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Overexpression of Cyclin-dependent Kinase-activating CDC25B Phosphatase in Human Gastric Carcinomas

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CDC25 phosphatases activate cyclin-dependent kinases by removing inhibitory phosphate groups on the molecules and positively regulate the cell cycle progression. The expression of CDC25A, B and C was examined in gastric carcinoma cell lines and gastric carcinoma tissues by northern blotting and immunohistochemistry. The gastric carcinoma cell lines expressed CDC25A, B and C mRNA at various levels. The expression levels of CDC25B were generally higher than those of CDC25A and C. Of the 40 gastric carcinomas, 70% of the tumors expressed CDC25B mRNA at higher levels than the corresponding normal mucosas, while 38% overexpressed CDC25A mRNA. The CDC25C expression was at very low or undetectable levels. No obvious correlation was detected between the expression of CDC25B and *p53* gene mutations. Immunohistochemically, CDC25-positive tumor cells were detected in 43 (78%) of 55 gastric carcinoma cases, of which 27 (49%) were strongly positive. Strong expression of CDC25B protein was associated with advanced stage and deep invasion. Furthermore, the incidence of strong expression was significantly higher in carcinomas with nodal metastasis than in those without metastasis. These findings suggest that overexpression of CDC25B may favor development and progression and may be an indicator of malignant behavior of gastric carcinomas.

Key words: Overexpression — CDC25B — Human gastric carcinoma

Abnormalities in cell cycle regulators allow uncontrolled cell growth and division, which may participate in carcinogenesis.[1,2] Cyclin/cyclin-dependent kinase (CDK) complexes are activated by phosphorylation by the CDK-activating kinase and progress the cell cycle, while the complexes are negatively regulated by a number of CDK inhibitors.[3–11] On the other hand, CDKs are inactivated by phosphorylation of threonine and tyrosine residues at positions 14 and 15, possibly by inhibitory kinases Weel and Mik1.[12–14] CDC25 phosphatases dephosphorylate the same residues and finally activate the CDKs.[15–19] In human cells, there are three CDK-activating phosphatases, CDC25A, B and C.[16,20] CDC25 functions at the G1/S phase and at the G2/M phase.[16,21] CDC25A is expressed early in the G1 phase of the cell cycle following serum stimulation of quiescent fibroblasts, while CDC25B and CDC25C are expressed at the G2/M boundary.[22–24] CDC25A or CDC25B, but not CDC25C, cooperates with either Ha-RAS^{G12V} or loss of RB 1 in oncogenic transformation.[25] CDC25B mRNA is highly expressed in a portion of primary breast cancers that correlates with less favorable prognosis and survival.[25] However, no study has been conducted to elucidate the role of CDC25 in the development and progression of human gastric carcinomas.

In the present study, we examined the expression of CDC25A, CDC25B and CDC25C in human gastric carcinoma cell lines and gastric carcinoma tissues and analyzed the correlation with clinicopathological features of the gastric carcinomas.

MATERIALS AND METHODS

Cell cultures Eight human gastric carcinoma cell lines were examined. TMK-1 (poorly differentiated gastric adenocarcinoma) was established in our laboratory.[26] KATO III and HSC-39 (signet ring cell carcinoma) were kindly provided by Dr. M. Sekiguchi (University of Tokyo, Tokyo) and Dr. K. Yanagihara (Hiroshima University, Hiroshima),[27] respectively. The MKN series (MKN-1, adenosquamous carcinoma; MKN-7, -28 and -74, well differentiated adenocarcinoma; MKN-45, poorly differentiated adenocarcinoma) were kindly provided by Dr. T. Suzuki (Fukushima Medical College, Fukushima). They were routinely maintained in RPMI-1640 (Nissui Co., Tokyo) supplemented with 10% fetal bovine serum (Whittaker M. A. Bioproducts Inc., MD) under 5% CO_2 in air at 37°C. For the experiment on treatment with interleukin-1 alpha (IL-1α) or epidermal growth factor (EGF), TMK-1 cells were grown to subconfluence in the above-mentioned medium. After 24 h of serum starvation, 10 units/ml IL-1α and 1 n*M* EGF were added. Human recombinant IL-1α was kindly donated by Dr. M. Yamada (Dainippon Pharmaceutical Co., Ltd., Osaka) and human recombinant EGF by Wakunaga Pharm. Co., Hiroshima. The cells were har-

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vested at 0 (control) time, and at 1 h, 3 h, 12 h and 24 h after the treatment, and RNAs were isolated.

