CDC25B and p53 Are Independently Implicated in Radiation Sensitivity for Human Esophageal Cancers

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ABSTRACT

Ionized radiation leads to G1 arrest and apoptosis by a p53-dependent pathway and G2-M arrest through a p53-independent pathway. In this study, we evaluated the role of cell cycle-regulating molecules in the sensitivity of cancer cells for radiation therapy. Forty-seven patients with squamous cell carcinomas of the esophagus had undergone radiation therapy, followed by surgical resection. They were classified as sensitive to radiation (SR, 14 cases) with no residual tumor in the surgical specimen or as resistant to radiation (RR, 33 cases) with viable residual tumors. Their cell cycle-related molecules, including p53, CDC25A, CDC25B, cyclin D1, cyclin B1, and Ki-67, p53 expression was negative in 71% (10 of 14) of SR and positive in 91% (30 of 33) of RR. The association was strong between high radiation sensitivity and negative p53 expression (P = 0.0001). CDC25B, which is not expressed in normal epithelium but is in the cytoplasm of esophageal cancers, was strongly expressed (2+) in 46% (6 of 14) of SR and in 6% (2 of 23) of RR. Thus, the sensitivity for radiation therapy was significantly correlated with CDC25B overexpression. With respect to CDC25A, cyclin D1, cyclin B1, and Ki-67, no statistically significant differences were found in their expressions between SR and RR tumors. p53 and CDC25B expressions showed no significant associations, and multivariate analysis revealed that both p53 and CDC25B are significant independent markers for predicting radiation sensitivity. CDC25B was revealed to be a novel predictor of radiation sensitivity in esophageal cancers. Because CDC25B is an oncogene, which affects G2-M progression, these results suggest the importance of a p53-independent G2-M checkpoint in radiation therapy.

INTRODUCTION

Apoptosis induced by DNA damage is one of the essential cell properties, which is preserved even in tumor cells. This property is used in various anticancer therapies, such as irradiation and chemotherapy. The extent of apoptosis correlates well with the response to radiation therapy and is affected by a variety of genes. The p53 gene plays a central role in radiation-induced apoptosis (1), because many studies have shown apoptosis to be increased by wild-type p53 but decreased by its mutation (2, 3). A signal transduction pathway initiated by irradiation involves the ATM3 gene, mutated in ataxia telangiectasia, located upstream of p53 (4, 5). ATM is activated by irradiation and phosphorylates p53 on serine 15 (6, 7). This results in p53 activation and stabilization by interfering with binding to Mdm2 (8). Finally, p53 up-regulates the transcription of GML (9) and Bax, which forms heterodimers with the antiapoptotic proteins Bcl-2, and consequently leads to apoptosis (10, 11).

Cell cycle arrest is another cell response, which occurs after DNA damage induced by irradiation. G1 arrest results from p21 suppression (13), a strong inhibitor for CDK2 and CDK4, both of which are required for transition from the G1 phase to the S phase (14). Recently, the mechanism of G2-M arrest after irradiation has been elucidated as follows. ATM activates Chk1, which phosphorylates CDC25 on serine-216 and inactivates it (15, 16). Because CDC25A activates CDC2 by dephosphorylation of the tyrosine 15 and threonine 14 residues, inactivation of CDC25 results in G2-M arrest through inactivation of the cyclin B1/CDC2 complex.

Although both apoptosis and cell cycle arrest are caused by irradiation, it is not known whether cell cycle arrest facilitates or suppresses apoptosis. There are a few reports of cell cycle arrest stimulating apoptosis. However, a decline of G2-M arrest attributable to p21 suppression (17, 18) or cyclin D1 overexpression (19) is associated with apoptosis. In addition, radiation-induced apoptosis has been reported to be increased by inhibition of G2-M arrest by caffeine (20) but to be decreased by anticancer drugs that cause G2-M arrest (21). These findings strongly suggest that cell cycle arrest is does not promote radiation-induced apoptosis but rather suppresses it. This can be explained as follows. Cell cycle arrest is indispensable for repairing DNA damaged by irradiation, and
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apoptosis results when DNA damage is not adequately repaired (22). Thus, the cell after irradiation can survive if cell cycle arrest and DNA repair occur, but apoptosis will take place if there is not sufficient cell cycle arrest.

