CIP2A Is Associated with Human Breast Cancer Aggressivity

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Abstract

Purpose: To investigate the clinical relevance of the recently characterized human oncoprotein cancerous inhibitor of protein phosphatase 2A (CIP2A) in human breast cancer.
Experimental Design: CIP2A expression (mRNA and protein) was measured in three different sets of human mammary tumors and compared with clinicopathologic variables. The functional role of CIP2A in breast cancer cells was evaluated by small interfering RNA–mediated depletion of the protein followed by an analysis of cell proliferation, migration, anchorage-independent growth, and xenograft growth.
Results: CIP2A mRNA is overexpressed (n = 159) and correlates with higher Scarff-Bloom-Richardson grades (n = 251) in samples from two independent human breast cancer patients. CIP2A protein was found to be overexpressed in 39% of 33 human breast cancer samples. Furthermore, CIP2A mRNA expression positively correlated with lymph node positivity of the patients and with the expression of proliferation markers and p53 mutations in the tumor samples. Moreover, CIP2A protein expression was induced in breast cancer mouse models presenting mammary gland–specific depletion of p53 and either BRCA1 or BRCA2. Functionally, CIP2A depletion was shown to inhibit the expression of its target protein c-Myc. Loss of CIP2A also inhibited anchorage-independent growth in breast cancer cells. Finally, CIP2A was shown to support MDA-MB-231 xenograft growth in nude mice.
Conclusions: Our data show that CIP2A is associated with clinical aggressivity in human breast cancer and promotes the malignant growth of breast cancer cells. Thus, these results validate the role of CIP2A as a clinically relevant human oncoprotein and warrant further investigation of CIP2A as a therapeutic target in breast cancer treatment. (Clin Cancer Res 2009;15(16):5092–100)

Breast cancer is the most common malignancy that affects women, with >1 million cases occurring worldwide annually. Further, breast cancer is the most important cause of cancer-related deaths in women. However, the understanding of the molecular mechanisms that maintain the malignant growth of breast cancer cells remains incomplete (1).

The oncogenic transformation of human cells requires the perturbation of a distinct set of oncogenes and tumor suppressors (2). It was recently shown that the tumor suppressor activity of protein phosphatase 2A (PP2A) prevents the transformation of human breast epithelial cells (3). The role of PP2A as a relevant breast cancer tumor suppressor was further strengthened by a recent study showing that somatic mutations occurred in one of the subunits of the functional PP2A trimer (PP2A Aβ) in 13% of human breast cancers and that PP2A trimers containing this mutation fail to suppress the oncogenic activity of RalA (4, 5). In

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Translational Relevance

Cancerous inhibitor of protein phosphatase 2A (CIP2A) is a recently identified human oncoprotein that inhibits c-Myc protein degradation in cancer cells. CIP2A has been found to be overexpressed in different human cancers, but its clinical relevance has not yet been established. In addition, the role of CIP2A in human breast cancer has not yet been studied. In this study, we show that CIP2A expression strongly correlates with aggressive characteristics of human breast cancer tumors (high Scarff-Bloom-Richardson grade, lymph node positivity, and expression of proliferation markers). Importantly, these results show for the first time that CIP2A expression is linked with clinical markers of aggressivity in human cancer. We also show that CIP2A depletion decreases the proliferation of human breast cancer cell lines and inhibits the growth of xenograft MDA-MB-231 cells *in vivo*. Altogether, these results warrant further investigation of CIP2A as a therapeutic target in the treatment of breast cancer.

addition to RalA inactivation, regulation of the proteolytic stability of the oncogenic transcription factor c-Myc is an important mechanism by which PP2A exerts its tumor suppressor activity (6, 7). PP2A-mediated dephosphorylation of serine 62 on c-Myc results in the ubiquitination and proteolytic degradation of c-Myc and thereby the inhibition of malignant cell growth and cellular transformation (6, 7). Increased protein stability of c-Myc has been detected in malignant cells isolated from hematologic cancers (8). On the other hand, the overexpression of c-Myc protein has been reported in 40% to 45% of human breast cancers, whereas the amplification of the *c-Myc* gene is observed only in 20% to 25% of human breast cancers (9, 10). These data suggest that, in addition to gene amplifications, the stabilization of c-Myc protein might contribute to its oncogenic activity in breast cancer.

We have recently characterized a human oncoprotein designed cancerous inhibitor of PP2A (CIP2A; ref. 11). CIP2A promotes c-Myc protein stability in human cancer cells by its capacity to inhibit PP2A activity directed toward serine 62 on c-Myc (11). Moreover, CIP2A promotes the proliferation and *in vivo* tumor growth of HeLa cells and cells derived from human head and neck squamous cell carcinomas (11). In addition, CIP2A was found overexpressed in tissue samples derived from human head and neck squamous cell carcinoma, human colon cancer, and human gastric cancer (11, 12). However, the clinical role of CIP2A and its association with disease progression is yet to be clearly defined. In addition, the role of CIP2A in human breast cancer has not been studied thus far.

Here we show that CIP2A expression correlates with the invasive and aggressive characteristics of human breast tumors [high Scarff-Bloom-Richardson (SBR) grade, lymph node–positive tumors, and high proliferation]. We also show that CIP2A depletion inhibits c-Myc expression as well as the proliferation and tumorigenic growth of human breast cancer cells.

