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Original Article

Overexpression of Cdc25C predicts response to radiotherapy and survival in esophageal squamous cell carcinoma patients treated with radiotherapy followed by surgery

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Abstract

Biomarker identification is crucial for the selection of patients who might benefit from radiotherapy. To explore potential markers for response and prognosis in patients with locally advanced esophageal carcinoma treated with radiotherapy followed by surgery, we evaluated the expression of cell cycle checkpoint-related proteins Chk2, Cdc25C, and Cyclin D1. A total of 56 patients with locally advanced esophageal squamous cell carcinoma were treated with radiotherapy followed by surgery. Pretreatment tumor biopsy specimens were analyzed for Chk2, Cdc25C, and Cyclin D1 expression by immunohistochemistry. High expression of Chk2, Cyclin D1, and Cdc25C was observed in 44 (78.6%), 15 (26.8%), and 27 (48.2%) patients, respectively. The median survival was 16 months (range, 3–154 months), with a 5-year overall survival rate of 19.6%. Overexpression of Chk2 was associated with smoking (P = 0.021), overexpression of Cdc25C was associated with patient age (P = 0.033) and tumor length (P = 0.001), and overexpression of Cdc25C was associated with pathologic complete response (P = 0.038). Univariate analysis demonstrated that overexpression of Cdc25C and pathologic complete response was associated with better survival. In multivariate analysis, Cdc25C was the most significant independent predictor of better survival (P = 0.014) for patients treated with radiotherapy followed by surgery. Overexpression of Cdc25C was significantly associated with pathologic complete response and better survival of patients with locally advanced esophageal cancer treated with radiotherapy followed by surgery. These results suggest that Cdc25C may be a biomarker of treatment response and good prognosis for esophageal carcinoma patients. Thus, immunohistochemical staining of Cdc25C in a pretreatment specimen may be a useful method of identifying optimal treatment for patients with esophageal carcinoma.

Key words Cdc25C, radiation response, survival, esophageal squamous cell carcinoma

Carcinoma of the esophagus remains a major public health problem and ranks sixth in terms of cancer mortality worldwide[1]. It is endemic in many parts of the world, particularly in developing countries like China and certain areas of African countries. In most countries in the West, where esophageal carcinoma is infrequent, incidence has dramatically increased over the last 20 years[2]. Despite advances in diagnostics and therapies, the 5-year survival rate remains lower than 20%[3]. The alarming increase in incidence in developed nations and the large patient population in developing nations have made therapeutic management of this disease an urgent issue.

Currently, the mainstay in treating esophageal carcinoma is still surgical resection[4]. However, more

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than 60% of patients are contraindicated to surgery for locally advanced disease or distant metastasis[5]. Thus, a multi-modal or comprehensive therapeutic strategy is required to treat this condition. Radiotherapy has been used extensively in treating locally advanced esophageal cancer. However, only a small percentage of patients benefit from radiotherapy and the outcomes vary greatly and unpredictably[6]. In recent years, the main obstacle of this approach is the lack of methods by which to select patients who are optimally suited for it. Pretreatment clinical parameters such as TNM classification, sex, age, and tumor differentiation are unable to predict the biologic behavior of patients who undergo preoperative radiotherapy[7]. Thus, to streamline treatment and spare patients from toxic and expensive therapies that are unlikely to benefit them, it is necessary to identify biomarkers that can predict tumor response and treatment outcomes before therapy.

Cell cycle checkpoints are control mechanisms that function to halt cell division and ensure the fidelity of cell reproduction. Dysfunction of checkpoints is considered a pathologic hallmark of neoplastic transformation and tumor progression[6]. Agents used for cancer treatment, such as cytotoxic chemotherapy and ionizing radiation, also activate cell cycle checkpoints through complex networks. Checkpoint signaling networks are highly conserved and can be categorized by the function of their components: sensors, proximal transducer kinases, mediators, distal transducer kinases, and effectors. Chk2, Cyclin D1, and Cdc25C are functional components of the cell cycle checkpoint network[8]. Furthermore, checkpoint-related proteins are important candidates for such parameters because they enhance apoptosis induced by cytotoxic treatment by facilitating cell cycle arrest[9].

The aim of this study was to determine the role of Chk2, Cyclin D1, and Cdc25C expression in terms of response rate and overall survival in locally advanced esophageal squamous cell carcinoma (ESCC) treated with radiotherapy followed by surgery. This was done by using immunohistochemical staining to explore the potential markers in patients with locally advanced esophageal cancer.

