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Genetic Status and Expression of the Cyclin-dependent Kinase Inhibitors in Human Gastric Carcinoma Cell Lines

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Deregulation of cyclin, cyclin-dependent kinases (CDKs) and their inhibitors could have a pivotal role in the development of diverse human cancers. We examined the genetic status and the expression of CDK inhibitors (*p21, p27, p16* and *p15*), CDK2 and cyclins (A, D1 and E) in eight gastric carcinoma cell lines, in comparison with the status of *p53* gene alterations. All the cell lines (except MKN-28) that contained a *p53* gene abnormality expressed very low or undetectable levels of *p21* mRNA, while the cell lines (MKN-45 and -74) with wild-type *p53* gene expressed high levels of *p21* mRNA. An inverse correlation was found between the level of *p21* mRNA and the expression of mRNAs for CDK2 and G1 cyclins. MKN-28 was an exception; it contained mutated *p53*, and expressed mRNAs for *p21*, CDK2 and G1 cyclins at high levels. Only MKN-45 and -74, with wild-type *p53*, expressed considerable levels of p21 protein. Homozygous deletion of the *p16* and *p15* genes was detected in two (MKN-45 and HSC-39) of the eight gastric carcinoma cell lines. p16 protein was not expressed in three cell lines (MKN-28, MKN-74 and KATO-III), as well as MKN-45 and HSC-39. Rearrangement of the *p15* gene was found in TMK-1. Rearrangement of the *p27* gene was detected in MKN-45, although the expression of p27 protein was well preserved in all the gastric carcinoma cell lines. The expression of pRb was also preserved in all the cell lines except KATO-III. No obvious correlation was observed between the *p53* gene status and the expression of *p27* and *p16*. These findings suggest that abnormal regulation of CDK2/cyclins and CDK inhibitors might be involved in deregulated growth of gastric carcinomas.

Key words: CDK inhibitors — CDK — Cyclin — Cell cycle — Gastric carcinoma cells

Deregulation of the G1/S transition in the cell cycle can cause unbridled cell division and may play a role in cancer development.[1] It appears that multiple cyclins and cyclin-dependent protein kinases (CDKs) regulate the G1/S phase transition.[2] Gene amplification and subsequent overexpression of cyclins D1 and E have been found in esophageal,[3] colorectal,[4] liver[5] and breast carcinomas.[6] We have also found frequent amplification of the cyclin E gene and increased cyclin-dependent protein kinase activity in gastric and colorectal carcinomas.[7,8] CDK activity is known to be controlled by a number of recently identified cellular inhibitors of CDK, such as $p21^{SDI1/WAF1/CIP1}$,[9–11] $p27^{KIP1}$,[12,13] $p16^{MTS1/INK4A}$ and $p15^{MTS2/INK4B}$.[14,15] Inactivation of these CDK inhibitors may bring about deregulation of the expression of cyclins, contributing to loss of cell cycle control.[1]

$p21^{WAF1/CIP1}$, identical to *SDI1*,[9] is a *p53*-inducible gene, whose product binds to cyclin/CDK complexes and inhibits the function of CDKs.[10,11] *p21* also inhibits DNA replication by interacting with PCNA.[16] *p21* promoter has a p53 binding site and the transcription is activated by wild-type *p53*, but not by mutated *p53*.[10,11] Alteration of the *p21* gene has been reported to be a rare event in human cancers, suggesting that mutational inactivation of *p21* is not involved in tumorigenesis.[17–19] In stomach carcinogenesis, alterations in multiple oncogenes and tumor suppressor genes are involved.[20] We have already reported that many gastric and colorectal carcinomas contain mutant *p53* gene.[21–23] However, some of them do not show any mutation in *p53* gene. It is still unclear how *p53* mutations lead to the development of cancer.

$p27^{KIP1}$, which exhibits sequence similarity with *p21*, was first identified as a negative growth regulator present in cells that were rendered quiescent by contact inhibition or in TGF-β-treated cells.[12,13,24] *p27* inhibits a wide variety of cyclin/CDK complexes including CDK2 and CDK4 complexes.[13] Many transformed cells are no longer contact-inhibited and fail to arrest in response to TGF-β treatment, which implies that *p27* function may be altered during oncogenesis.[1] However, this remains speculative, and few mutations of the *p27* gene have been reported in human cancers.[25–27]

Two highly homologous cdk inhibitors, $p16^{MTS1/INK4A}$ and $p15^{MTS2/INK4B}$, both located on chromosome 9p21 encode related proteins that primarily inhibit the activ-