Materials and Methods

Tumor samples and clinical material. Tumor samples used for the mRNA expression analysis as well as protocols for mRNA extraction and real-time PCR have been described previously (13, 14). To summarize, a total of 159 primary breast carcinomas and 5 normal breast tissues were analyzed in this study. These carcinomas were obtained from patients who had undergone initial surgery between 1989 and 2001 at the Cancer Research Center of Val d'Aurelle in Montpellier, the Bergonié Institute in Bordeaux, or the Department of Obstetrics and Gynecology of Turin. Informed consent was obtained from the patients before surgery. The patients' age at diagnosis varied from 27 to 92 years (mean,

Table 1. Summary of characteristics of the breast cancer patients (mRNA expression study; Fig. 1)

Tumors of the quantitative PCR study (159 patients)	No. patients
Histologic type	
IDC	82
ILC	27
IDC + intraductal comedo carcinoma	20
IDC + micropapillary	9
IDC + ILC	8
Mucinous	3
Papillary	3
Tubular	3
Ductal carcinoma *in situ*	1
Other	3
SBR grade	
1	27
2	88
3	43
Not defined	1
Stage	
I	34
IIa	63
IIb	45
IIIa	2
Unknown	15

Tumors of the microarray study (251 patients)	No. patients
Estrogen receptor status	
Positive	213
Negative	34
Unknown	4
Progesterone receptor status	
Positive	190
Negative	61
Elston SBR modified grade	
1	67
2	128
3	54
Unknown	2
Lymph node status	
Positive	84
Negative	158
Unknown	9
Patient age (y)	
<50	51
50-65	83
>65	117
p53 status	
Wild-type	193
Mutant	58

Table 2. Primers used for quantitative real-time PCR

Primer	Sequence (5′-3′)
36B4-fwd	GTCACTGTGCCAGCCCAGAA
36B4-rev	TCAATGGTGCCCCTGGAGAT
β-actin-fwd	CCAACCGCGAGAAGATGA
β-actin-rev	CCAGAGGCGTACAGGGATAG
CIP2A-fwd	GAACAGATAAGAAAAGAGTTGAGCATT
CIP2A-rev	CGACCTTCTAATTGTGCCTTTT
HPRT1-fwd	ACGTCTTGCTCGAGATGTGAT
HPRT1-rev	TGTAATTCCAGCAGGTCAGCAA

63 years; median, 65 years). For the 159 patients, the median follow-up time was 65.9 months. The tumors were sampled from patients at stage I (21%), stage IIa (40%), stage IIb (28%), and stage IIIa (1%), whereas 9% of the tumors were at an unknown stage (Table 1). Two tumors presenting approximately three times higher CIP2A expression than the next highest expressing tumor in the rest of the group (23.1 and 19.1 compared with 6.9) were excluded from the study because they were not within a normal distribution of the values. Fresh tissues were formalin-fixed and paraffin-embedded immediately after surgical removal. Frozen sections were stained with H&E to select samples consisting of at least 50% tumor cells and to establish the histologic type and the histologic grade (Table 1).

RNA extraction and purification. Frozen breast samples were homogenized using the Fast-Prep System from Q-Biogene. Briefly, ~40 mg frozen tissues were broken up in lysing buffer on a lysing matrix for 40 s. Total RNA was extracted and cleaned up from the lysate using the Qiagen RNeasy Mini Kit. The RNA purity and integrity were controlled by using a Bioanalyzer 2100 (Agilent). Only RNAs with a score of 8 to 10 were included in this study.

cDNA synthesis. After DNase treatment, 1 μg total RNA was incubated with 250 ng random hexamer for 10 min at 70°C. Total RNA was reverse transcribed in a final volume of 20 μL containing 1× first-strand buffer, 0.1 mol/L DTT, 10 mmol/L deoxynucleotide triphosphate, and 200 units SuperScript reverse transcriptase. The samples were incubated at 25°C for 10 min, and then at 42°C for 1 h. The reverse transcriptase was finally inactivated by heating at 70°C for 15 min.

PCR amplification. CIP2A primers have been designed using the Universal ProbeLibrary for Humans from Roche Applied Science (forward primer 5′-GAACAGATAAGAAAAGAGTTGAGCATT-3′ and reverse primer 5′-CGACCTTCTAATTGTGCCTTTT-3′). The quantification was based on the standard curve method. The data were normalized using the expression median of three reference genes (36B4, HPRT1, and β-actin; primer sequences are indicated in Table 2). Oligonucleotides were obtained from Proligo. For quantitative real-time PCR, 2 μL of diluted reverse transcription reaction samples (1/15) were added to 13 μL of a PCR mixture made up of 7.5 μL of 2× SYBR Green PCR Master Mix (Applied Biosystems), 0.075 μL of each primer at a concentration of 100 μmol/L, and RNase-free water. The thermal cycling conditions comprised an initial step at 50°C for 2 min and a denaturation step at 95°C for 10 min followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. All PCRs were carried out using an ABI Prism 7000 Sequence Detection System (Applied Biosystems). The specificity of each primer couple was shown by a dissociation curve analysis. To generate a calibration curve, a serially diluted cDNA mixture was used as a standard and quantified for each primer set. The standard concentration was plotted against the cycle number at which the fluorescence signal increased above the background (threshold) value (Ct value). The amplification efficiency [E (%) = $(10^{(1/-s)} - 1) * 100$ (s = slope)] of each standard curve was determined and appeared to be >95% and <105% over a wide dynamic range.

Histologic staining. CIP2A protein was detected using a rabbit anti-CIP2A antibody (ref. 15; diluted 1:1,000) and an anti-mouse peroxidase polymer. 3,3′-Diaminobenzidine was used as the chromogen (ImmunoVision), and hematoxylin was used as a counterstain. Human breast cancer tissue microarrays (n = 33) were used for immunohistochemical stainings. The staining methods have been described previously (16). These samples were independent from other samples used in this study.