Patients and Methods

Patients and specimens

Between November 1990 and November 2007, a total of 56 patients with locally advanced esophageal carcinoma were selected for this study. All patients were required to meet the following criteria: (a) histologically confirmed ESCC; (b) preoperatively staged with locally advanced (cT3, cN0/+, cM0) disease; (c) preoperative radiotherapy and surgery were the only treatment procedures; (d) Eastern Cooperative Oncology Group (ECOG) performance status 0–2; and (e) no other severe medical disease. Patients with distant metastasis, except to the supraclavicular and celiac lymph nodes, were excluded. Each patient underwent the following staging procedures: physical examination, chest radiography, computed tomography (CT) of the chest and upper abdomen, esophagography, esophagogastroduodenoscopy, and hematologic and biochemical profiles. Endoscopic ultrasound was used for some patients when necessary. Tissue samples collected during biopsy and surgery were formalin-fixed and paraffin-embedded. All hematoxylin and eosin (HE)–stained slides were reviewed for the confirmation of diagnosis. This research protocol was approved by the Institutional Review Board of the Cancer Hospital, Peking Union Medical College (Beijing, China).

Treatment

Radiotherapy

All patients underwent external beam radiotherapy using a 6-MeV high-energy linear accelerator. The initial treatment volume included the primary tumor and enlarged lymph nodes. Two-dimensional or three-dimensional treatment plans using CT scans were performed. The median radiation dose of 42.9 Gy (36 to 70 Gy, 2 Gy per fraction, 5 days per week) was delivered with a 3- or 4-field technique.

Surgery

Two to five weeks after the completion of radiotherapy, restaging and surgery was performed. Each patient underwent esophagectomy and systematic mediastinal lymphadenectomy. An experienced pathologist with no knowledge of patient outcome examined each resected specimen to assess the pathologic radiation response. The pathologic response was assigned to one of two categories: no residual carcinoma (pathologic complete response, pCR) or the presence of cancer cells (pathologic partial response, pPR or <pCR). Patients having no residual cancer in the resected specimen were considered radiosensitive.

Follow-up

Follow-up evaluations were performed every 3–4 months after surgery until the study was finished or the patient died. Follow-up evaluations included clinical examination, barium swallow, chest radiography, and CT scans of the chest and abdomen. Esophagogastroduodenoscopy and biopsies were performed when indicated.

Immunohistochemistry for cell cycle checkpoint-related proteins

Immunohistochemical staining of formalin-fixed, paraffin-embedded tumor tissue obtained by endoscopic biopsy before radiotherapy was done according to standardized protocols. The post-radiation surgical specimen was used to determine the pathologic radiation response using light microscopy. After initial deparaffinization, rehydration was performed through graded alcohols. The sections were microwaved in 10 mmol/L citrate buffer (pH 6.1) to unmask antigenic epitopes and were then immersed in methanol containing 0.3% hydrogen peroxide for 20 min to block the endogenous peroxidase activity. This was followed by incubation in 2.5% blocking serum to reduce nonspecific binding and then incubation overnight at 4°C with primary antibodies: rabbit anti-human monoclonal antibodies against Chk2 (dilution, 1:500; MBL), Cyclin D1 (dilution, 1:50; Santa Cruz;), and Cdc25C (dilution, 1:250; Epitomics). After rinsing three times with PBS, the bound antibody was detected using a goat horseradish peroxidase kit with diaminobenzidine as the chromogen. For a negative control, PBS was used in place of primary antibody.

Immunohistochemistry analysis was carried out without previous knowledge of patient survival. Sections were blindly assessed by two pathologists by using light microscopy. Immunostains were scored semiquantitatively by assessing the nuclear staining intensity and percentage of positive cells. In each case, the intensity (weak, moderate, or strong) and percentage of neoplastic immunoreactive cells were evaluated. Tumors were scored as follows: score 0, no appreciable staining or staining in <5% of neoplastic cells; score 1+, tumors with faint/barely appreciable incomplete nuclear staining in 5%–25% of neoplastic cells; score 2+, tumors with weak to moderate complete nuclear staining or containing 25%–50% of neoplastic cells with moderate incomplete nuclear staining; score 3+: strong immunoreactivity of the entire nucleus in >50% of neoplastic cells or containing >50% of neoplastic cells with strong nuclear immunoreactivity. Tumors classified as 0 or 1+ were considered negative, and those classified as 2+ or 3+ were considered positive. Any discrepancies were resolved with a multihead microscope.

