CIP2A Is Overexpressed in Gastric Cancer and Its Depletion Leads to Impaired Clonogenicity, Senescence, or Differentiation of Tumor Cells

Wenjuan Li,1,3 Zheng Ge,3 Cheng Liu,3 Zhifang Liu,2 Magnus Björkholm,3 Jihui Jia,1 and Dawei Xu3

Abstract

Purpose: Cancerous inhibitor of protein phosphatase 2A (CIP2A) is an oncogenic factor stabilizing c-MYC protein and driving cellular transformation. We determine whether CIP2A expression can serve as marker for gastric cancer and investigate the mechanism underlying CIP2A-mediated transformation and cell proliferation.

Experimental Design: Normal and malignant gastric tissues derived from 37 patients with gastric cancer were analyzed for CIP2A expression using reverse transcription-PCR and immunohistochemical staining. Gastric and other cell lines with different p53 and pRB backgrounds were used to inhibit CIP2A expression using small interfering RNA and then examined for clonogenic potentials, senescence, or differentiation.

Results: CIP2A mRNA was present in 34 of 37 (90%) of tumor specimens but absent in 27 of 37 (73%) of matched normal gastric mucosa. In 10 adjacent normal tissues with detectable CIP2A mRNA, 6 of them exhibited much weaker levels of CIP2A compared with their corresponding tumors. Thus, a total of 32 (87%) gastric cancer samples overexpressed CIP2A. CIP2A protein expression was readily detectable in the tumor tissues but absent in normal gastric mucosa. Depleting CIP2A expression substantially inhibited growth and clonogenic capabilities of tumor cell lines independently of p53 and pRB pathways. Gastric cancer–derived AGS cells underwent senescence following the inhibition of CIP2A expression. Moreover, CIP2A depletion triggered partial differentiation of leukemic HL60 cells.

Conclusion: CIP2A in tumor cells is required for sustained proliferation by preventing cell growth arrest, senescence, or differentiation and its expression is significantly (P < 0.001) discriminatory between normal and cancerous gastric tissue.

Gastric cancer is one of the most common malignancies worldwide and ranks second in terms of global cancer-related mortality (1, 2). Helicobacter pylori infection has been shown to initiate the pathogenesis of gastric cancer by causing a chronic gastritis, the precursor to all the pathophysiologic abnormalities characteristic of gastric carcinogenesis (1, 3, 4). However, these precursor lesions only develop in a proportion of infected subjects and do not necessarily progress into invasive cancers. The host genetic, bacterial virulence, environmental, and many other factors have been implicated in affecting the gastric oncogenic process, but the underlying molecular mechanism is poorly understood (1, 3). Moreover, because of lack of reliable early diagnostic markers, the prognosis of gastric cancer remains poor. Therefore, better defining the pathogenesis of gastric cancer, looking for useful biomarkers, and exploring novel therapeutic targets for treatment are urgently demanding tasks (1–3).

A hallmark of cancer is unlimited cellular proliferation due to the aberrant expression of key factors regulating cell cycle progression, apoptosis, senescence, and differentiation (5). One of these molecules, named cancerous inhibitor of protein phosphatase 2A (PP2A; CIP2A), has recently been identified to stabilize c-MYC protein by inhibiting its degradation mediated by PP2A in cancer cells and to be required for malignant cell growth (6). When overexpressed, CIP2A transforms immortalized human cells. Furthermore, CIP2A was observed to be highly expressed in human neck and head carcinomas and colon cancers (6). Taken together, CIP2A functions as an oncoprotein contributing to malignant transformation of human cells. In the present study, we compared CIP2A expression between normal and malignant gastric tissues to determine whether CIP2A could serve as a diagnostic marker for gastric cancer; moreover, we sought to investigate the...
mechanism behind CIP2A-mediated tumorigenesis by evaluating its role in clonogenic capability, cellular senescence, and differentiation.

Materials and Methods

Patients and tissue specimens. Thirty-seven patients with primary gastric cancer were included in the study, which was approved by the local ethics committee. The patients underwent gastrectomy at Qilu Hospital, Shandong University and Shandong Provincial Hospital, between 2006 and 2007. After surgery, the tumor specimens and distant normal gastric mucous tissues (>5 cm from the margin of the tumor) were collected and stored at -80°C until use. None of the patients enrolled in the study had received chemotherapy or radiotherapy before surgery, and there was no evidence of any other malignancies. The diagnosis of all gastric cancers was histopathologically confirmed by examination of surgical specimens. For each tumor, histology, differentiation status, tumor size (diameter), invasiveness, and regional and distant metastases (pathologic tumor-node-metastasis classification) at the time of operation were evaluated. Detailed patient and disease characteristics are documented in Table 1.