Tissue samples For molecular analysis, 40 cases of gastric carcinoma were used. The primary tumor tissues and the corresponding normal mucosas were surgically resected, frozen immediately in liquid nitrogen and stored at −80°C. We confirmed microscopically that each tumor tissue specimen consisted mainly of carcinoma tissue and non-neoplastic mucosa did not exhibit any tumor cell invasion or show significant inflammatory involvement. For immunohistochemistry, 55 surgically resected gastric carcinomas were used. Tissues were fixed in 10% buffered formalin and embedded in paraffin. One or two representative slides of the primary tumors, which included superficial, central and deep areas, from each case were analyzed. Histological types, staging and depth of tumor invasion were classified according to the criteria of the Japanese Research Society for Gastric Cancer.[28]

Northern blot analysis RNAs were extracted by the standard guanidium isothiocyanate/cesium chloride method. Aliquots (5 μg) of poly(A)$^+$ selected RNA were electrophoresed on 1.0% agarose/formaldehyde gels and blotted onto nitrocellulose filter membranes. The filters were baked for 2 h at 80°C under vacuum and hybridized with ^{32}P-labeled probes using the random hexamer priming method. After hybridization, filters were washed under stringent conditions and exposed for autoradiography to Fuji RX films with intensifying screens at −80°C.[29] The following ^{32}P-labeled cDNA probes were used. The 0.66 kb *Bgl* II and *Nde* I CDC25A fragment of pBS KS$^-$, the 0.71 kb *Nae* I and *Sac* I CDC25B fragment of pBS KS$^-$ and the 0.91 kb *Eco*R I and *Bgl* II CDC25C fragment of pBS KS$^-$ were kindly provided by Dr. A. Nagata (University of Tokyo, Tokyo).[30] G3PDH cDNA probe was applied as an internal control.

Western blot analysis The protein samples were prepared and western blotting was carried out as described.[31] Aliquots (100 μg) of protein were subjected to 10% polyacrylamide gel electrophoresis followed by electroblotting onto a nitrocellulose filter. Anti-human CDC25B rabbit polyclonal antibody (sc-326) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). For detection of the immunocomplex, the ECL western blotting detection system (Amersham, Aylesbury, UK) was used.

Immunohistochemistry A modification of the immunoglobulin enzyme bridge technique (ABC method) was employed as described elsewhere.[32] Deparaffinized tissue sections were immersed in methanol containing 0.03% hydrogen peroxide for 30 min to block endogenous peroxidase activity. The section was then incubated with normal goat serum (diluted 1 : 20) for 30 min to block the non-specific antibody binding sites. The sections were treated consecutively at room temperature with anti-human CDC25B rabbit polyclonal antibody (diluted 1 : 100) for 2 h, biotinylated anti-rabbit IgG goat serum (diluted 1 : 100, Vector, Burlingame, CA) for 30 min, and avidin DH-biotinylated horseradish peroxidase complex (Vectastain ABC kit, Vector) for 30 min. Peroxidase staining was performed for 10–15 min using a solution of 3,3′-diaminobenzidine tetrahydrochloride in 50 m*M* Tris-HCl (pH 7.5) containing 0.001% hydrogen peroxide. The sections were weakly counterstained with hematoxylin. The specificity of the immunostaining was confirmed by an absorption test using the antigenic peptide (Santa Cruz Biotechnology, sc-326P).

For superficial carcinomas, entire stained sections were observed. For large tumors of advanced cases, at least ten fields including superficial, central and deeply invasive areas were observed and the number of stained cells and the staining intensity were estimated. The immunoreactivity was graded as − to +++ as follows: −, almost no positive cells; +, 5–25% of tumor cells showed weak to moderate immunoreactivity; ++, 25–50% of tumor cells showed moderate immunoreactivity or 10–50% of the tumor cells showed intense immunoreactivity; +++, over 50% of tumor cells showed intense immunoreactivity. Typical cases with staining grades +++ and ++ are shown in Fig. 4, a and b, respectively. Grades ++ and +++ were regarded as strongly positive.