Microarray reanalysis. Data from Affymetrix U133B arrays hybridized with mRNA from 251 breast tumors (17) were reprocessed using the R language (R Development core team) and the MAS5 algorithm implemented in the Bioconductor package *affy*. A boxplot of CIP2A expression (probe set 231855_at) was drawn using R by dividing the samples into groups based on respective clinical parameters. Ki-67 and proliferating cell nuclear antigen (PCNA) status was estimated from the expression data itself. The Affymetrix probe sets and cutoffs for dividing tumors into "high"/"low"–expressing groups were 212020_s_at >500 units for Ki-67 and 201202_s at >1,500 units for PCNA. Other clinical data were derived from the published information (17). The statistical significance of differences between groups was assessed using a Mann-Whitney test implemented in R.

Small interfering RNA transfections. Double-stranded small interfering RNA (siRNA) oligonucleotides (50 nmol per 35 mm plate; CIP2A: 5′-CUGUGGUUGUGUUUGCACUTT-3′; scrambled: 5′-UAACAAUGAGAGCACGGCTT-3′) were transfected with Oligofectamine reagent (Invitrogen) according to the manufacturer's instructions.

Antibodies. The following antibodies were used: rabbit polyclonal anti-CIP2A (11); mouse monoclonal anti-human Ki-67, clone Mib-1 (DAKO); rat monoclonal anti-mouse Ki-67, clone TEC-3 (DAKO); mouse monoclonal anti-c-Myc, clone 9E10 (BD Pharmingen); mouse monoclonal anti-β-actin, clone AC-74 (Sigma); rabbit polyclonal anti-phospho-Akt (Santa Cruz Biotechnology); and rabbit polyclonal anti-phospho-MEK (Cell Signaling technology).

Cell proliferation assay. MDA-MB-231 or T47D cells were transfected on 96-well plates with CIP2A or scrambled siRNA for 3 days, and the number of living cells was subsequently analyzed by using a CellTiter 96 Non-Radioactive Cell Proliferation Assay (Promega) following the manufacturer's instructions.

Anchorage-independent soft-agar growth. MDA-MB-231 cells (10×10^3) were seeded in a 6-well plate 48 h after siRNA transfection. Soft-agar assays were done in medium containing 10% fetal bovine serum as described previously (11). The number and size of colonies were analyzed using ImageJ 1.38x software from microscopy images (magnification, ×35).

In vivo *tumor formation.* MDA-MB-231 cells were transfected with CIP2A or scrambled siRNAs for 72 h, and down-regulation of CIP2A protein expression was confirmed from parallel samples before injection. siRNA-transfected cells (2×10^6) mixed with Matrigel (BD Bioscience) were injected into the mammary fat pad of an immunocompromised mouse. Twenty-four injections were done for each condition (siCIP2A or siSCR). The size of the palpable tumors was evaluated every third day by the use of a precision instrument and the tumor weight was analyzed at the end of the experiment.

Migration assay. MDA-MB-231 cells (10×10^4) were cultivated on a monolayer and treated for 3 days with the indicated siRNA in a 24-well plate. Then, a wound (2 mm width) was scratched into confluent cultures of siCIP2A- or siSCR-treated MDA-MB-231 cells. Randomly marked wound regions with an identical width were studied, and wound closure (the percentage of closed scratch area) was measured using ImageJ 1.38x software from microscopy images (magnification, ×35).

Results

CIP2A expression correlates with human breast cancer aggressivity. To study CIP2A expression in human breast cancer, 159 previously characterized human mammary tumors and 5 normal breast samples (refs. 13, 14; Table 1) were evaluated for CIP2A mRNA expression by real-time PCR analysis. We

found that CIP2A is significantly overexpressed in human mammary tumors when compared with normal tissue (P = 0.027; Fig. 1A). Regarding histologic subtypes of breast cancer, statistically significant overexpression of CIP2A, compared with normal breast samples, was found in invasive ductal carcinoma (IDC; P = 0.027), invasive lobular carcinoma (ILC; P = 0.012), and IDC with intraductal comedo carcinoma (P = 0.032; Fig. 1B). Importantly, mucinous carcinomas, which are mammary tumors with a good prognosis, displayed CIP2A mRNA expression at a level that is comparable with expression levels in normal breast samples and significantly lower than in the invasive IDC, ILC, and IDC with intraductal comedo carcinoma tumors (P < 0.044; Fig. 1B).

The above results suggest that CIP2A expression may be linked with human breast cancer aggressivity. To support this, analysis of the data set shown in Fig. 1A and B revealed that CIP2A expression correlates with a higher tumor grade of ILC; SBR grade 2 and 3 samples presented significantly higher CIP2A expression levels (P = 0.011) than grade 1 lobular carcinomas (Fig. 1C). In IDC, CIP2A expression levels correlated with higher SBR grades. However, this correlation was not quite statistically significant (P = 0.066; data not shown). On the other hand, no association existed between CIP2A expression and the SBR grade of the tumors derived from IDC with intraductal comedo carcinoma (P = 0.663; data not shown).