Cell lines and culture conditions. Human gastric cancer cell lines AGS, HGC-27, KATO-III, and BGC-823, cervical cancer line HeLa, colon cancer line HCT116 with wild-type p53 (HCT116p53+/+) and its variant HCT116 with p53 deletion (HCT116p53/-; ref. 7), and leukemic cell line HL60 were cultured at 37°C, 95% air, 5% CO2 in RPMI 1640 (Life Technologies) containing 10% FCS, 100 units/mL penicillin, and 2 mmol/L L-glutamine. For serum starvation, the cells were washed with PBS twice and incubated in FCS-free medium for 72 h.

Small interfering RNA treatment. Chemical modified Stealth small interfering RNA (siRNA) targeting CIP2A and control siRNA were bought from Invitrogen. The sequence for the CIP2A siRNA was 5'-GACAACUGUCAAGUGUACCACUCUU-3'. The cells were transfected with siRNA using LipofectAMINE 2000 (Invitrogen) according to the manufacturer’s protocol.

Table 1. Patient and tumor characteristics and CIP2A mRNA expression in gastric cancers and adjacent normal mucosa

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<th>No.</th>
<th>Age (y)/sex</th>
<th>Size (cm)</th>
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<th>CIP2A (normal mucosa)</th>
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*Stages according to International Union Against Cancer/tumor-node-metastasis classification.

-1neg, negative, no visible specific CIP2A bands; +, positive with weak to medium signals; ++, positive with strong signals.

The relative level of CIP2A mRNA was calculated as described in Materials and Methods.
RNA extraction, reverse transcription-PCR, and real-time quantitative PCR. Total cellular RNA in the tissue specimens and in cells with different treatments was extracted using the Trizol (Invitrogen) or ULTRASPEC kit (Biotec Lab.) according to the manufacturer’s protocol. cDNA was synthesized using random primers (N6) (Pharmacia) and MMLV reverse transcriptase. The PCR primers used in the study were as follows: sequences specific for CIP2A mRNA: 5'-CCA-TATGCTCACTCAGATGATGT-3' (forward) and 5'-GTGTATCATCTCCA-CAGAGAGTT-3' (reverse). The above primer pairs cross intron/exon boundaries in the CIP2A gene; thus, the resultant PCR products do not represent genomic DNA contamination. The PCR conditions were optimized to make sure a linear amplification of each target: PCR for CIP2A was done by running 26 and 32 cycles (95°C for 15 s, 60°C for 45 s, and 72°C for 60 s) for cell lines and gastric samples, respectively. β2-microglobulin expression was used as a control for RNA loading and reverse transcription efficiency and amplified. PCR products were resolved in 2% agarose gels, stained with ethidium bromide, and visualized in UV light.

Real-time quantitative PCR (RT-QPCR) was carried out in an ABI7700 sequence detector (Applied Biosystems) using Pre-made Gene Expression Assays (Applied Biosystems) primers and probes for CIP2A (HS00978142_m1). Levels of CIP2A mRNA were expressed as the ratio versus human β2-microglobulin (HS00187842_m1) based on the CT values.

Western blot. Total cellular proteins were extracted with radio-immunoprecipitation assay lysis buffer as described [8]. Proteins (20 μg) were resolved by SDS-PAGE and transferred to an intracellular membrane. The membranes were probed with the specific antibodies against CIP2A (Novus Biologicals), p16, c-MYC, p53 (Santa Cruz Biotechnologies), and p21 (BD) followed by anti-mouse or rabbit horseradish peroxidase–conjugated IgG and developed with the enhanced chemiluminescence method (ECL). β-actin served as a loading control.

Immunohistochemistry. Immunohistochemical analyses were done as described earlier (9). Following deparaffinization and antigen unmasking, the antibody against CIP2A (Novus Biologicals) was added onto the sections and incubated for 45 min at room temperature. Subsequently, biotinylated rabbit anti-mouse IgG (H+L), alkaline phosphatase–conjugated avidin-biotin complex, and Vector Red Alkaline Phosphatase Substrate (Vector Laboratories) were employed to visualize CIP2A antibody binding. The slides were finally counterstained with hematoxylin. The negative control was done in parallel using the mouse IgG instead of CIP2A antibody.

Colony formation assay. The cells were transfected with control or CIP2A siRNA for 72 h. Thereafter, cells were planted into six well-plates (800 per well for KATO-III and 300 per well for the rest of the cell lines) and incubated for 10 days. Plates were stained with Giemsa and the number of colonies with more than 50 cells was counted.