Statistical analyses

Overall survival (OS) was calculated using the Kaplan-Meier method. OS was defined as the time from enrollment in the study until death. When the date of death was not available, the last follow-up date was used. The differences between the survival curves were determined using the log-rank test. A comparison of clinicopathologic characteristics was evaluated with Fisher's exact test. The Cox proportional hazards regression model was used to determine the joint effects of several variables on survival. All analyses were done with SPSS for Windows 13.0 software. A P value less than 0.05 was considered statistically significant.

Results

Patient characteristics

Between November 1990 and November 2007, 56 patients with locally advanced esophageal cancer were treated with radiotherapy followed by surgery. The clinicopathologic characteristics of the patients are listed in Table 1. All patients had squamous cell carcinoma, and the majority of patients were male. The median age of the patients at registration was 56.3 years (range, 38–79 years). Among the 56 patients, 35 were smokers and 36 were alcohol drinkers. Twelve patients had a family history of cancer. Tumors were located at cervical (8/56), upper (28/56), median (17/56), and inferior (3/56) portions. Pretreatment staging (cTNM version 6) demonstrated that 8 patients had stage II disease and 48 patients had stage III disease. After careful pretreatment evaluation, all patients underwent radiotherapy followed by surgery. After treatment, pCR and pPR were achieved in 10 patients (18%) and 46 patients (82%), respectively (Table 2). Follow-up was completed in May 2008, and the median follow-up time was 19 months.

Overexpression of Cdc25C was associated with radiation response in ESCC

High expression of Chk2, Cyclin D1, and Cdc25C was observed in 44 (78.6%), 15 (26.8%), and 27 (48.2%) patients, respectively (Figure 1). Overexpression of Chk2 was associated with smoking (P = 0.021), and overexpression of Cdc25C was associated with patient age (P = 0.033) and tumor length (P = 0.001). Moreover, overexpression of Cdc25C was significantly associated with pCR (P = 0.038). There was no significant association between the overexpression of Cyclin D1 and any clinicopathologic characteristics (Table 2).

Overexpression of Cdc25C was associated with patient outcome in ESCC

The median OS of all patients was 16 months (range, 3–154 months), with a 5-year OS rate of 19.6% (Figure 2). Four patients were lost to follow-up and were considered dead. In univariate analysis, overexpression

Table 1. Patient characteristics of 56 patients with esophageal carcinoma

Characteristic	No. of patients
Sex	
Male	47
Female	9
Age (years)	
Median	56.3
Range	38–79
ECOG performance status	
0	8
1	33
2	15
Location	
Cervical	8
Superior	28
Median	17
Inferior	3
Preoperative TNM stage	
II	8
III	48

ECOG, Eastern Cooperative Oncology Group.

Table 2. Associations between Chk2, Cyclin D1, and Cdc25C and clinicopathologic characteristics

Characteristic	No. of patients	Cdc25C staining			Cyclin D1 staining			Chk2 staining		
		Positive	Negative	*P*	Positive	Negative	*P*	Positive	Negative	*P*
Sex				0.228			1.000			0.180
Male	47	21	26		13	34		35	12	
Female	9	6	3		2	7		9	0	
Age (years)				0.033			0.643			0.609
⩽ 56	29	10	19		7	22		22	7	
>56	27	17	10		8	19		22	5	
Smoking				0.112			0.815			0.021
Smoker	35	14	21		9	26		24	11	
Non-smoker	21	13	8		6	15		20	1	
Alcohol				0.061			0.686			0.506
Drinker	36	14	22		9	27		27	9	
Non-drinker	20	13	7		6	14		17	3	
Family history				0.889			0.715			0.263
Positive	12	6	6		4	8		8	4	
Negative	44	21	23		11	33		36	8	
Tumor length (cm)				0.001			0.233			0.609
⩽ 6	29	20	9		10	19		22	7	
> 6	27	7	20		5	22		22	7	
Dose (Gy)				0.440			0.082			0.470
⩽ 40	42	19	23		14	28		34	8	
>40	14	8	6		1	13		10	4	
Response				0.038			0.259			0.433
pCR	10	8	2		1	9		7	3	
pPR	46	19	27		14	32		37	9	

