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Frequent Amplification of the Cyclin E Gene in Human Gastric Carcinomas

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We searched for genetic alterations of the cyclin D1 and cyclin E genes in 45 human gastric carcinoma tissues. Expression of cyclin E mRNA and protein was also analyzed in eight of them by Northern and Western blots and immunohistochemical staining. The cyclin E gene was amplified 3–10 fold in seven gastric cancer tissues (15.6%), of which six were advanced gastric cancers. All of the cases with the cyclin E gene amplification displayed lymph node metastasis. Moreover, the case with the gene amplification overexpressed the cyclin E mRNA and protein. One of eight gastric cancer cell lines, MKN-7, shared the cyclin E gene amplification, and all of the gastric cancer cell lines expressed high levels of the cyclin E mRNA and protein even without gene amplification. Amplification of the cyclin D1 gene was not observed in any of the gastric carcinoma tissues or gastric carcinoma cell lines. These results suggest that the gene amplification and overexpression of cyclin E play an important role in the abnormal growth and progression of gastric carcinoma.

Key words: Cell cycle – G1 cyclins – G1/S transition

Recent evidence indicates that multiple cyclins and cyclin-dependent protein kinases (cdks) regulate the G1/S phase transitions.[1,2] Cyclin D1, functionally forming a complex with cdk4/cdk6, is involved in G1/S transition and may play a role in oncogenesis.[1–3] The expression of cyclin E and the abundance of the cyclin E-cdk2 complex are maximal at the G1/S transition.[4,5] Cyclin D1 accumulates earlier in G1 phase than cyclin E, but in continuously proliferating cell populations their levels may oscillate only minimally.[1–5] Alteration in cell cycle regulators and subsequent deregulation of the cell cycle can cause unbridled cell division, contributing to cancer development.[2]

Chromosome segment 11q13, including the cyclin D1 gene and the growth factor genes *hst*-1 and *int*-2,[6] is well known to be amplified in carcinomas of the head and neck, esophagus, breast and liver.[7–12] Although the most striking abnormality found in the expression of G1 cyclins in several carcinomas, including breast and colorectal carcinomas,[13,14] is that of cyclin E,[15,16] the chromosomal localization of the cyclin E gene is unknown.

We report here frequent amplification of the cyclin E gene in human gastric carcinoma.

Eight human gastric carcinoma cell lines were employed in this study. TMK-1 cell line (poorly differentiated gastric adenocarcinoma) was established in our laboratory.[17] 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),[18] respectively. The other four human gastric carcinoma cell lines of 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 grown in RPMI-1640 (Nissui Co., Tokyo) supplemented with 10% fetal bovine serum (Whittaker M. A. Bioproducts Inc., Maryland) under conditions of 5% CO_2 in air at 37°C.

A total of 45 cases of gastric carcinoma were examined. Forty-five primary tumor tissues and the corresponding normal mucosas were surgically resected, frozen immediately in liquid nitrogen and stored at −80°C until use. We confirmed microscopically that the tumor tissue specimen consisted mainly of carcinoma tissue and that non-neoplastic mucosa did not exhibit any tumor cell invasion or show significant inflammatory involvement.

DNAs were extracted by the phenol-chloroform method after treatment with sodium dodecyl sulfate (SDS) and proteinase K. DNAs were digested with a restriction enzyme under the conditions recommended by the supplier. The completely digested DNAs (10 μg) were subjected to electrophoresis on 0.8% agarose gel and blotted onto nitrocellulose filter membrane. Filters were baked for 2 h at 80°C under vacuum. Hybridization, washing and autoradiography were performed as described.[19]

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RNAs were extracted by the standard guanidium isothiocyanate/cesium chloride method.[19] Five μg of poly (A)$^+$ selected RNA was electrophoresed on 1.0% agarose/formaldehyde gel and blotted onto a nitrocellulose filter membrane. Filters were baked for 2 h at 80°C under vacuum. Hybridization, washing and autoradiography were performed as described.[19]

The following ^{32}P-labeled cDNA probes were used. The 1.3 kb *Not* I cyclin D1 fragment of pBS KS$^-$ and the 2.5 kb *Eco*RI cyclin E fragment of pBS KS$^-$ were kindly provided by Dr. S. I. Reed (The Scripps Research Institute, California).[20]

The protein samples were prepared and Western blotting was carried out as described.[21] Fifty μg protein was subjected to 10% polyacrylamide gel electrophoresis followed by electroblotting onto a nitrocellulose filter. Anti-human cyclin E mouse monoclonal antibody was purchased from Pharmingen, California. For detection of the immunocomplex, the ECL Western blotting detection system (Amersham, Aylesbury) was used.