β-Galactosidase staining. β-Galactosidase (β-Gal) staining of cells was done as described (10, 11). Briefly, the cells grown in 24-well plates were transfected with control or CIP2A siRNA. After 72 h, the cells were rinsed with PBS once, fixed in 3% of formaldehyde for 5 min, and incubated with freshly prepared β-Gal staining solution at 37°C overnight (10, 11).

Statistical analyses. The difference in CIP2A mRNA expression between tumors and adjacent normal gastric tissues as detected using conventional reverse transcription-PCR (RT-PCR) and RT-QPCR was analyzed using χ² and Mann-Whitney U tests, respectively. The comparison of foci numbers between control and CIP2A-depleted cells was made using a Student’s t test. All the tests were two-tailed and computed using SigmaStat 3.1 software (Systat Software). P values < 0.05 were defined as statistical significance.

Results

Overexpression of CIP2A in cancerous gastric tissues. Normal and cancerous gastric specimens were analyzed for CIP2A mRNA expression using conventional RT-PCR and RT-QPCR. Both of the assays gave rise to similar results in most cases (Table 1; data not shown). The presence of CIP2A transcripts was found in 34 of 37 (90%) of cancer samples (Table 1; Fig. 1A), whereas only 10 of 37 (27%) normal gastric mucosa exhibited detectable CIP2A mRNA expression. There was a highly significant difference in CIP2A expression between gastric cancer samples and their corresponding normal gastric tissues (conventional RT-PCR, χ² = 29.7, P < 0.001; RT-QPCR, Mann-Whitney U test, P < 0.001). Among the CIP2A-positive normal samples, 6 of them had significantly lower levels compared with those in their corresponding tumors. Taken together, the overexpression of CIP2A transcripts occurred in 32 of 37 (87%) gastric tumor specimens (Table 1; Fig. 1A). There was no clear association between presence of CIP2A mRNA and age, gender, tumor burden, or differentiation status.

We further analyzed the CIP2A protein level in 10 available gastric cancer specimens and their corresponding normal mucosa using an immunohistochemical approach (Fig. 1B-D). Consistent with its mRNA expression profile, CIP2A protein in 80% (8 of 10) of cancer samples was readily detectable with positive cell fractions from 11% to 100% (Fig. 1C and D).
A significant heterogeneity in CIP2A signal intensity was observed in the same tumor. CIP2A protein was present in both cytoplasmic and nuclear compartments of tumor cells but a much stronger signal seen in the former one (Fig. 1C and D). The adjacent normal gastric mucosa exhibited negative CIP2A staining, although rare epithelial cells (<5%) were found to be positive for CIP2A protein expression and those positive cells were predominately localized at the proliferation zones of crypts (Fig. 1B; data not shown).

**Proliferation-regulated CIP2A expression.** c-MYC expression is highly proliferation regulated: induced in actively proliferating cells but silent in quiescent or growth-arrested cells (12). Given the fact that CIP2A targets c-MYC protein for its stabilization, we hypothesize that CIP2A expression may be associated with cellular proliferation status, too. To probe this, we incubated the AGS and HCT116 cells in FCS-free medium for 72 h and then analyzed CIP2A mRNA and protein levels. As expected, a substantial decrease in CIP2A expression was seen in serum-depleted cells compared with those grown in FCS-containing medium (Fig. 2). With a longer serum starvation (1 week), CIP2A mRNA became undetectable (data not shown). The finding suggests that CIP2A is a proliferation-regulated gene.

Diminished clonogenic potentials of tumor cells mediated by the depletion of CIP2A expression independently of p53 and pRB pathways. It has recently been shown that CIP2A is required for sustained growth of HeLa cells and its inhibition leads to cell growth arrest or defective foci formation (6). We thus sought to determine whether this is the case in other tumor cells and, if so, whether p53 and/or pRB checkpoint pathways are involved in such effects. For this purpose, we employed the CIP2A-specific siRNA to knock down CIP2A expression in the following cell lines with different p53 and pRB backgrounds: gastric AGS cells with wild-type p53 and pRB (13), cervical HeLa cells with inactivation of both p53 and pRB (14), HCT116 cells with wild-type p53 and pRB (HCT116p53+/+), and their variant HCT116 with the targeted deletion of p53 but containing intact pRB (HCT116p53-/-; ref. 7). The cells were first transfected with siRNA targeting CIP2A and left for 72 hours. Thereafter, the cells were replated into six well-plates at 300 per well and incubated for 10 days. Efficient silence of CIP2A expression in all these cells was verified using RT-PCR and Western blot analyses (Fig. 3A). Consistent with a recent observation (6), the depletion of CIP2A in HeLa cells led to a significant reduction in foci numbers as well as sizes (control versus CIP2A siRNA: 144 ± 25 versus 78 ± 9, P < 0.001, Student’s t test). Similarly, the potential of colony formation was substantially diminished in other three cell lines AGS, HCT116p53+-/+ and HCT116p53-/- following CIP2A knock down (control versus CIP2A siRNA, P < 0.001 in all the cell lines: AGS 44 ± 5 versus 16 ± 3, HCT116p53-/+ 80 ± 10 versus 38 ± 6, and HCT116p53-/- 103 ± 6 versus 34 ± 4; Fig. 3B). Moreover, as expected, the down-regulation of c-MYC expression was observed in all four cell lines treated with CIP2A siRNA (Fig. 3C).