To further substantiate the observed correlation of CIP2A expression with the increase in breast cancer aggressivity, the expression levels of CIP2A were correlated with several markers of tumor progression in a published microarray data set of 251 human breast tumors (17). Again, CIP2A mRNA expression was found to correlate with a higher SBR grade of the tumor (grade 3 versus 1 and grade 3 versus 2; P < 0.001; Fig. 1D; Table 3). Using

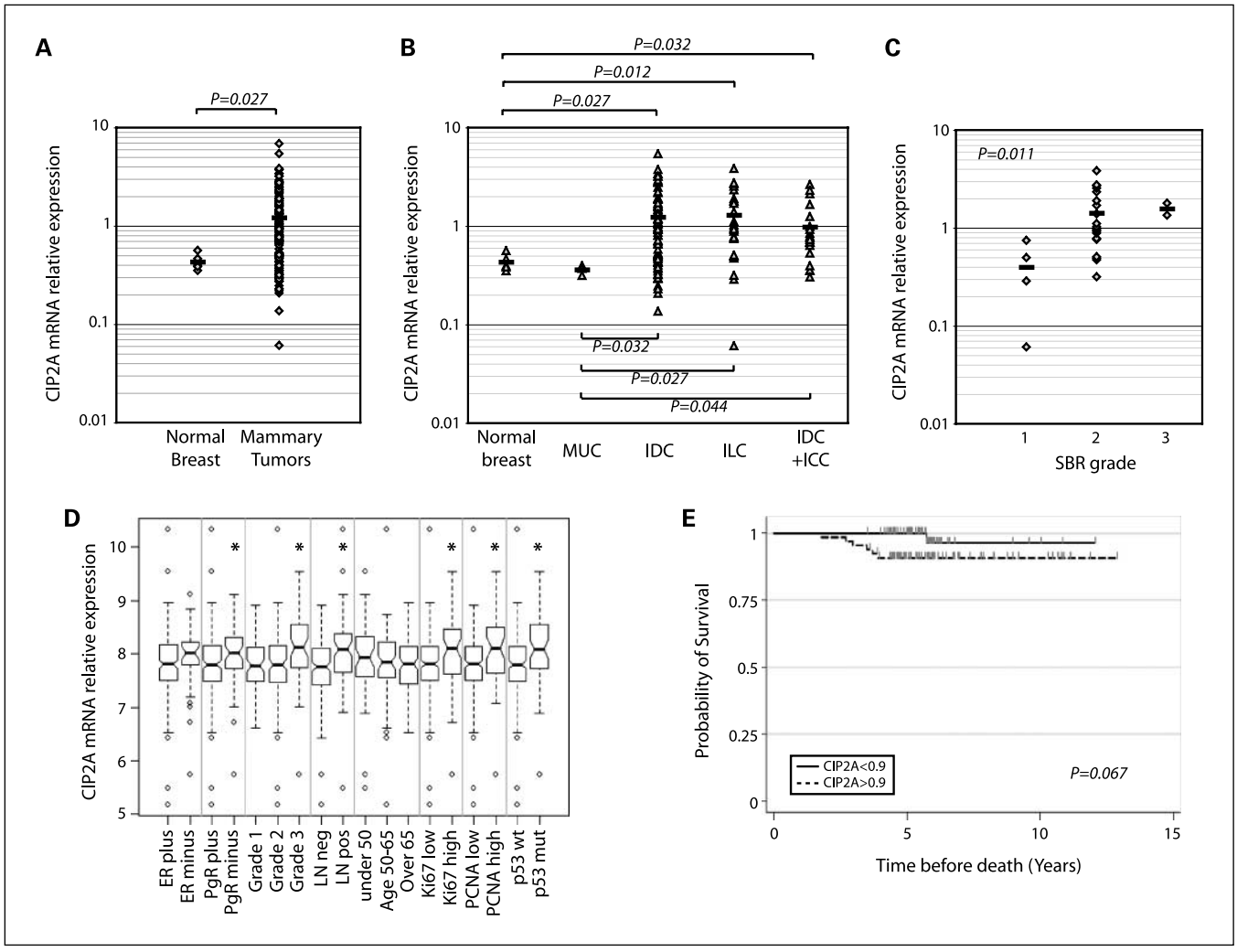

Fig. 1. CIP2A expression correlates with breast cancer aggressivity. *A,* real-time PCR analysis for CIP2A mRNA from 159 human mammary tumors and 5 normal breast samples. Two-sample Wilcoxon (Mann-Whitney) rank-sum test was used for statistical analysis. *Small bold line,* average. *B,* CIP2A mRNA expression in the indicated breast cancer tumor types. *MUC,* mucinous carcinoma. Two-sample Wilcoxon (Mann-Whitney) rank-sum test was used for statistical analysis. *Small bold line,* average. *C,* correlation of CIP2A mRNA expression and SBR grade of ILC from the tumors presented in *A* and *B*. Mann-Whitney rank-sum test was used for statistical analysis. *Small bold line,* average. *D,* correlation of CIP2A expression with the indicated clinical parameters on the microarray data from 251 human mammary tumors (17). The statistical significance of differences between groups was assessed using a Mann-Whitney test implemented in R. *, P < 0.05 (exact values are indicated in Table 3). *E,* Kaplan-Maier curves of the overall survival of the patients from the quantitative-PCR analysis (*A* and *B*). Tumors are separated in two groups based on their CIP2A expression levels compared with the median value (0.9) for the total tumor material. *Solid line,* tumors with a lower CIP2A expression compared with the median (<0.9; 77 tumors, 48.4% of the tumors); *dashed line,* tumors with a higher CIP2A expression than the median (>0.9; 82 tumors, 51.6% of the tumors); *vertical ticks,* censored patients.

Table 3. Correlation between CIP2A expression and the clinicopathologic variables of the tumors from the Miller et al. microarray data (*P* values of a regular two-sided Wilcoxon rank-sum test, Mann-Whitney test was used)

	P
Estrogen receptor + vs -	0.130
Progesterone receptor + vs -	0.024
Grade 1 vs 3	<0.001
Grade 2 vs 3	<0.001
Lymph node - vs +	<0.001
Age (y) <50 vs >65	0.129
Low vs high Ki-67	0.009
Low vs high PCNA	0.018
p53 wild-type vs mutant	<0.001

the same microarray data set, we also investigated the association of CIP2A with other clinicopathologic variables and found that CIP2A mRNA expression associates with progesterone receptor negativity ($P = 0.024$), lymph node positivity ($P < 0.001$), p53 mutation ($P < 0.001$), and expression of the proliferative markers Ki-67 ($P = 0.009$) and PCNA ($P = 0.018$; Fig. 1D; Table 3). However, there was no significant association between CIP2A expression and patient age or estrogen receptor status (Fig. 1D; Table 3).