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ity of cyclin D/CDK4 and cyclin D/CDK6 complexes.[1,28,29] pRb phosphorylation by cyclin D/CDK4 is inhibited by *p16 in vitro*.[28] Alterations of the *p16* and *p15* genes have been reported in cell lines from many different tumor types.[14,15] Deletions and mutations of *p16* and *p15* genes have been documented in 85% of pancreatic adenocarcinomas and 52% of esophageal squamous cell carcinomas.[14,30] Loss of *p16*, overexpression of D-type cyclins and loss of Rb have similar effects on tumorigenesis.[31,32]

In the present study, we examined the status of *p21*, *p27*, *p16* and *p15* genes and the expression of *p21*, *p27*, *p16*, *p15*, CDK2, G1 cyclins and Rb in human gastric carcinoma cell lines with or without *p53* gene mutation in order to determine which alteration among them is most commonly implicated in the abnormal cell growth of gastric cancer.

MATERIALS AND METHODS

Cell cultures Eight human gastric carcinoma cell lines were used. TMK-1 cell line (poorly differentiated gastric adenocarcinoma) was established in our laboratory.[33] KATO-III and HSC-39 cell lines (signet ring cell carcinoma) were kindly provided by Dr. Sekiguchi (University of Tokyo, Tokyo) and Dr. K. Yanagihara (Hiroshima University, Hiroshima),[34] respectively. The other five human gastric carcinoma cell lines of the MKN series (MKN-1, adenosquamous carcinoma; MKN-7, MKN-28 and MKN-74, well-differentiated adenocarcinoma; MKN-45, poorly differentiated adenocarcinoma) were kindly provided by Dr. T. Suzuki (Fukushima Medical College, Fukushima). The status of *p53* gene in these cell lines has already been reported.[21] They were routinely grown in RPMI-1640 (Nissui Co., Tokyo) containing 10% fetal bovine serum (Whittaker M.A.Bioproducts Inc., Walkersville, MD) under conditions of 5% CO_2 in air at 37°C.

DNA preparation and Southern blot analysis DNAs were extracted by the phenol-chloroform method after treatment with sodium dodecyl sulfate and proteinase K. DNAs were digested with a restriction enzyme under the conditions recommended by the manufacturers. 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.[35]

RNA preparation and Northern blot analysis RNAs were extracted by the standard guanidium isothiocyanate/cesium chloride method.[35] Five micrograms of poly $(A)^+$ selected RNA was electrophoresed on 1.0% agarose/formaldehyde gels 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.[35]

cDNA probes The cDNA fragments encoding *p21* was kindly provided by Dr. A. Noda.[9] The cDNA fragment encoding *p27* was kindly provided by Dr. J. Massague.[12] The cDNA fragments encoding *p16* and *p15* were kindly provided by Dr. T. Nobori.[15] The cDNA fragment encoding cyclin A was kindly provided by Dr. C. Brechot.[36] The cDNA fragments encoding cyclins D1 and E were kindly provided by Dr. S. I. Reed.[37] The cDNA fragment encoding CDK2 was kindly provided by Dr. M. Meyerson.[38] The β-actin probe was purchased from Oncor Inc., Gaithersburg, MD.

Preparation of extracts and Western blot analysis The protein samples were prepared and Western blotting was carried out as described.[8] The protein (50 μg) was subjected to 12% polyacrylamide gel electrophoresis followed by electrotransfer onto a nitrocellulose filter. Anti-human *p21* mouse monoclonal antibody (mAb) (EA-10) was purchased from Oncogene Science, Inc., Uniondale, NY. Anti-human CDK2 rabbit polyclonal antibody (15536E), anti-human cyclin E mouse mAb (14591A), anti-mouse *p27* rabbit polyclonal antibody (15596E), anti-human *p16* rabbit polyclonal antibody (15126E) and anti-human Rb mouse mAbs (G3-245) were purchased from Pharmingen, San Diego. Anti-human cyclin A rabbit polyclonal antibody (06-138) and anti-human cyclin D1 rabbit polyclonal antibodies (06-137) were purchased from Upstate Biotechnology, Inc., Lake Placid, NY. For detection of the immunocomplex, the ECL Western blotting detection system (Amersham, Aylesbury, UK) was used.