Although many biological markers for radiation sensitivity have been reported, p53 may be the only reliable marker for predicting radiation sensitivity both in vitro and in vivo (23, 24). p53 is involved in both apoptosis and cell cycle arrest, but its effect on apoptosis may override that on cell cycle arrest, even if cell cycle arrest might partially inhibit apoptosis. On the other hand, rapidly growing tumors are known to be more sensitive to radiation therapy than slowly growing ones (25). Disorders of the cell cycle and cell cycle-regulating molecules are ubiquitous characteristics of cancer cells. Taken together, the molecules that down-regulate the cell cycle checkpoint and stimulate cell growth should be involved in increasing sensitivity to radiation therapy. In the present study, to investigate the implication of cell cycle checkpoints in sensitivity to clinical radiation therapy, we evaluated the expression of representative cell cycle regulating molecules, including not only p53 but also cyclin D1, Ki-67, cyclin B1, and CDC25B using immunohistochemical techniques.

### MATERIALS AND METHODS

**Patients and Samples.** The subjects were 47 patients with SCC of the esophagus who had undergone chemoradiation therapy, followed by esophagectomy in the Department of Surgery II, Osaka University Medical School. Histological examination of the removed esophagus revealed residual viable cancer cells in 33 cases, which were classified as RR, but no residual cancer cells were found in the remaining 14 cases, which were classified as SR. Their biopsy specimens from before CRT were obtained and subjected to immunohistochemistry. Surgical specimens were used for immunohistochemistry and Western blot for 48 RR cases, including an additional 15 RR cases for which pretreatment biopsy samples were not available, and 43 cases not subjected to preoperative treatment.

**Preoperative Adjuvant Therapy.** Patients received a total dose of 40 or 60 Gy over 4 or 6 weeks, 2 Gy a day, five fractions a week, in combination with continuous infusion of 5-fluorouracil (300 mg/m² per day) and a single infusion of cisplatin (10 mg/body per day) on each day. A 10-MeV high energy linear accelerator was used to deliver radiation to the mediastinum and the neck. The toxicities and their treatments were described previously (26).

**Immunohistochemical Staining Procedures.** Immunostaining for CDC25A, CDC25B, p53, cyclin B1, cyclin D1, and Ki-67 was performed using streptavidin-peroxidase complex methods described previously (27). In brief, 4-μm paraffin sections were deparaffinized, and endogenous peroxidase was blocked with 0.3% H2O2 in methanol. The sections were heated in 0.01 M citrate buffer (pH 6.0), heated for 45 min at 95°C, then left covered with 10% normal goat or rabbit serum for 20 min at room temperature. These prepared sections were incubated overnight at 4°C with primary antibodies (CDC25A: sc-97, 0.5 μg/ml (Santa Cruz Biotechnology, Santa Cruz, CA); CDC25B: sc-326, 2.0 μg/ml (Santa Cruz); p53: DO-7, dilution 1:50 (Novocastra Laboratories, Newcastle, United Kingdom); cyclin B1: clone 7A9, dilution 1:20 (Novocastra); cyclin D1: M-20, 0.5 μg/ml (Santa Cruz); Ki-67: clone Ki-S5, 1.0 μg/ml (DAKO, Carpinteria, CA)). They were then processed by the biotin-streptavidin method using a kit according to the manufacturer’s instructions (Histo-Fine SAB-PO kit, Nichirei, Japan). The color was developed with diaminobenzidine tetrahydrochloride supplemented with 0.02% hydrogen peroxide, and the nuclei were counterstained with Mayer’s hematoxylin.

**Evaluation of Staining.** The immunoreactivities of CDC25A and CDC25B were classified according to the frequency of the positively stained cells as follows: negative (−), <10%; weak (+) from 10 to 50%; and strong (2+), >50%. Concerning p53 and cyclin D1 staining, when >10% of the cancer cells showed positive staining, the tumors were evaluated as positive (+; Refs. 27 and 28). We calculated cyclin B1 and Ki-67 indices as the percentage of cells with nuclear staining of the total number of tumor cells.