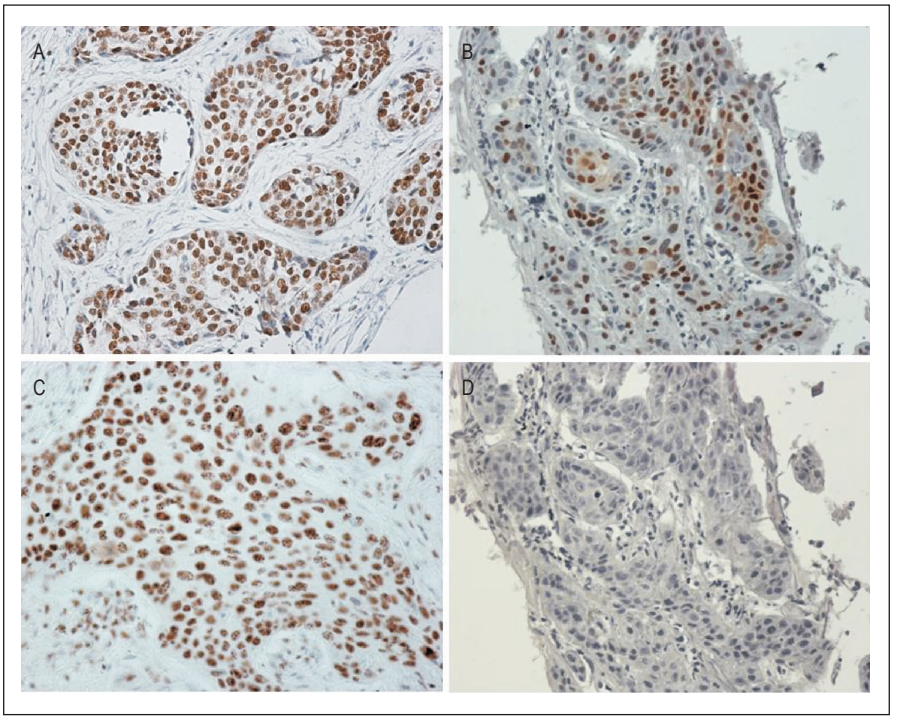

Figure 1. **Immunohistochemical staining of cell cycle checkpoint-related proteins in esophageal cancer (×200).** Representative images show high expression of checkpoint proteins in the nuclei of esophageal cells in pretreatment biopsy specimens. A, Chk2; B, Cyclin D1; C, Cdc25C; D, negative control.

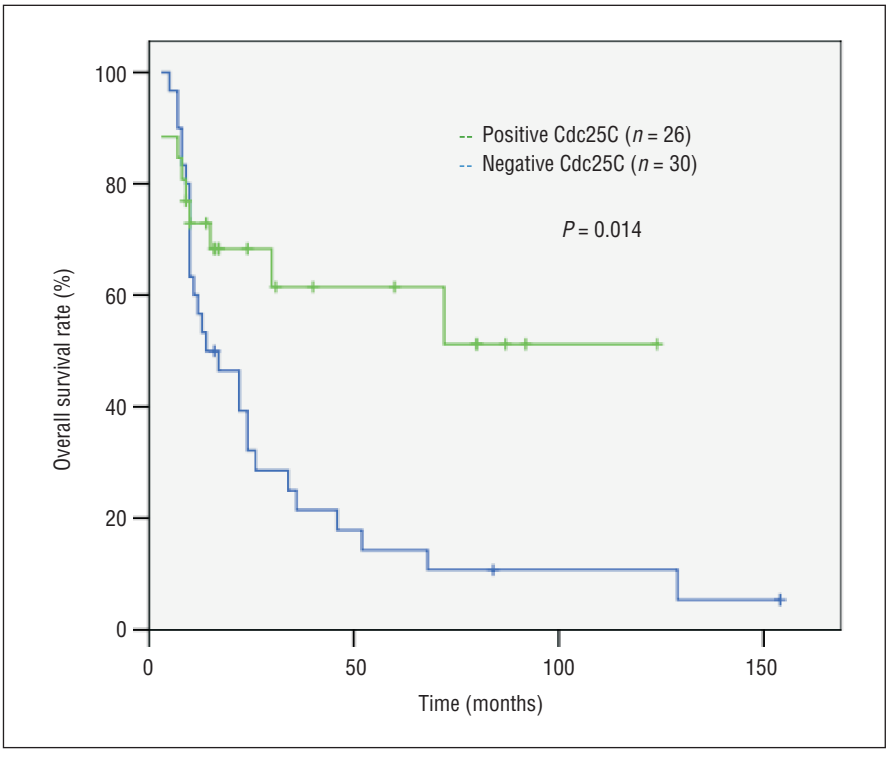

Figure 2. **Kaplan-Meier survival plots for patients with different Cdc25C expression status.** Patients with positive Cdc25C staining had a better chance of survival than those with negative Cdc25C staining (P = 0.014).

of Cdc25C in specimens from patients with ESCC was associated with better OS (median, 17 months versus 13 months; P = 0.014). Radiation response in this series of patients was also associated with OS (P = 0.015). However, other parameters showed no association with OS. High expression of Chk2 (P = 0.147) or Cyclin D1 (P = 0.142) was not associated with patient outcome (Table 2). In multivariate analysis, Cdc25C was the most

significant independent predictor of better OS (P = 0.019) for ESCC patients treated with radiotherapy followed by surgery (data not shown).