RESULTS

The expression of mRNAs for *p21*, CDK2 and G1 cyclins The results of Northern blot analysis of *p21*, CDK2 and cyclins A, D1 and E are shown in Fig. 1. The same filter was reprobed with each of the cDNA probes including β-actin probe. All the cell lines but one that contained *p53* gene abnormality expressed very low or undetectable levels of *p21* mRNA. *p53* gene is mutated in these cell lines except KATO-III, whose *p53* is completely deleted. *p21* mRNA was expressed at medium to high levels in MKN-28, -45 and -74 cells, among which MKN-45 and -74 have the wild-type *p53* gene. As to the expression of CDK2 and cyclins, MKN-74 cells with wild-type *p53* gene and a high level of *p21* expressed extremely low levels of CDK2 and cyclins. MKN-45 cells with wild-type *p53* gene and a moderate level of *p21* expression expressed relatively low levels of CDK2 and cyclins. The cell lines with mutated *p53* gene and a low level of *p21* expressed CDK2 and cyclins at moderate levels. In general, the levels of *p21* expression were related to the

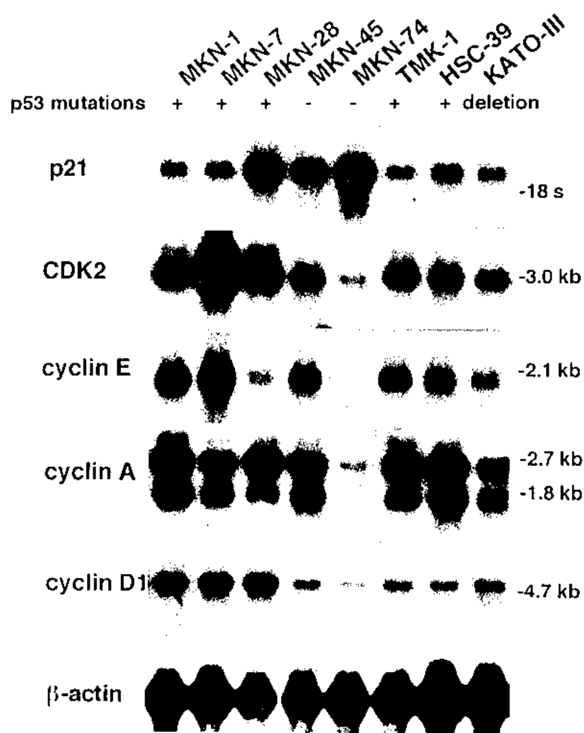

Fig. 1. The expression of mRNAs for *p21*, CDK2, cyclins A, D1 and E in gastric carcinoma cell lines. Five microgram of poly $(A)^+$-selected RNA was subjected to Northern blot analysis as described in "Materials and Methods." β-Actin probe was applied as an internal control. The status of *p53* gene abnormalities is shown in the second row. +; cells with mutated *p53* gene: −; cells with wild type *p53* gene: *p53* gene is completely deleted in KATO-III. Right ordinate, molecular weight marker size.

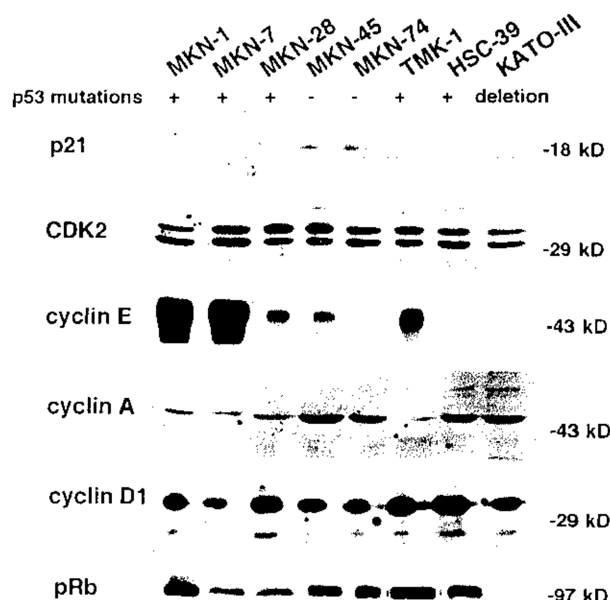

Fig. 2. Western blot analysis of *p21*, CDK2, cyclins A, D1, E and pRb in gastric carcinoma cell lines. Fifty μg of protein was subjected to Western blot analysis as described in "Materials and Methods." The status of *p53* gene abnormalities is shown in the second row. +; cells with mutated *p53* gene: −; cells with wild-type *p53* gene: *p53* gene is completely deleted in KATO-III. Right ordinate, molecular weight marker size.

status of *p53* gene abnormalities, and an inverse correlation was found among the levels of *p21*, CDK2 and cyclins. Although MKN-28 expressed *p21* mRNA at a high level, the expressions of CDK2 and cyclins were well preserved. No gross alterations of the *p21* gene were detected (data not shown).