Tumor tissues were fixed in 10% neutral formalin and embedded in paraffin. For immunohistochemistry, a modification of the immunoglobulin enzyme bridge technique (ABC method) was used as described previously.[22] Deparaffinized tissue sections were immersed in methanol containing 0.03% hydrogen peroxide for 30 min to block the endogenous peroxidase activity and incubated in a microwave oven for 10 min. The section was then incubated with normal horse serum (diluted 20:1) for 30 min to block the non-specific antibody binding sites. The sections were treated consecutively at room temperature with anti-human cyclin E mouse monoclonal antibody (diluted 1:250) for 1.5 h, biotinylated anti-mouse IgG horse serum (diluted 1:100, Vector, California) 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 counterstained with 0.1% hematoxylin.

DNAs from 45 human primary gastric carcinoma tissues and corresponding non-neoplastic mucosas were digested with restriction enzymes and analyzed for genetic abnormalities of cyclins D1 and E by Southern blotting. Seven (15.6%) of the 45 primary gastric carcinoma tissues had amplification of the cyclin E gene

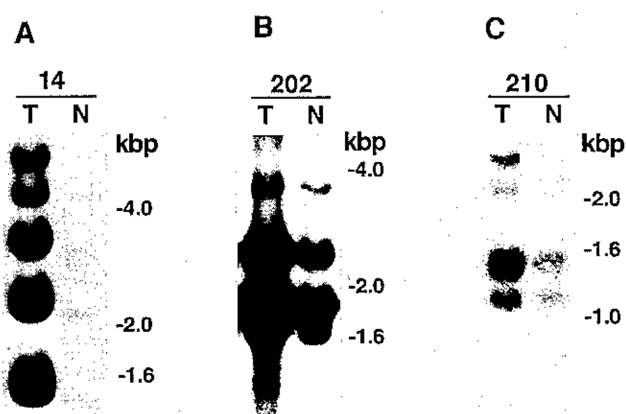

Fig. 1. Amplification of cyclin E gene in gastric carcinoma tissues in Cases 14, 202 and 210. Ten μg of genomic DNA was digested with the restriction enzymes indicated and electrophoresed through a 0.8% agarose gel. DNA was blotted onto a nitrocellulose filter membrane and hybridized with ^{32}P-labeled cyclin E probes as described in "Materials and Methods." T, gastric carcinoma tissue; N, non-neoplastic mucosa. In A, DNAs from tissues were completely digested with *Pst*I; In B, *Hin*dIII; In C, *Msp*I. Cases 14, 202 and 210 show 10-, 3- and 3-fold amplification of the cyclin E gene, respectively. Right ordinate, molecular weight marker sizes.

Table I. Clinical Features of the Patients with an Amplified Cyclin E Gene

Patient No.	Age/sex	Histology[a]	Depth of invasion[a]	Nodal metastasis	Stage[a]	Prognosis	Amplification of cyclin E gene
9	73/M[b]	well	sm	positive	II	27 mo, death[c]	3×
11	69/F	poorly	ss	positive	II	unknown	3×
14	63/M	well	ss	positive	IV	4 mo, death	10×
24	65/M	poorly	ss	positive	III	7 mo, death	3×
202	65/M	well	se	positive	III	24 mo, survive	3×
210	65/M	well	ss	positive	II	unknown	3×
528	79/M	well	ss	positive	II	21 mo, survive	5×

a) According to the criteria of the Japanese Classification of Gastric Carcinoma.[30] Well, well differentiated adenocarcinoma including papillary and tubular adenocarcinoma; poorly, poorly differentiated adenocarcinoma including signet ring cell carcinoma and mucinous adenocarcinoma; sm, submucosa; ss, subserosa; se, serosa.
b) M, male; F, female.
c) mo, month.

(representative cases; Cases 14, 202 and 210 shown in Fig. 1). Table I shows clinical features of the patients with an amplified cyclin E gene. To examine the expression of cyclin E, Northern and Western blot analyses and immunohistochemical staining were performed on 8 of the 45 primary gastric carcinoma cases. Representative results are shown in Fig. 2 (Southern, Northern and Western blot analyses). The case with gene amplification (Case 528) synchronously overexpressed cyclin E mRNA and protein ranging in size from 20 kDa to 50 kDa. An increased expression of cyclin E mRNA was detected in all tumor tissues and one of them (Case 526) displayed overexpression of cyclin E protein even without gene amplification. In all of the cases with overexpression of cyclin E protein (Cases 526 and 528), immunoreactivity to cyclin E was found in the nucleus, but cytoplasmic staining was not seen (Fig. 3, B and C). The other cases without overexpression of cyclin E protein showed no immunoreactivity to cyclin E (representative case; Case 525 shown in Fig. 3A).