Discussion

Surgical resection is still the mainstay treatment for patients with localized esophageal carcinoma. The definition of a standard therapy for locally advanced esophageal carcinoma remains a clinical controversy[10]. Radiotherapy is usually used to treat this disease empirically by downstaging the tumor, increasing resectability, and alleviating symptoms[11]. However, not all patients benefit from this treatment. In some conditions, radiotherapy even harms or leads to death of the patient. Thus, biomarkers that can predict radiation response and outcomes before treatment are needed.

Cell cycle checkpoints allow progression through the cell cycle or induce arrest in response to DNA damage so that the cell has time to repair the damaged DNA. The inability to properly repair such damage in cancer cells leads to genetic instability and fragile to death[12]. Accumulating evidence has revealed that a complex damage response pathway regulates the fate of cells after ionizing radiation—cell cycle arrest or apoptosis[13]. Chk2 is a serine/threonine kinase that regulates cell cycle checkpoints after it is activated by phosphorylation at Thr68. When activated after ionizing radiation, Chk2 can phosphorylate (and thereby activate) its downstream target proteins, such as Cdc25C. Cdc25C belongs to a protein phosphatase family including Cdc25A, Cdc25B, and Cdc25C, which each activate the Cyclin-dependent kinases at different points of the cell cycle. Cdc25C phosphatase in particular mediates cellular entry into mitosis by activating the Cyclin-dependent kinase Cdc2/Cyclin B1 complex [8,14]. Cyclin D1 also plays an important role in regulating the progression of cells through the G_1 phase of the cell cycle[15]. In esophageal carcinoma, several studies have demonstrated that amplification and overexpression of Cyclin D1 are good predictors of prognosis for esophageal carcinoma patients[16-18]. However, the prognostic value of Chk2 and Cdc25C in esophageal carcinoma was unknown. We evaluated the expression of Chk2, Cdc25C, and Cyclin D1 under the hypothesis that abnormalities in these cell cycle checkpoint–related proteins may be associated with response to radiotherapy, ultimately having distinct effects on the survival of patients with locally advanced esophageal cancer.

Cyclin D1 is the most frequently investigated cell cycle checkpoint–related protein in esophageal carcinoma. A SNP of Cyclin D1 gene predicts response to neoadjuvant radiotherapy and prognosis in rectal cancer[19]. Moreover, Cyclin D1 expression was reported to be a possible predictor of sensitivity to chemoradiotherapy for esophageal squamous cell carcinoma[20]. Unfortunately, in our study, high expression of Cyclin D1 was not associated with radiation response or prognosis in esophageal carcinoma. Furthermore, although overexpression of Chk2 was associated with smoking in our series, Chk2 was not associated with radiation response or patient outcome.

The most important finding of the current study was the prognostic significance of Cdc25C overexpression. pCR has been associated with a better outcome than pPR (<pCR)[21]. In the present study, overexpression of Cdc25C was associated with radiation response. Moreover, overexpression of Cdc25C was the most significant predictor of better OS in multivariate analysis, surpassing that of pCR.

As essential cell cycle regulators, the Cdc25 phosphatases are currently considered potential targets for the development of novel therapeutic approaches[22]. In addition, Cdc25A and Cdc25B are purportedly related to prognosis in ovarian cancer patients[23]. Cdc25A was also reported to be a prognostic factor for ESCC[24]. However, to the best of our knowledge, the role of Cdc25C as a prognostic factor has not been reported in esophageal carcinoma so far. Our study demonstrates that overexpression of Cdc25C may predict radiation response and better survival of patients with ESCC. This may be attributable to enhanced cell cycle checkpoint arrest, preventing proliferation and activating apoptosis in cells damaged by radiotherapy[9,25]. However, this study is a retrospective analysis with a relatively small sample size. Further prospective studies with large numbers of patients are warranted to confirm the role of Cdc25C overexpression as a prognostic factor for esophageal carcinoma.

Taken together, overexpression of Cdc25C was significantly associated with better survival of patients with locally advanced esophageal carcinoma treated with preoperative radiotherapy. If the prognostic significance of the Cdc25C overexpression is validated by further prospective studies with a larger number of patients, relatively simple immunohistochemical staining for Cdc25C in a pretreatment endoscopic biopsy specimen may provide valuable information for the oncologist to select patients likely to benefit from radiotherapy or alternative treatment.

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