The correlation between CIP2A mRNA expression and overall survival of the patients was studied in the samples shown in Fig. 1A and B. Patients were separated into two groups based on the median value of their CIP2A expression (0.9): the "low-expressing" group contained patients with CIP2A expression levels lower than the median of 0.9 and the "high-expressing" group contained patients with higher CIP2A expression than the median. During the first 5 years, no deaths occurred in the group of patients with low CIP2A expression, whereas six patients died due to breast cancer in the population with high CIP2A expression during the same period (Fig. 1E). Although the difference in overall survival of patients with high or low CIP2A-expressing tumors was not quite statistically significant ($P = 0.067$), patients with high CIP2A expression showed worse survival during the first 5 years post-surgery ($P = 0.016$; Supplementary Fig. S1).

These results show that CIP2A is overexpressed in invasive human mammary carcinomas. Importantly, these findings show for the first time that CIP2A expression correlates with the tumor grade and lymph node positivity in human cancer patients. Moreover, the association of CIP2A expression with markers of increased cellular malignancy (Ki-67 and PCNA expression) further supports the role of CIP2A in promoting the aggressive behavior of breast cancer.

CIP2A protein expression in breast cancer. The above results show the overexpression of CIP2A mRNA in human mammary tumors. To confirm these findings, we also wanted to study CIP2A protein status in breast cancer. CIP2A is a cytoplasmic protein (11, 12, 15). As shown in Fig. 2A, normal mammary tissue did not express detectable levels of cytoplasmic CIP2A, whereas high cytoplasmic expression of CIP2A protein was observed in human breast carcinomas. In a tissue array of 33 human mammary tumors, clear cytoplasmic CIP2A positivity was observed in 39% of the tumors (Fig. 2B). To further evaluate the observation of CIP2A expression in breast cancer tissue, CIP2A and Ki-67 protein expression was analyzed in a well-defined mouse model of breast cancer presenting a mammary gland–specific deletion of p53 and either BRCA1 or BRCA2 (*K14cre*;*Brca1*$^{F/F}$;*p53*$^{F/F}$ or *K14cre*;*Brca2*$^{F/F}$;*p53*$^{F/F}$; refs. 18, 19). All analyzed tumors (10 of 10) displayed strong CIP2A staining in epithelial carcinoma cells, whereas no CIP2A positivity was observed in the normal mammary gland tissue (Fig. 2C). In concordance with the correlation of CIP2A and Ki-67 mRNA expression in human breast cancer material (Fig. 1D), CIP2A-positive cancer cells displayed clear nuclear expression of Ki-67 in both breast cancer mouse models (Fig. 2C). These results, taken together with the observed correlation of p53 mutation and CIP2A mRNA expression in human tumor material (Fig. 1D), further suggest that CIP2A may be involved in mammary gland tumorigenesis induced by the inhibition of p53 tumor suppressor activity.

CIP2A promotes c-Myc protein expression and cell proliferation in breast cancer cells. Our group and others have recently shown that CIP2A promotes c-Myc protein stability in HeLa, head and neck squamous cell carcinoma, and gastric cancer cells (11, 12, 15). Here, we show that the depletion of CIP2A in MDA-MB-231 and T47D breast cancer cell lines results in the inhibition of c-Myc steady-state protein levels (Fig. 3A and B). The specificity of the siRNA used for CIP2A depletion has been reported previously (11). Importantly, CIP2A depletion did not inhibit the phosphorylation of Akt or MEK (Fig. 3A). We have shown previously that CIP2A depletion in HeLa cells inhibits bromodeoxyuridine incorporation but does not induce programmed cell death (11). Here, CIP2A depletion inhibited the number of living MDA-MB-231 and T47D breast cancer cells cultured under high-serum conditions as measured by MTT assay (Fig. 3C). As CIP2A depletion has been shown to inhibit bromodeoxyuridine incorporation but not induce apoptosis (ref. 11; data not shown), we conclude that the observed decrease in the number of living cells in response to CIP2A depletion is most likely due to the inhibition of proliferation. A positive correlation between CIP2A expression and lymph node positivity in human breast cancer (Fig. 1D) suggests that CIP2A may be involved in regulating the migration properties of breast cancer cells. However, we did not observe any significant effect of CIP2A depletion on MDA-MB-231 cell migration in a standard scratch wound assay (Fig. 3D).

CIP2A depletion inhibits tumor growth of MDA-MB-231 cells. The ability of cells to grow and form colonies on semisolid agar (anchorage-independent growth) is a hallmark of malignantly transformed cells. To study the role of CIP2A on the malignant growth of breast cancer cells, MDA-MB-231 cells were transfected with scrambled or CIP2A siRNA, and their capacity to form colonies on semisolid agar was evaluated. For this purpose, we first studied the efficiency of CIP2A depletion by a single transfection of siRNA. A very potent reduction of CIP2A protein expression was still found after 10 days (Supplementary Fig. S2). We found that CIP2A depletion resulted in a statistically significant decrease of the anchorage-independent growth of MDA-MB-231 cells 14 days post-transfection (Fig. 4A). To study whether CIP2A is required for breast cancer tumor growth, MDA-MB-231 cells transfected with either CIP2A or scrambled siRNA were injected into the mammary fat pad of athymic mice and tumor growth was monitored over 31 days. Beginning at day 18 after injection, CIP2A depletion