RESULTS

Expression of CDC25 in gastric carcinoma cell lines The expression of CDC25A, B and C mRNAs in 8 gastric carcinoma cell lines is shown in Fig. 1a. The same filter was reprobed with each of the cDNA probes, including G3PDH cDNA probe. Most of the gastric carcinoma cell lines expressed CDC25A, B and C mRNA at various levels. The expression levels of CDC25B were generally higher than those of CDC25A and C. CDC25B was expressed at high levels in TMK-1, KATO III, MKN-1 and MKN-28 cells, but its expression levels were very low in MKN-45 and MKN-74 cells, both of which possess wild-type *p53* gene.[33] We confirmed the expression of CDC25B protein by western blotting (Fig. 1b). The expression levels of CDC25B protein agreed with the results of northern blot analysis. CDC25B protein was strongly expressed in HSC39, KATO III, MKN-1 and MKN-28, but very weakly expressed in MKN-45 and 74 cells. We searched for gross alterations of the CDC25 genes in gastric carcinoma cell lines by Southern blot analysis. Neither gene amplification nor rearrangement of the CDC25 genes was found in any of the gastric cancer cell lines (data not shown).

TMK-1 cells were treated with IL-1α or EGF and the expression of CDC25B was examined by northern blot analysis. We have confirmed that these cytokines are growth stimulators for TMK-1 cells.[34, 35] Both IL-1α and EGF slightly induced the expression of CDC25B mRNA

Fig. 1. Expression of CDC25A, B and C in gastric carcinoma cell lines. a, Northern blot analysis: Poly $(A)^+$-selected RNA (5 μg) was subjected to northern blot analysis as described in "Materials and Methods." G3PDH cDNA probe was applied as an internal control. b, Western blot analysis: Protein (100 μg) was subjected to western blot analysis as described in "Materials and Methods." The status of *p53* gene abnormalities is shown in the second row. +, cells with mutated *p53* gene; −, cells with wild type *p53* gene; the *p53* gene is completely deleted in KATO III.[33]

Fig. 2. Effect of IL-1α and EGF on CDC25B expression in TMK-1 cells. Poly $(A)^+$ RNA was prepared from the cells after treatment with 1 n*M* IL-1α or 1 n*M* EGF for the indicated periods and analyzed as described in the legend to Fig. 1. The signal intensity was measured by densitometric scanning and relative expression levels were presented using that of control, 0 (h) as a standard (1.0).

Fig. 3. Expression of CDC25A, B and C in gastric carcinoma tissues. Northern blot analysis was performed as described in the legend to Fig. 1. T, gastric carcinoma tissues; N, non-neoplastic mucosa. The status of *p53* gene abnormalities is shown in the second row. Mutant, cases with mutated *p53* gene; Wild type, case with wild type *p53* gene; blank space, *p53* gene status not examined.[36]

Table I. Immunohistochemical Detection of CDC25B in Gastric Carcinoma Tissues

	Case no.	CDC25B-positive cases[b] (%)		
		positive	strongly positive	
Histological type				
well-differentiated[a]	33	25 (76)	11 (33)	c)
poorly differentiated[a]	22	18 (82)	16 (73)	
Stage[a]				
1	28	20 (71)	7 (25)	c)
2	2	2 (100)	2 (100)	
3	11	10 (91)	8 (73)	
4	14	11 (79)	10 (71)	
Depth of invasion[a]				
m	26	18 (69)	6 (23)	c)
sm, mp	2	2 (100)	1 (50)	
ss/se/si	27	23 (85)	20 (74)	
Lymph node metastasis				
negative	28	20 (71)	7 (25)	c)
positive	27	23 (89)	20 (74)	
Total	55	43 (78)	27 (49)	

a) According to the criteria of the Japanese Classification of Gastric Carcinoma.[28]
b) Positive including grades of +, ++ and +++; strongly positive including grades of ++ and +++.
c) Significant difference by Fisher's exact test ($P<0.05$).

by TMK-1 cells (Fig. 2). Maximal levels of CDC25B mRNA were found 12 h after IL-1α treatment and 1 h after EGF treatment.