The expression of proteins for *p21*, CDK2, G1 cyclins and pRb Next we examined the expression of *p21*, CDK2 and G1 cyclins at protein levels (Fig. 2). MKN-45 and -74 cells with wild-type p53 expressed p21 protein, whereas p21 protein was not detectable in any of the cell lines with p53 mutations. The levels of expression of CDK2 and cyclin D1 protein were almost the same among all the cell lines. The cyclin E protein was expressed at high levels in MKN-1 and -7 cells, which expressed cyclin E mRNA at high levels. The expression level of cyclin A varied depending on the gastric carcinoma cell lines. MKN-74 cells expressed cyclin A protein at high level, although the level of cyclin A mRNA was very low. We also examined the expression of Rb to investigate associations with the status of CDK inhibitors, CDK2, G1 cyclins and phosphorylated Rb. All the gastric carcinoma cell lines except KATO-III expressed certain levels of pRb of 110 kD in size. We confirmed that the bands of 110 kD corresponded to hyperphosphorylated Rb by comparison with the migration of both hyperphosphorylated form and underphosphorylated form (data not shown and Ref. 39).

Genetic status and expression of the *p27* gene In order to evaluate genetic alteration of *p27*, Southern blot analysis was performed on DNAs from 8 gastric carcinoma cell lines (Fig. 3). No gross deletion or rearrangement was found within the *p27* gene in any of the gastric carcinoma cell lines except MKN-45 cells. In MKN-45 cells, the major band was located in a position corresponding to faster migration, indicating rearrangement or a polymorphism of the *Eco*RI site of the *p27* gene. However, the expression of p27 protein was observed in all the gastric carcinoma cell lines.

Genetic status and expression of the *p16* and *p15* genes DNAs from 8 human gastric carcinoma cell lines were

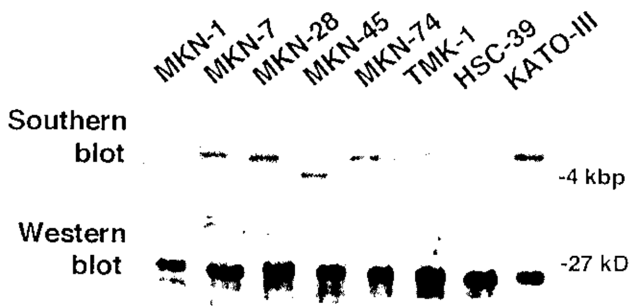

Fig. 3. Southern and Western blot analyses of *p27* in gastric carcinoma cell lines. Ten μg of DNA digested with restriction enzyme *Eco*RI was analyzed by Southern blotting as described in "Materials and Methods." The expression of *p27* was analyzed by Western blotting as described in "Materials and Methods." Right ordinate, molecular weight marker size.

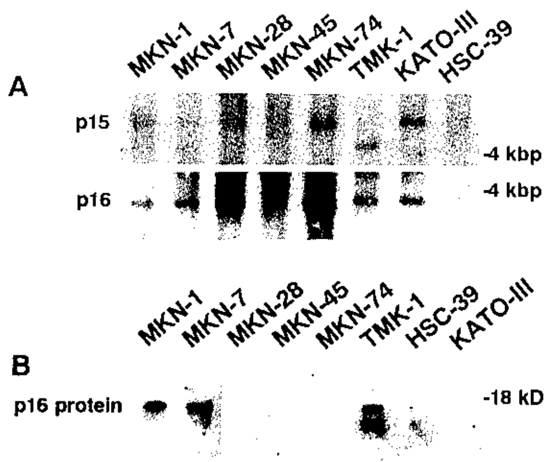

Fig. 4. Homozygous deletion of the *p16* and *p15* genes and subsequent expression of *p16* in gastric carcinoma cell lines. A, DNAs were digested with restriction enzyme *Eco*RI and analyzed by Southern blotting as described in "Materials and Methods." B, The expression of *p16* was analyzed by Western blotting as described in "Materials and Methods." Right ordinate, molecular weight marker size.

digested with *Eco*RI and analyzed by Southern blotting to visualize abnormalities of the *p16* and *p15* genes. MKN-45 and HSC-39 cells did not show any signals of *p16* and *p15* (Fig. 4A). We then digested the DNAs with *Bam*HI, conducted a Southern blot analysis and obtained the same result; no signals (data not shown). Lack of p16 protein expression was consistently detected in MKN-28, MKN-74 and KATO-III, as well as MKN-45 and HSC-39 cells (Fig. 4B). We concluded that the *p16* and *p15* genes were homozygously deleted. In TMK-1 cells, the faster migrating signal of *p15* was detected, implying that the *p15* gene is rearranged or that there is a polymorphism of the *Eco*RI site (Fig. 4A).