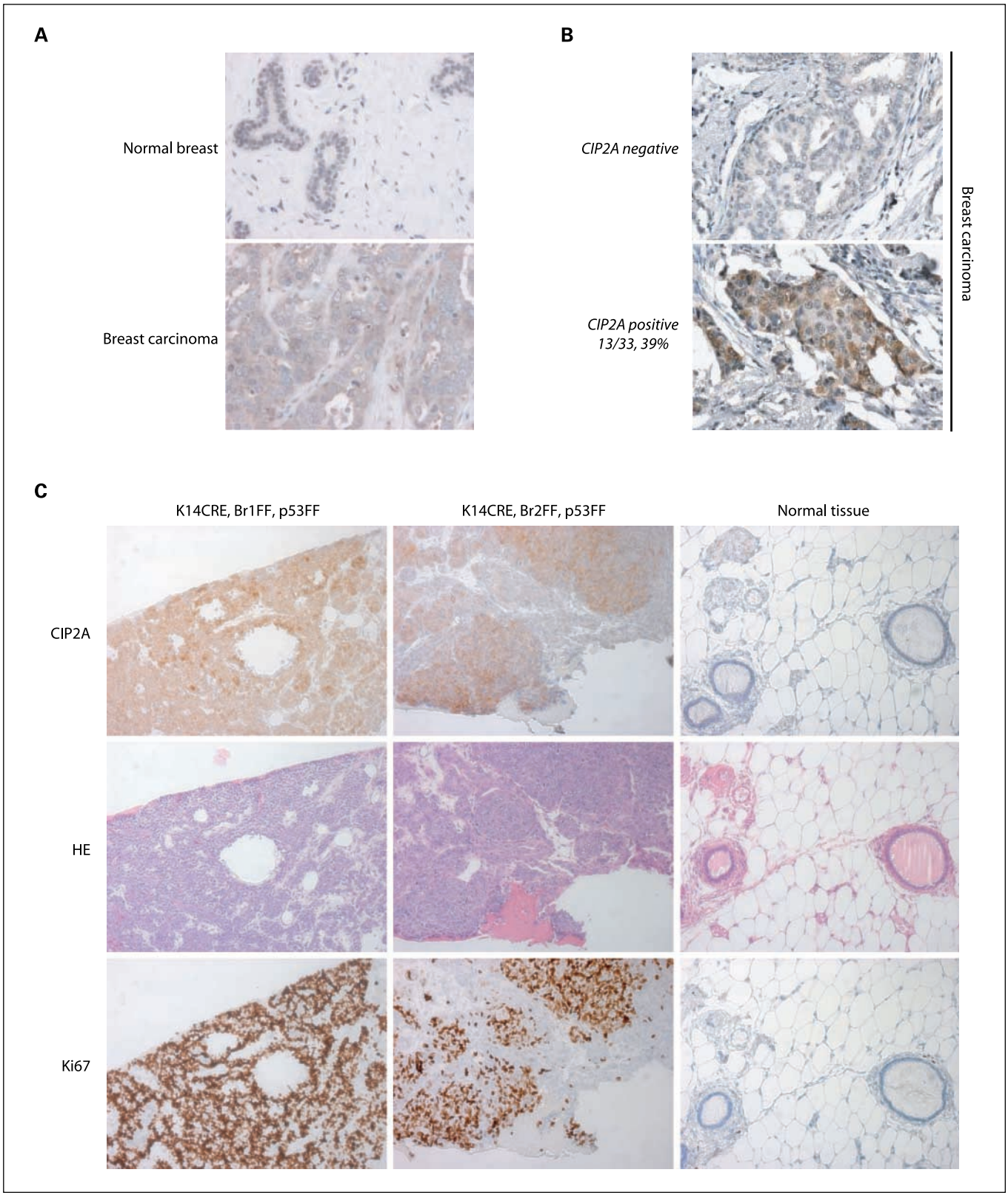

Fig. 2. CIP2A protein expression in human breast cancer. *A,* CIP2A immunoreactivity in normal breast and breast cancer tissue was evaluated by immunohistochemistry. As reported previously in other tissue types (11), CIP2A antibody displayed nonspecific nuclear staining of normal tissue, whereas clearly distinguishable cytoplasmic staining was observed for breast cancer tissue. *B,* CIP2A immunostaining of human breast cancer tissue array. Cytoplasmic CIP2A expression was found in 39% of the tumors analyzed. *C,* CIP2A and Ki-67 immunostaining of mouse mammary tumors. CIP2A and Ki-67 expression was studied by immunohistochemistry on mammary tumor sections from two different breast cancer mouse models, *K14cre*;*Brca1*$^{F/F}$;*p53*$^{F/F}$ or *K14cre*;*Brca2*$^{F/F}$;*p53*$^{F/F}$. Representative samples of 10 tumors per group are shown, and all display strong CIP2A and Ki-67 staining in the tumor cells. Magnification, ×20.

resulted in a significant reduction in tumor volume (Fig. 4B) as determined by the ANOVA for repeated-measures test, which indicates the difference in global evolution of xenograft growth over time. In addition, CIP2A depletion resulted in a significant decrease in the tumor volume and weight as measured at the end of the experiment at day 31 (Fig. 4C and D). Together, these results show that CIP2A promotes malignant growth of human breast cancer cells.

Discussion

CIP2A was recently identified as a human oncoprotein based on its capacity to transform human immortalized cells, its overexpression in human head and neck squamous cell carcinomas and colon cancer, and its capacity to promote tumor growth (11). However, the clinical role of CIP2A and its association with disease progression has not yet been established.