Among eight gastric carcinoma cell lines, one (MKN-7) exhibited gene amplification of cyclin E (Fig. 4). This cell line showed overexpression of both mRNA and protein of cyclin E. The other cell lines with a single copy of the cyclin E gene also overexpressed both mRNA and protein of cyclin E, and their levels were well correlated. All the cell lines also expressed cyclin E proteins of various sizes. It has been reported that some breast carcinomas overexpressed cyclin E protein ranging in size from 35 kDa to 50 kDa, and the alterations in cyclin E expression became progressively more severe with increasing stage and grade of the breast cancer.[15] There-

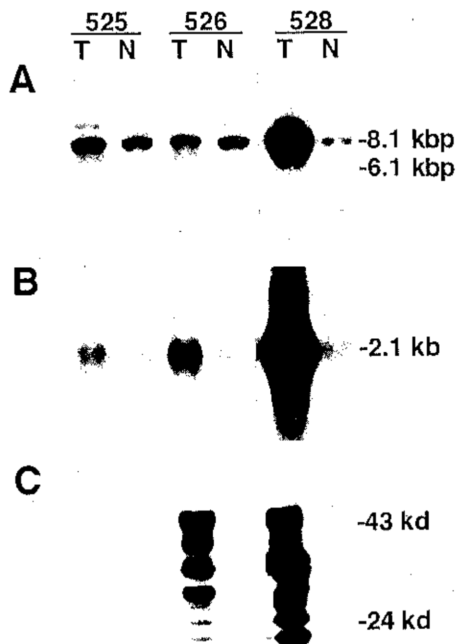

Fig. 2. Gene amplification and overexpression of cyclin E in gastric carcinoma tissues. Southern blot (A), Northern blot (B) and Western blot (C) analyses of cyclin E were performed as described in "Materials and Methods." Right ordinate, molecular weight marker sizes. In A, DNAs from tissues were completely digested with *Eco*RI.

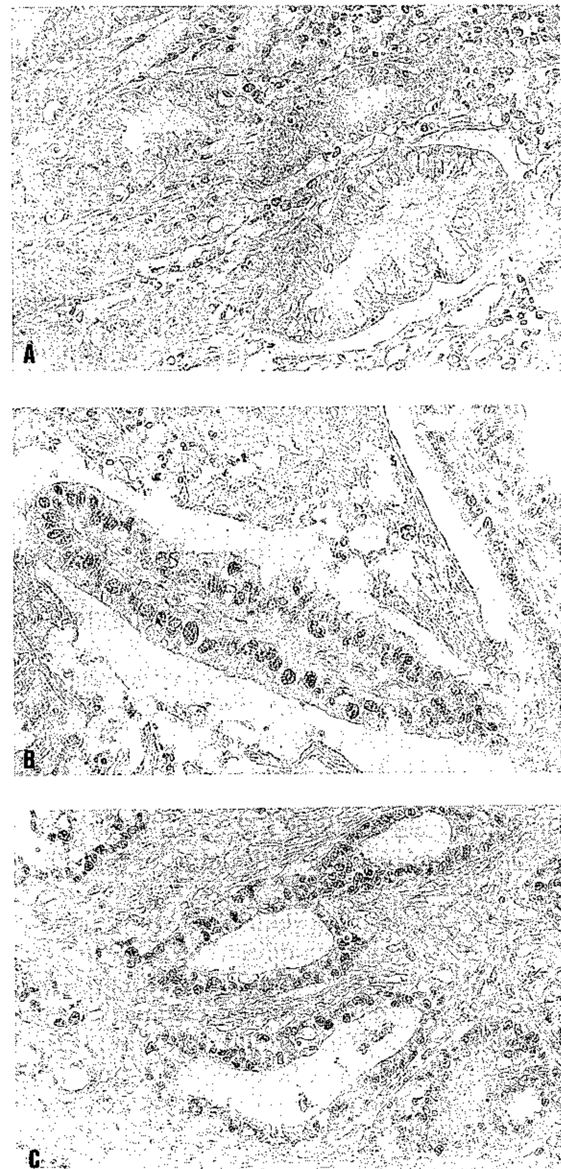

Fig. 3. Immunohistochemical staining of cyclin E in human primary gastric carcinoma tissues. A: Case 525. Neither nuclear nor cytoplasmic staining of cyclin E was observed, ×200. B: Case 526. Moderate nuclear staining of cyclin E was found without cytoplasmic staining, ×400. C: Case 528. Moderate immunoreactivity to cyclin E was seen in the nucleus, ×200.