Expression of CDC25 in gastric carcinoma tissues We next examined the expression of CDC25A, B and C mRNAs in gastric carcinoma tissues and corresponding non-neoplastic mucosa by northern blotting. Representative results of eight gastric carcinoma cases are shown in Fig. 3. Apparent overexpression of CDC25B in carcinoma tissues was found in many cases (cases 244, 245, 246, 248, 249), while three cases (cases 244, 248, 249) expressed CDC25A at higher levels in the tumors than in non-neoplastic tissues. The CDC25C expression was at very low or undetectable levels. Twenty-eight (70%) of the 40 gastric carcinomas expressed CDC25B mRNA at higher levels than the corresponding normal mucosas, while 12 (38%) of the 32 cases tested overexpressed CDC25A mRNA. The T/N ratio (mRNA level of tumor tissue/that of normal tissue) of the CDC25B expression tended to be higher in poorly differentiated adenocarcinomas (average±SE; 2.4±2.2) than in well differentiated adenocarcinomas (1.7±0.9). Since most of these cases were advanced carcinoma cases, the relationship, if

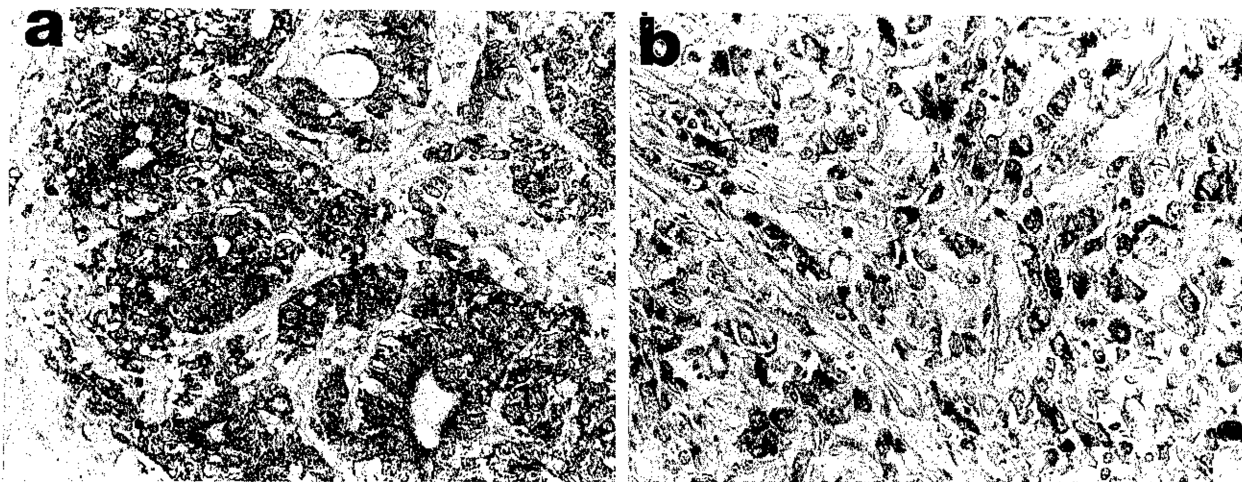

Fig. 4. Immunostaining of CDC25B in gastric carcinomas. a, Well-differentiated adenocarcinoma (staining grade +++, ×120). Most of the carcinoma cells show intense immunoreactivity to CDC25B. CDC25B is localized in the cytoplasm. b, Poorly differentiated adenocarcinoma (staining grade ++, ×192). About 40% of the tumor cells are strongly positive to CDC25B.

any, between the expression of CDC25B and tumor stage or invasiveness could not be evaluated.

As a correlation between CDC25B expression and *p53* gene status was suggested in gastric carcinoma cell lines (Fig. 1), we analyzed the expression of CDC25B mRNA in the cases with and without *p53* gene mutations. The *p53* gene status of the 20 cases had been reported previously.[36] However, 7 (58%) of the 12 carcinomas with mutated *p53* gene exhibited overexpression of CDC25B, while 5 (63%) of the 8 carcinomas without *p53* mutation revealed overexpression, so that there was no obvious correlation between the expression of CDC25B and *p53* gene mutations. As in the cell lines, no gross alterations of the CDC25 genes, such as amplification or rearrangement, were detected in any of the cases (data not shown).