We show here that CIP2A is overexpressed in the most common types of human mammary carcinomas compared with normal breast. Importantly, CIP2A overexpression was observed at both mRNA and protein levels by using two independent sets of human breast cancer material (Figs. 1A and 2B). Moreover, CIP2A expression correlates with the aggressive characteristics of the tumors (high SBR grade, lymph node–positive tumors, and high Ki-67 and PCNA levels; Fig. 1C and D). At this point, the difference in the overall survival of patients with high or low CIP2A-expressing tumors does not advocate the use of CIP2A as a prognostic marker ($P = 0.067$). However, our observation that high CIP2A expression did predict a worse prognosis for the patients at a 5-year follow-up (Fig. 1E; Supplementary Fig. S1) indicates that the predictive role of CIP2A in association with other genes overexpressed in high-grade human breast cancers should be further evaluated in the future by studying more patient samples. It is also possible that the lack of a significant survival effect is due to the relatively long overall survival of patients in the studied cohort, which is likely due to the fact that most of the studied tumors were from patients with early stages of disease (Table 1).

On the molecular level, we show that CIP2A depletion results in the reduction of c-Myc protein levels in two distinct human breast cancer cell lines. Interestingly, the combination of our results together with the previously published studies reveals that *c-Myc* amplification and CIP2A overexpression correlate with the shared features of human mammary tumors. Indeed, both *c-myc* amplification and CIP2A expression correlate with a higher tumor grade and with lymph node positivity (refs. 20, 21; Fig. 1C and D). Similarities in clinical roles of *c-myc* amplification and CIP2A expression, together with the role of CIP2A in promoting c-Myc protein expression (Fig. 3A and B), clearly strengthen the functional link between these two human oncoproteins. The role of CIP2A in promoting c-Myc expression in breast cancer cells is an important observation in the light of previous evidence that overexpression of c-Myc protein has been observed in the majority of human breast cancer patient

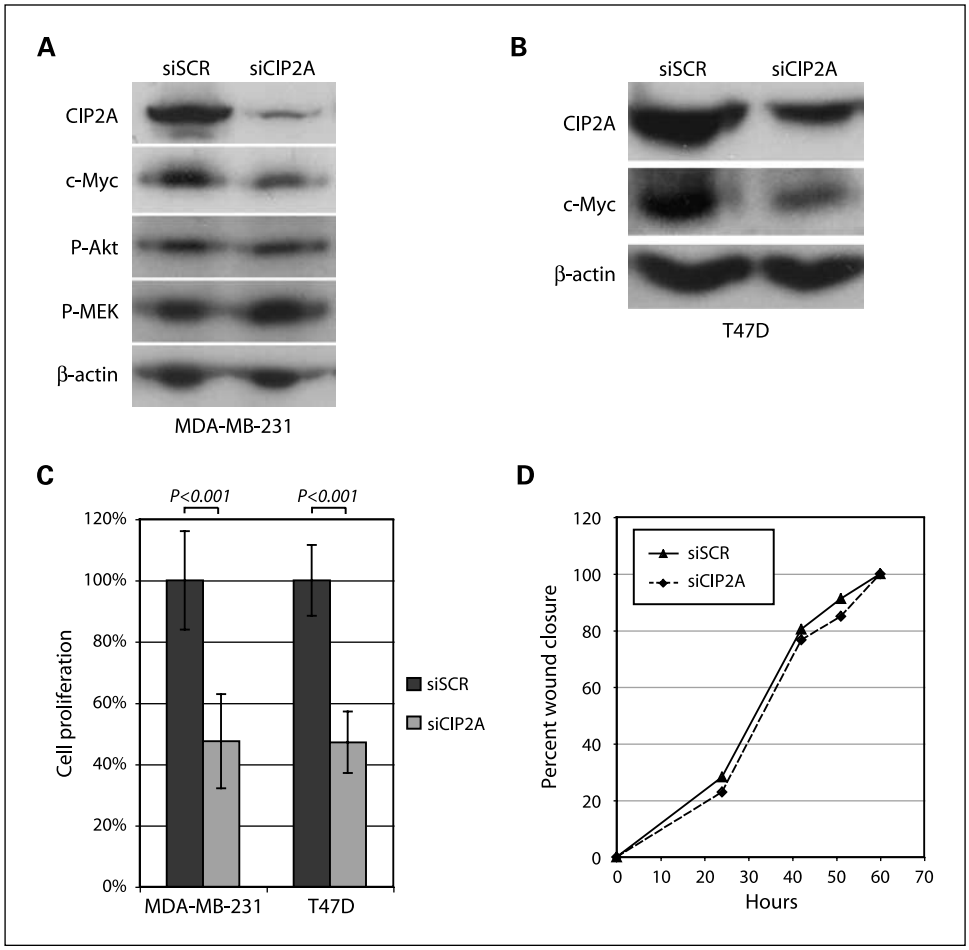

Fig. 3. CIP2A promotes c-Myc expression and cell proliferation in breast cancer cells. Western blot analysis of MDA-MB-231 (*A*) or T47D (*B*) cells treated with CIP2A or scrambled siRNA for 72 h. The expression and phosphorylation status of the indicated proteins was studied by previously validated antibodies. Representative results of three to five independent experiments with similar results. *C,* cell proliferation assay of MDA-MB-231 or T47D cells treated with CIP2A or scrambled siRNA for 3 d. Mean ± SD of three independent experiments with six replicates. Kruskal-Wallis equality of populations test was used for statistical analysis. *D,* scratch wound assays of MDA-MB-231 cells. Cells were treated for 3 d with the indicated siRNA. Subsequently, a 2 mm scratch wound was introduced into confluent cultures and the wound closure (percentage of closed scratch area) was measured in photographs at the time points indicated.