DISCUSSION

In the present study, we first examined the relationship between *p53* mutations and the expression of *p21*, cyclins and CDK in human gastric carcinoma cells.

Gastric carcinoma cell lines with mutated *p53* expressed *p21* mRNA at low levels, while they expressed considerable levels of mRNAs of CDK2 and G1 cyclins. In contrast, MKN-45 and -74, with no *p53* mutation, showed a high level of expression of *p21* mRNA and decreased expression of mRNAs of CDK2 and G1 cyclins. These findings indicate that the status of *p53* gene is an important determinant for the expression of *p21*, and *p21* may negatively regulate the expression of CDK2 and G1 cyclins. In the case of the cell lines without *p53* mutation, there is a possibility that cell cycle regulating molecules other than those of the *p21*/CDK2 pathway, such as CDK4 and CDK6,[28,40] may be involved in the abnormal cell proliferation. On the other hand, the expressions of CDK2 and cyclins A and D1 were well preserved in MKN-74 cells, although these cells expressed extremely low levels of the mRNAs. In addition, all the cell lines except KATO-III expressed pRb protein. These findings suggest that the expression of CDK2 and cyclins might also be regulated post-transcriptionally.

MKN-28 cell line was an exception in having a mutated *p53* gene but expressing *p21* mRNA at a high level. *p53*-independent pathways for the regulation of *p21* expression may exist in this cell line. Recent studies have shown that various agents, such as differentiation factors and growth factors including TGF-β1, induce the expression of *p21* mRNA.[41,42] We have also found that *p21* is induced by TGF-β1 through a *p53*-independent pathway in gastric carcinoma cell line TMK-1.[39] This should be taken into account to explain the discrepancy between *p53* and *p21*. On the other hand, although the level of *p21* was high, considerable levels of CDK2, cyclins A and D1 were expressed in MKN-28 cells, suggesting functional abnormality of *p21*. However, no point mutations of *p21* gene were detected in its open reading frame in gastric carcinoma cell lines (unpublished data). So far, only one Burkitt's lymphoma cell line has been reported to contain *p21* gene mutation.[19] In addition, *p21* knockout mouse does not show increased cancer susceptibility, unlike *p53* knockout mouse, suggesting that mutational inactivation of *p21* may not be involved in tumorigenesis.[43] At present, it remains unclear why *p21* was induced but the expressions of CDK2 and cyclins were not suppressed in

MKN-28 cells. Although the function of *p27* is similar to that of *p21*, altered expression of *p27* was not observed in any of the gastric carcinoma cell lines examined.

In Southern blot analysis of the *p27* and *p15* genes, altered bands suggesting rearrangements or polymorphisms of these genes were found in MKN-45 and TMK-1. However, no obvious correlation with the expression of *p27* was detected in these cell lines. Further studies are needed to clarify the precise abnormalities of these genes and their significance for gastric carcinogenesis.

In the present study, homozygous deletion of the *p16* and *p15* genes was detected in 2 of 8 (25%) gastric carcinoma cell lines. It is well known that the *p16* gene is deleted in a variety of tumor cell lines,[14, 15] although genetic alteration is less frequent in primary tumors.[44, 45] Similarly, we have found no genetic alteration of *p16* or *p15* in primary gastric carcinoma (data not shown). Recently, it has been reported that abnormal methylation of the 5′ CpG island of *p16* gene blocks transcription in many cancers.[46, 47] The present study also demonstrated that MKN-28, -74 and KATO-III did not express *p16*, although no genetic alteration was detected. These findings suggest that homozygous deletion as well as abnormal methylation may be linked with inactivation of *p16*.

Recently, it has been reported that pRb-negative cells contain intact *p16*, while *p16*-negative cells contain intact pRb.[48, 49] Therefore, pRb-positive tumors might require decreased amounts of functional *p16* to achieve levels of CDK4 activity sufficient for Rb inactivation.[50] We have already reported that the expression of pRb is inversely correlated with the *p16* expression in esophageal carcinoma cell lines.[51] However, all the cell lines examined except KATO-III expressed pRb of about 110 kD, which corresponds to phosphorylated form, implying a discrepancy between *p16* and pRb in KATO-III cells. Therefore, it is not likely that the expression of *p21* in gastric carcinoma cells was affected by Rb status.

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