Immunohistochemical analysis of CDC25B in gastric carcinomas Immunohistochemistry was performed on 55 gastric carcinoma tissues of various stages to analyze the relation with clinicopathological parameters. The incidence of CDC25B expression in gastric carcinomas is summarized in Table I. CDC25B-positive carcinoma cells were detected in 43 (78%) gastric carcinomas, among which 27 (49%) expressed CDC25B strongly (grades ++ and +++). CDC25B-immunoreactivity was localized in the cytoplasm of the tumor cells (Fig. 4). The incidence of strong positivity to CDC25B was higher in well-differentiated adenocarcinomas than in poorly differentiated adenocarcinomas ($P<0.05$). The incidence of strong CDC25B expression was significantly higher in the advanced cases (stages 2–4) than in stage 1 ($P<0.05$). As to the depth of tumor invasion and lymph node metastasis, a similar correlation was detected. Strong expression of CDC25B was observed in more than 50% of the carcinomas invading the submucosa or further, whereas it was detected in 23% of the carcinomas limited to the mucosa, its incidence being significantly different between the two ($P<0.05$). Furthermore, the incidence of strong expression was significantly higher in carcinomas with nodal metastasis (74%) than in those without metastasis (25%) ($P<0.05$). Almost no immunoreactivity to CDC25B was detected in non-neoplastic gastric mucosa, including foveolar epithelia, fundic glands and pyloric glands.

DISCUSSION

As we have shown in this paper, the expression levels of CDC25B mRNA were higher than those of CDC25A and C mRNA in gastric carcinoma cell lines. CDC25B mRNA was overexpressed in 70% of the gastric carcinomas, and CDC25A mRNA was overexpressed in 38% of them. This is consistent with the findings in primary breast cancers.[25] Therefore, the overexpression of CDC25, especially CDC25B, might favor the development and progression of gastric carcinomas as well. Furthermore, strong expression of CDC25B was detected in various cell lines of different origins (oral, esophagus and colon), in addition to gastric and breast cancer cell lines (Kudo and Yasui, unpublished observation). Increased expression of CDC25B might be a common event in a wide variety of human cancers.

In gastric carcinoma cell lines, MKN-45 and -74, with the wild-type *p53* gene, expressed very low levels of CDC25B mRNA and protein. In these cell lines, p21 and

wee1, negative regulators of the cell cycle, were expressed at high levels.[37] However, the growth rates of these cell lines are not very different from those of other cell lines with increased expression of CDC25B. The cell cycle progression is regulated by a balance of negative and positive regulators. The expression of cyclin H in MKN-45 and -74 is at high levels. Abnormalities in target molecules for CDKs, such as RB, may also favor the anomalous growth of these cell lines.

Although a correlation between increased expression of CDC25 and *p53* gene status was suggested in gastric carcinoma cell lines, it was not found in the gastric carcinoma cases. This is consistent with the finding that CDC25B overexpression is not associated with *p53* gene mutations in breast cancers and CDC25 does not show oncogenic character in *p53*-null cells.[25] The expression and function of CDC25B seem to be *p53*-independent.

The most interesting result in this study is that the immunostaining of CDC25B protein was closely associated with tumor stage, invasiveness and nodal metastasis. The incidence of strongly CDC25B-positive tumors was significantly higher in cases of stages 2–4, deeply invasive cases and cases with lymph-node metastasis. The tumor cells in the metastatic foci showed similar immunoreactivity to those in the primary sites (data not shown). Although we do not know at present how overexpressed CDC25B brings about high metastatic potential, increased expression of CDC25B could predict malignant behavior of gastric carcinomas. We should examine the significance of CDC25B as a prognostic factor.

As to the mechanism of overexpression of CDC25B found in gastric carcinomas, it is unlikely that genetic alterations such as amplification and rearrangement are involved. Gastric carcinomas overexpress various growth factors/cytokines and their receptors that favor biological malignancy. Both IL-1α and EGF are autocrine growth factors for gastric carcinoma cells.[34, 35, 38] We found slight induction of the expression of CDC25B mRNA by these factors in TMK-1 cells. There must be other strong inducers of CDC25B expression. Recently, it has been reported that activation of the cell cycle via *ras/raf*1 pathways is partly mediated by CDC25.[39] CDC25A and B act cooperatively in *myc*-driven cell cycle activation and/or apoptosis.[40] The relationship between the overexpression of CDC25B and *ras* mutations or *myc* amplification should be examined in gastric carcinomas. Furthermore, since some chromosomal abnormalities are known to be caused by cell cycle checkpoint defects, it is of interest to examine the association of CDC25B overexpression and chromosomal abnormalities in gastric carcinomas.

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