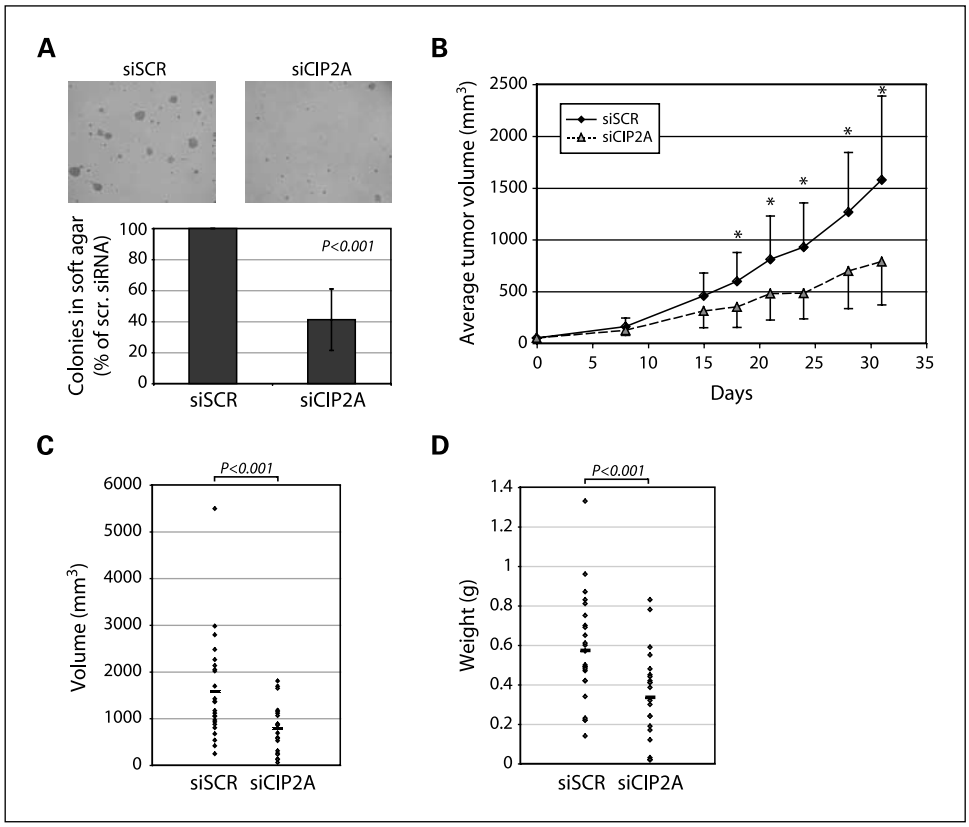

Fig. 4. CIP2A promotes tumor growth of MDA-MB-231 cells. *A,* anchorage-independent growth on soft agar of MDA-MB-231 cells transfected with CIP2A or scrambled siRNA. *Top,* representative phase-contrast microscopy images of the indicated cultures; *bottom,* number of colonies measured 15 d after replating by ImageJ software. Average ± SD of four experiments. Kruskal-Wallis test was used for statistical analysis. *B,* xenograft growth of MDA-MB-231 cells treated with CIP2A or scrambled siRNA. Cells were treated with the siRNA for 3 d. Two million cells were subsequently injected with Matrigel into the mammary fat pad of Hsd: athymic nude mice. Mean ± SE of tumor volumes at the indicated time points. Multiple-sample ANOVA for repeated-measures test was used (*, $P < 0.0001$). *C,* final volume of tumors from the experiment shown in *B* at day 31. Kruskal-Wallis test was used for statistical analysis. *Small bold line,* average. *D,* weight of tumors from the experiment shown in *B* at day 31. Kruskal-Wallis test was used for statistical analysis. *Small bold line,* average.

samples, whereas *c-Myc* gene amplification occurs only in a far smaller fraction of cancers (9, 10). Taken together, these results suggest that, along with other mechanisms that stabilize c-Myc protein (6, 22), CIP2A expression may be important for sustaining the malignant behavior of breast cancers. Importantly, our data show that although CIP2A depletion inhibited c-Myc protein expression, it did not markedly alter the activity of either extracellular signal-regulated kinase or Akt/protein kinase B pathway, supporting previously reported data that CIP2A depletion does not stimulate general PP2A activity (11). However, based on the data presented here, we cannot exclude the possibility that, in addition to its role in regulating c-Myc protein expression, CIP2A could promote breast cancer aggressivity by regulating other, yet to be defined signaling pathways.

The current treatment for breast cancer includes traditional regimens such as surgery and radiotherapy, whereas the only molecularly targeted drugs for breast cancer treatment today are the HER2-targeting antibody trastuzumab, the antiestrogen drugs such as tamoxifen, and the humanized anti–vascular endothelial growth factor antibody bevacizumab in limited cases. In this regard, there is an urgent need to identify novel potential target proteins for breast cancer therapy. The results shown in this study support the potential role for CIP2A as a target protein for future cancer therapies. Importantly, CIP2A expression is very low in most human tissues (11). In this study, we further showed that CIP2A protein is not detectable in human and mouse mammary gland tissues (Fig. 2A and C). This suggests that CIP2A targeting may not have severe side effects that could limit the efficacy of such a therapy.

In conclusion, the results of this study show that CIP2A is associated with human breast cancer aggressivity. Importantly, these results provide the first indications for the clinical relevance of this recently characterized human oncoprotein in human breast cancer. Based on the functional characteristics and cancer-specific expression of CIP2A, it is evident that the role of CIP2A in promoting the aggressivity of breast cancer and other types of human malignancies deserves further attention.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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