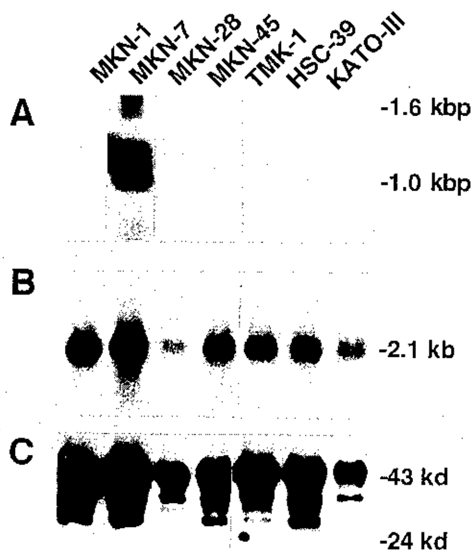

Fig. 4. Gene amplification and overexpression of cyclin E in gastric carcinoma cell line MKN-7. Southern blot (A), Northern blot (B) and Western blot (C) analyses of cyclin E were performed as in Fig. 2. Right ordinate, molecular weight marker sizes. In A, DNAs from tissues were completely digested with *Eco*RI.

fore, the expression of cyclin E by gastric carcinoma cell lines might be quantitatively and qualitatively abnormal. However, we can not rule out the possible degradation of cyclin E protein at present.

No amplification of the cyclin D1 gene was found in any of the gastric carcinoma tissues and cell lines examined so far (data not shown).

Gastric cancers involve amplification of multiple oncogenes encoding tyrosine kinase receptor, such as c-*met*, K-*sam* and c-*erb*B-2.[23-27] The amplification of these genes correlates well with tumor progression, metastasis and poor prognosis of the patients.[28] In this study, we found that the cyclin E gene was amplified in about 15% of gastric carcinomas. The overexpression of cyclin E was detected in all gastric carcinomas with gene amplification and in some of the carcinomas without gene amplification. These results suggest that abnormalities in cyclin E may be involved in stomach carcinogenesis. The accumulation of cyclin E and cyclin A proteins and induction of cdk2 kinase in the late G1 phase, which coincides with the timing of the restriction point protein, is necessary for moving cells toward the S phase.[29] The deregulation of this process may lead to abnormal proliferation and transformation. Clinicopathological features of gastric carcinomas with cyclin E gene amplification are summarized in Table I. Except for one case, all of the cases with gene amplification were advanced gastric cancers. No mucosal gastric cancers or stage I cancers had the gene amplification. Next, we compared the histology and nodal metastasis of the tumor between the cases with cyclin E gene amplification and the cases without cyclin E gene amplification. Histologically, 5 (33%) of 15 well differentiated adenocarcinomas showed cyclin E gene amplification, whereas only 2 (7%) of 30 poorly differentiated adenocarcinoma had the gene amplification. Moreover, although all the cases with the amplified cyclin E gene revealed nodal metastasis, 23 (61%) of 38 cases without the amplified cyclin E gene revealed nodal metastasis. Therefore, cyclin E gene amplification is often associated with advanced well differentiated stomach cancer. It remains to be elucidated whether metastatic tumors show amplification of the gene. It is also an important question whether cyclin E gene amplification is associated with the amplification of other oncogenes, such as c-*erb*B-2 and c-*met*, which play a role in the progression of gastric cancer.

On the other hand, the amplification of the cyclin D1 gene was not detected in any of the gastric carcinomas. This is quite different from the findings observed in esophageal squamous cell carcinomas, where coamplification of the cyclin D1 gene with *hst*-1 and *int*-2 occurs in 50% of primary tumors and 100% of metastatic tumors.[7] These findings overall indicate that the cyclins involved in the development and progression of cancer are different between gastric adenocarcinoma and esophageal squamous cell carcinoma. The amplification of the cyclin E gene and subsequent abnormal overexpression may have implications for development and progression of gastric adenocarcinoma.

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