyzing the relationship between radiosensitivity and gene function (1–10). For some molecules, there are discrepant results relating function and radiosensitivity. For example, p53 was reported to correlate with radiosensitivity (1), but in contrast, it was reported separately to correlate with radioresistance (2). Another study has demonstrated no correlation between p53 and radiosensitivity or radioresistance (3). The complicated mechanisms of action to irradiation may lead to these different results. To escape from the damage caused by irradiation, many complex pathways may be altered, and therefore many molecules appear to play important roles. Our study demonstrated that the expression status of HDGF was associated with sensitivity to irradiation in both cell lines and clinical esophageal cancer. The relationship between FGF and radioresistance was reported (11, 12). We examined the relation between bFGF expression and radioresistance in three established cell lines, but there was no relation. Consequently, HDGF may play a role in reaction to irradiation independent of the bFGF pathway. However, the precise mechanism has not been determined. What genes mediate signals to HDGF and by what means HDGF is regulated are still not clarified. We will further investigate the function of HDGF in its action related to X-ray irradiation and the response of other molecules that are related to radiosensitivity to estimate the likely response to radiotherapy before treatment and provide clinical applications for such molecular studies.

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References


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