**Immunoblot Analysis.** The tissue samples were minced and homogenized with lysis buffer [50 mM Tris (pH 8.0), 150 mM NaCl, 0.5% NP40, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml apronin, and 2 μg/ml leupeptin], and the extract was centrifuged at 14,000 rpm for 25 min at 4°C. Aliquots (100 μg) of supernatant proteins were separated by 10% PAGE, followed by electroblotting onto a polyvinylidene difluoride membrane.
Antihuman CDC25B rabbit polyclonal antibody (Santa Cruz) was used at a concentration of 1.0 μg/ml. Detection of the protein bands was performed using the Amersham ECL detection system (Amersham, Arlington Heights, IL) according to the manufacturer’s instructions.

Statistical Analysis. For statistical evaluations, Student’s t test and Spearman’s rank correlation test were used. P < 0.05 was accepted as statistically significant. This analysis was carried out using the StatView J 4.5 software statistical package (Abacus Concepts, Inc., Berkeley, CA).

RESULTS

Expression of CDC25B. CDC25B was not expressed in interstitial tissue including fibroblasts, lymphocytes, smooth muscles, and vessels. In the normal stratified squamous epithelium, CDC25B was not detected in the basal and parabasal layers but slightly detected in the granular and keratinizing layers (Fig. 1a). In the preradiation biopsy samples of cancerous tissue, CDC25B was strongly expressed in the cytoplasm of cancer cells (Fig. 1c) and classified as (+) in 17.0% (8 cases), (++) in 46.8% (22 cases), and (−) in 36.2% (17 cases) according to the frequency of the positive cells (Table 1). In the cancer tissues obtained by surgical treatment, CDC25B tends to be expressed more in the deep infiltrating parts than in the superficial parts (Fig. 1d).

Immunohistochemical findings for CDC25B were confirmed by immunoblot analysis using representative surgical specimens with distinct CDC25B expression (Fig. 2). A band with strong intensity was located at Mr 63,000, which corresponds to the molecular weight of CDC25B. In the preradiation biopsy samples of cancerous tissue, CDC25B was faintly detected in the granular and keratinizing layers (Fig. 1a), whereas in CDC25B (−) tumor samples, a strong band appeared, whereas in CDC25B (+) tumors and normal tissues, the sample bands were faint or absent. Cell extract from HeLa cells was used as a positive control. N, normal tissues; T, esophageal cancer.

Expression of Other Cell Cycle-related Molecules. We and others have reported the expression of p53, a tumor suppressor gene, and cyclin D1, an oncogene, which were stronger in human esophageal cancers than in noncancerous tissues. The frequency of p53-positive (+) expression was 72.3% (34 cases) in this series (Table 1) and slightly higher than in previous reports, probably because of advanced clinical stages being more frequently included in preoperative irradiation cases. Cyclin D1 displayed overexpression (+) in 48.9% (23 cases; Table 1). CDC25A was not expressed in noncancerous epithelium, whereas it was overexpressed frequently in esophageal cancer cells and classified as: +, 19 cases (40.4%); +, 12 cases (25.5%); and −, 16 cases (34.1%; Table 1; Fig. 1, g and h). Cyclin B1 were specifically expressed in the cytoplasm or nuclei of cells at the G2-M phase, and Ki-67 was expressed in the nuclei of all proliferating cells, except the G0 phase (Fig. 1, e and f). Because both cyclin B1 and Ki-67 were expressed to some extent in all normal and cancer tissues, they were evaluated as an indices representing the percentage of positively stained cells. These indices of cyclin B1 and Ki-67 in esophageal cancers averaged 18.5 ± 2.0% and 38.0 ± 3.9%, respectively, which were significantly higher than in the noncancerous esophageal epithelium (8.3 ± 1.7% and 10.8 ± 1.9%, respectively).

Radiation Sensitivity and Cell Cycle-regulating Molecules. The expression of cell cycle-related molecules was compared with the radiation sensitivity of tumors classified as RR (33 cases) and SR (14 cases), according to the presence of residual cancer cells.