Given the above observations, we then determined the response of other three gastric cancer cell lines to CIP2A depletion: HGC-27 and BGC-823 cells with mutant p53 and KATO-III with null p53 (15–17). As shown in Fig. 3B, all these siRNA-treated cells exhibited defective foci formation (control versus CIP2A siRNA: HGC-27 138 ± 17 versus 63 ± 25, KATO-III 113 ± 24 versus 42 ± 7, P < 0.001; BGC-823 165 ± 15 versus 134 ± 12, P = 0.01; Fig. 3B). The down-regulation of c-MYC protein expression was seen in CIP2A-depleted KATO-III and BGC-823 cells (Fig. 3C). However, c-MYC protein was undetectable in both control and treated HGC-27 cells (Fig. 3C), although these cells did express c-MYC mRNA (data not shown).

We further examined whether the expression of p53 and its target p21 was altered in the CIP2A-depleted cells. As shown in Fig. 3C, there were no clear increases in p53 and p21 in all the cells treated with CIP2A siRNA compared with the control cells. In addition, to corroborate the above results, we determined the phosphorylation of p53 at Ser15, a marker for p53 activation, using its specific antibody. The phosphorylated p53 signal at this residual was undetectable in both AGS and HCT116p53-/- cells (data not shown), in good accordance with unchanged total p53 levels.

**Senescence of AGS cells induced by the depletion of CIP2A.** CIP2A depletion results in growth arrest rather than increased apoptosis (ref. 6; data not shown). To explore potential mechanisms behind the CIP2A depletion-mediated growth arrest, we asked whether cellular senescence could be induced in the tumor cells treated with CIP2A siRNA. For this purpose, we stained the cells with β-Gal, a specific marker for senescent cells (10). In control AGS cells, less than 5% of them were β-Gal-positive, whereas up to 30% of CIP2A siRNA-treated cells were stained with β-Gal (Fig. 4A and B). In contrast, all other cells line transfected with CIP2A siRNA (HeLa, HCT116p53+-/+; HCT116p53-/-; HGC-27, KATO-III, and BGC-823) did not undergo senescence as verified by negative β-Gal staining (data not shown).

Because p21 expression was at similar levels between control and siRNA-treated AGS cells, we determined another cyclin-dependent kinase inhibitor p16 that is known to play a crucial role in inducing cellular senescence (18–21). No significant differences in p16 expression were observed between them (Fig. 3C). Other cell lines exhibited largely equal amounts of p16 expression or no alterations in control and CIP2A-depleted cells (Fig. 3C). Taken together, neither p21 nor p16 is required for CIP2A depletion-induced senescence and growth arrest.

**Partial differentiation of leukemic HL60 cells triggered by CIP2A depletion.** As differentiation arrest contributes to...
malignant transformation, we thus determined whether CIP2A is involved in the regulation of cellular differentiation. Leukemic HL60 cells with differentiation potential were transfected with CIP2A siRNA and then examined for morphologic changes after 72 hours. As shown in other tumor cells, c-MYC expression was down-regulated in the CIP2A-depleted HL60 cells (Fig. 4C). More than 95% of control cells were myeloid blasts, whereas ~30% of CIP2A-depleted cells acquired characteristics of late promyelocytes with increased volumes and decreased nuclei/cytoplasm ratio (Fig. 4D-G). However, CD11b, a specific surface marker expressed on matured myeloid cells (12, 22), remained negative in both control and siRNA-treated cells (data not shown). Thus, CIP2A depletion led to partial differentiation of HL60 cells.

Discussion

As one of the most common human malignancies, gastric cancer remains a challenging disease, and the molecular pathways implicated in gastric cancer pathogenesis are still poorly understood (1–3). Moreover, the contribution of molecular biology to the development of novel diagnostic markers and new targeted therapies observed in other common cancers such as breast, colon, or lung is lagging far behind in gastric cancer. Thus, one of the major purposes of this study was to address the above issues.