p53 expression was not found in 10 of 14 SR cases (71.4%) but was found in 30 of 33 cases (90.9%). Positive p53 expression, which is regarded as an accumulation of abnormal protein, was strongly associated with resistance to radiation therapy (P < 0.001). There was no difference of tumor size between p53 (−) and p53 (+) tumors (39.1 ± 5.6 mm versus 44.0 ± 4.9 mm; P = 0.5851). The frequencies of strongly positive (+), weakly positive (++), and negative (−) CDC25B expression were 42.9% (6 of 14), 35.7% (5 of 14), and 21.4% (3 of 14) in SR cases but were 6.1% (2 of 33), 51.5% (17 of 33), and 42.4% (14 of 33) in RR cases. Thus, overexpression of CDC25B was significantly associated with radiation sensitivity (P = 0.0168). CDC25A (2+) was observed in 35.7% (5 of 14) of SR and 42.4% (14 of 33) of RR cases. Cyclin D1 was overexpressed in 57.1% (8 of 14) of SR and 45.5% (15 of 33) of RR cases. The cyclin B1 index was 17.0 ± 2.6% and 18.8 ± 1.8% in SR and RR, and the Ki-67 index was 40.5 ± 5.2% and 36.9 ± 3.3%, respectively (Table 2). Thus, no difference were observed for the cyclin D1, cyclin B1, and Ki-67 status in SR and RR. The cyclin B1/Ki-67 index was calculated to evaluate the proportion of G2-M cells and classified as high (>0.7), moderate (0.4 to 0.7), and low (<0.4). The frequencies of high, moderate, and low cyclin B1/Ki-67 index were 7.1, 42.9, and 50.0% in the SR group but 24.2, 51.6, and 24.2% in the RR group. Thus, RR cases showed higher cyclin B1/Ki-67 index than SR cases, although the difference was not statistically significant (P = 0.0575). Cyclin B1/Ki-67 index was lower in CDC25B (2+; 33.2%) than in CDC25B (+; 59.0%) or CDC25B (−; 56.0%).
Although this difference was not statistically significant, it might suggest the involvement of CDC25B in G_{2}-M progression in vivo.

**Expression in Surgical Specimens.** In RR cases, the expressions of p53 and CDC25B were also examined in surgical specimens and compared with those in preradiation biopsy samples. The expression of p53 was the same, except in two cases. However, in 19 cases (57.6%), the expression of CDC25B was inconsistent; 15 cases showed stronger staining in preradiation samples than in residual tumors, whereas 4 cases showed stronger staining in the residual tumors.

As mentioned above, CDC25B expression could be underestimated in the biopsy sample because its expression was stronger in the deep infiltrating parts. Thus, CDC25B expression of the whole tumor was evaluated for a total of 48 RR cases with postradiation residual tumors (including additional 15 cases without preoperative biopsy) and 43 cases without radiation (Table 3). The trend in Table 1 is more evident here; thus, the frequency of CDC25B (2+) was 4.2% in the former and 51.2% in the latter, and that of CDC25B (−) was 45.8 and 14.0%, respectively (P < 0.001).

**Markers for Predicting Radiation Sensitivity.** Coexpression evaluation of p53 and CDC25B could be used for predicting radiation sensitivity, because there was no correlation between the expression of p53 and CDC25B (Table 4). Thus, all four cases with p53 (−)/CDC25B (2+), but only 2 of 20 cases with p53 (+/−)/CDC25B (+/−) showed SR (Table 5). Multivariate analysis by the logistic regression model, including p53, CDC25B, and cyclin B1/Ki-67 indices, revealed that p53 and CDC25B were independent significant markers for predicting radiation sensitivity (P < 0.0001 and P = 0.0076, respectively).

**DISCUSSION**

We evaluated the role of cell cycle-regulating molecules in the sensitivity of radiation therapy for human esophageal cancers by immunohistochemical methods and found p53 and CDC25B to be significant independent markers for predicting radiation sensitivity. Although many studies have reported on the implications of p53, this is the first report of CDC25B as a marker for radiation sensitivity based on an in vivo study.

CDC25 is a family of protein phosphatases, which dephosphorylates and activates CDKs. Three members of the cdc25 gene family have been identified in human cells (29, 30). CDC25A activates CDK2 and facilitates the G_{1} checkpoint (31), whereas CDC25B and CDC25C promote G_{2}-M transition by activating the CDC2/cyclin B1 complex (16, 32, 33). Recent studies have shown the frequent overexpression of CDC25A and CDC25B, but not of CDC25C, in various types of cancers (34–36). The current study showed consistent CDC25B and CDC25A overexpression in esophageal SCC, thus suggesting their role as an oncogene that triggers G_{2}-M or G_{1}-S transition.

Cell death after irradiation was roughly classified into “interphase death” and “mitotic death” (37). The latter was strongly associated with G_{2}-M arrest, which is necessary to allow time for DNA repair to escape from apoptosis. Recently, the mechanism of G_{2}-M arrest after irradiation has been revealed to involve many molecules, such as ATM, cyclin B1, CDC2, Chk1, and CDC25. Among them, only the alteration of CDC25B has been observed frequently in various cancers. We investigated CDC2 and CDC25C expression by immunohistochemistry, but they were relatively invariant among esophageal cancers and not associated with radiation sensitivity (data not shown). According to this scheme, the high activity of CDC25B theoretically can prevent G_{2}-M arrest after irradiation. Therefore, overexpression of CDC25B in esophageal cancers might reduce the duration of G_{2}-M arrest, which would thus not allow enough time for the repair of DNA damage caused by irradiation, and result in increased apoptosis and sensitivity to radiation therapy.

The implication of G_{2}-M arrest in radiation sensitivity has not yet been demonstrated in clinical human samples in vivo. The number of cyclin B1-positive cells, which are only found in the G_{2}-M phase, was not correlated with radiation sensitivity. Because the portion of cells resting in the G_{0} phase was relatively high and varied among solid tumors (38), the relative proportion of the G_{2}-M phase in proliferating cells was demonstrated as the cyclin B1/Ki-67 index. Interestingly, the cyclin B1/Ki-67 index tended to be higher in tumors with radiation resistance. This might be interpreted as showing that G_{2}-M progression in these tumors was slower than that in the others, would provide a longer time for DNA repair, and eventually might lead to escape from apoptosis. p53 is partly involved in this pathway by sequestering CDC25 through up-regulation of the 14-3-3 protein (39). However, in general, cells that lack wild-type p53 still demonstrate strong G_{2}-M arrest, implying that p53 is involved more in G_{1} arrest than in G_{2}-M arrest.

Because CDC25B was expressed more strongly in deep infiltrating parts than in superficial parts, according to immunohistochemical tests, its expression might be underestimated in biopsy specimens. For example, CDC25B (2+) was observed in 51% of
the surgical specimens of esophageal cancers (Table 3); however, only half of them were evaluated as CDC25B (2+) with preoperative biopsy samples (data not shown). We compared CDC25B expression in surgical specimens with or without preoperative CRT (Table 3). The frequency of complete disappearance of tumors by CRT was observed in, at most, 20–40% of the cases (26). We concluded that a remarkably low level of CDC25B expression after CRT might imply that its expression is reduced during CRT. However, it is not clear whether this results from the clonal selection of cells with low CDC25B expression or suppression of the expression by radiation therapy.

G1 progression and total cell proliferation were also evaluated by the investigation of cyclin D1 and Ki-67 expression. Cyclin D1, which is frequently overexpressed in SCC of the esophagus (40), is the most crucial regulator of the G1 checkpoint (41). There have been a few reports showing cyclin D1 overexpression to be associated with high susceptibility for radiation therapy in vitro (42, 43). Such a trend was observed but was not statistically significant in this study. Rapidly growing tumors have been reported to be more radiosensitive in clinical samples (25). The total cell proliferative activity was evaluated by Ki-67 expression, which was more radiosensitive in clinical samples (25). The Ki-67 index was slightly higher in RR than in SR tumors; however, the difference was not statistically significant in the present study.

Table 4 Relationship between CDC25B and p53 expression

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<th>CDC25B expression</th>
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<th>−</th>
<th>−/−</th>
<th>−/+</th>
<th>Total</th>
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<td>34</td>
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<td>+</td>
<td>4</td>
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<td>−</td>
<td>4</td>
<td>6</td>
<td>3</td>
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<td>13</td>
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<tr>
<td>Total</td>
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<td>22</td>
<td>17</td>
<td>13</td>
<td>47</td>
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*p* Spearman’s rank correlation test: *P* = 0.1207.

Table 5 Relationship between pattern of p53/CDC25B expression and radiosensitivity

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<th>−/−</th>
<th>−/+</th>
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<td>18</td>
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*Fourteen cases.
Thirty-three cases.

results can be directly used for the treatment for esophageal cancers to predict the effect of CRT and select patients for this treatment. Furthermore, our results suggest the use of compounds that can up-regulate G2-M transition, such as caffeine, as radiosensitizers especially in patients with low CDC25B expression. Thus, clinical application of these biological markers is promising and of urgent priority.

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CDC25B and p53 Are Independently Implicated in Radiation Sensitivity for Human Esophageal Cancers
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