CIP2A, an endogenous PP2A inhibitor, has recently been identified as an oncprotein (6). Like the RAS oncogene, CIP2A is required for anchorage-independent cell growth and malignant transformation of human cells. Clinical studies further showed that CIP2A was overexpressed in head and neck squamous cell carcinoma and colon cancer (6). Given these findings, CIP2A is likely a widespread oncogenic factor in human malignancies. Consistently, the results presented in this report show that CIP2A mRNA is overexpressed in 32 of 37 tumor specimens derived from patients with gastric cancer. Therefore, CIP2A assay may serve as a diagnostic marker for gastric cancer. Of course, the present finding needs to be confirmed by evaluating CIP2A expression in bigger cohorts of patients with gastric cancer.

It has been shown that CIP2A inhibits PP2A activity toward the oncogenic transcription factor c-Myc Ser62 and thereby stabilizes the c-Myc protein by preventing its proteolytic degradation (6). Given a critical role for c-MYC in promoting cell proliferation, it is conceivable that CIP2A-mediated c-MYC stabilization is required for sustained growth of tumor cells. Indeed, we found that the depletion of CIP2A expression led to lower levels of c-MYC expression and substantially diminished...
foci formation of all the tested tumor cell lines, except HGC-27, in good accordance with a recent observation (6). However, it has been established that c-MYC is incapable of converting normal human cells into malignant ones; thus, the transforming capacity of CIP2A is likely beyond its ability to stabilize the c-MYC protein (5, 23). Consistent with this hypothesis, we did observe that HGC-27 cells, despite lack of detectable c-MYC protein expression, similarly exhibited defective foci formation on CIP2A depletion as did other cells. Given broad tumor suppression activities of PP2A (24), it is likely that CIP2A may play more active roles in oncogenesis by interfering with PP2A function in addition to its up-regulatory effect on c-MYC expression. This issue calls for further investigations.

Recent in vitro and in vivo data have accumulated that cellular senescence functions as a potent tumor suppressor (21, 25–28), and we thus tested a potential role for CIP2A in regulating cellular lifespan. A senescent phenotype was readily observed in gastric cancer–derived AGS cells following the inhibition of CIP2A expression. This result suggests that CIP2A overexpression may prevent cellular senescence, thereby promoting malignant transformation and sustained cell growth.

However, several issues remain unclear. First, the defective foci formation was seen in all the cell lines transfected with CIP2A siRNA, but senescence was induced only in AGS cells. Whether the CIP2A depletion-mediated senescence is cell type specific requires further analyses. Secondly, p53 downstream effector p21 and the pRB upstream factor p16 play key roles in inducing cellular senescence program (18–21, 25, 29); however, we failed to observe enhanced p21 and p16 expression in AGS cells with CIP2A inhibition. A potential link between CIP2A depletion and senescence induction remains to be defined. A recent study showed that the deletion of one copy of the c-MYC gene resulted in premature senescence of normal human fibroblasts (30). Furthermore, cellular senescence has been shown to be an important mechanism underlying tumor regression following c-MYC inactivation in tumor cells (31). It will be of interest to elucidate a role for the c-MYC down-regulation in cellular senescence in CIP2A-depleted AGS cells. Finally, in addition to senescence occurring in AGS cells, the CIP2A depletion-mediated growth arrest seems p53 and pRB independent too, and more studies are required to reveal underlying mechanisms.

Intriguingly, CIP2A depletion led to partial differentiation of leukemic HL60 cells, although the effect was not robust. It was shown previously that lowering c-MYC expression in HL60 cells triggered differentiation of HL60 cells (32); therefore, it is likely that the induction of HL60 cell differentiation results from the down-regulation of c-MYC protein due to CIP2A inhibition.

![Fig. 4. Senescence or partial differentiation of malignant cells triggered by CIP2A depletion.](image-url)
Based on this finding, overexpression of CIP2A, as seen in tumor cells, may interfere with cellular differentiation process, thereby contributing to malignant transformation. Overall, our study shows that (a) CIP2A mRNA is widespread in gastric cancer but absent in most normal gastric tissues and (b) the depletion of CIP2A leads to defective foci formation, senescence, or differentiation of malignant cells. Thus, CIP2A may be implicated in diagnosis and therapy of gastric cancer.

References
CIP2A Is Overexpressed in Gastric Cancer and Its Depletion Leads to Impaired Clonogenicity, Senescence, or Differentiation of Tumor Cells

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