

# Expression of cytoplasmic p16 and anion exchanger 1 is associated with the invasion and absence of lymph metastasis in gastric carcinoma

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**Abstract.** The tumor suppressor p16 is a negative regulator of the cell cycle, commonly believed to act in the nucleus. We recently found that p16 protein is expressed in the cytoplasm of gastric cancer cells, concomitantly with anion exchanger 1 (AE1). The aim of this study was to analyze the significance of cytoplasmic p16 and its relationship to AE1 in the progression of gastric cancer. Expression of p16 and AE1 was examined by immunohistochemical analysis in 196 patients; 98 with early gastric cancer and 98 with advanced gastric cancer. The relationship between cytoplasmic p16 and clinicopathological features, and the relationship between cytoplasmic p16 and AE1, were analyzed statistically. Expression of p16 was observed in the nucleus in early stage gastric cancer, but was located mainly in the cytoplasm in advanced cancer cells. Furthermore, cytoplasmic expression of p16 was correlated with AE1 expression, and both were associated with the absence of lymph metastasis in gastric cancer. In conclusion, cytoplasmic immunoreactivity of p16 appears to be a good prognostic indicator in advanced gastric cancer. Co-localization of p16 and AE1 predicts a lack of metastasis in gastric cancer. The role of cytoplasmic p16 and AE1, and the mechanisms involved in the progression of gastric cancer, warrant further investigation.

## Introduction

The p16 gene, which maps to chromosome 9p21, encodes a protein of 156 or 148 amino acids. The p16 protein interacts with CDK4/6, which was originally identified from a yeast two hybridization (YTH) screen using CDK4 as the bait protein (1,2). Overexpression of p16 inhibits CDK4/6 activity, leading to hypophosphorylated pRb, reduced E2F activity and arrest in the G<sub>1</sub> phase of the cell cycle. Thus, the main biological function of p16 is the negative regulation of the cell cycle via the p16/CDK4/cyclin D/Rb pathway (3,4). Subsequent studies have led to the understanding that the p16 protein might be a general regulator, involved in other cellular signaling pathways through its interactions with proteins other than CDK4/6.

Meanwhile, enormous effort has been expended in investigating biological behavior in a wide variety of human malignancies and transformed cell lines. Through these investigations, the functional inactivation of p16 has been observed (5,6). Homozygous deletions and transcriptional silencing by promoter hypermethylation have been found in many cancer cells; these may be mechanisms of p16 inactivation (7,8), suggesting that the inactivation of p16 may be a crucial event in the development of human tumors. However, the exact mechanisms involved in the inactivation of p16 have not been elucidated. Recent studies revealed that the p16 protein was expressed in the cytoplasm in some tumors, suggesting that cytoplasmic distribution of p16 might indicate an inactive form of the protein. The mechanisms involved and the significance of the intracellular localization of the p16 protein are not clear (9-11).

Human anion exchanger 1 (AE1, also called Band 3) is normally expressed in the RBC plasma membrane. Its truncated form, lacking the N-terminal 69 amino acid residues, is also expressed in the kidney. AE1 mediates the exchange of Cl<sup>-</sup> for HCO<sub>3</sub><sup>-</sup> across the plasma membrane, and is thereby involved in the regulation of intracellular pH and volume (12-14). AE1 is composed of three functional domains. The 45-kDa N-terminal domain and the acidic C-terminal tail

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(approximately 40 residues) of AE1 are both located in the cytoplasm. The central 55-kDa transmembrane domain spans the bilayer 12-14 times and is responsible for anion transport activity. The N-terminal region of the protein binds ankyrin and other erythrocyte cytoskeletal elements. The C-terminus affects anion transport activity and is likely involved in the three-dimensional structure of AE1 protein (15-17).

AE1 is a novel binding partner of p16. This relationship was identified by YTH using the C-terminal 112 residues of AE1 as bait (18). Subsequent studies have confirmed the interaction of AE1 with p16 *in vivo* and *in vitro*. Functional analysis has shown that the function of AE1 differs in gastric cancer cells as compared with normal cells. Silencing the expression of AE1 in gastric cancer cells results in the release of p16 to the nucleus and subsequent cell death. This suggests a mechanism for the cytoplasmic localization of p16, but the details remain to be investigated (19).

To verify the relationship between cytoplasmic p16 and AE1 in the progression of gastric cancer, the expression of p16 and AE1 was examined by immunohistochemical analysis in 98 early stage gastric cancers and in 98 advanced stage gastric cancers. The relationships between both cytoplasmic p16 and clinicopathological features and cytoplasmic p16 and AE1 were analyzed.

## Materials and methods

**Specimens.** A total of 196 patients with gastric cancer treated between 2001 and 2007 at Renji Hospital, Shanghai Jiao Tong University School of Medicine were studied retrospectively. Ninety-eight of these patients had early stage gastric cancer, and 98 had advanced stages of the disease. The University Ethics Committee approved the research protocol. Follow-up data were obtained from medical records.

**Immunohistochemical staining.** Tumor specimens were fixed in 4% neutralized formaldehyde, embedded in paraffin and sliced into 4- $\mu$ m sections, which were stained with hematoxylin and eosin (H&E). Histological subtypes of gastric carcinoma were defined in terms of Lauren's classification, and the staging for each gastric carcinoma was evaluated according to TNM staging guidelines. Anti-human p16 (ZJ11, 1:70, Maixin-Bio) or AE1 (BIII-136, 1:300, Sigma) monoclonal antibodies were used to detect the expression of p16 or AE1 protein. Deparaffinized sections were treated with 0.3% H<sub>2</sub>O<sub>2</sub> for 10 min to block endogenous peroxidase, and were subjected to antigen retrieval by citric acid (pH 6.0). Each specimen was washed 3 times for 5 min in PBS (pH 7.4). After overnight incubation with primary anti-p16 or AE1 antibodies at 4°C, the sections were incubated for 15 min at room temperature with horseradish peroxidase-labeled polymer conjugated with secondary antibody (Max-Vision™ Kits) and were incubated with diaminobenzidine for 5 min. Control experiments were performed using the same protocols and time exposures. All sections were evaluated by two independent observers (W.-Q. Xu and L.-J. Song) unaware of the disease outcome. Distinct staining was considered to be positive, regardless of the staining intensity. Less than 10% expression was considered to represent loss (-), and 11-25%, 26-50%, and >51% expression were designated +, ++, and +++ respectively.

**Cell culture.** Human normal gastric mucosae (Ges-1) and gastric cancer cell lines (MKN45, SGC7901 and AGS) were obtained from the Shanghai Cell Bank of the Chinese Academy of Sciences. Cells were cultured in DMEM, supplemented with 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin and 10% fetal bovine serum in a humidified atmosphere of 5% CO<sub>2</sub> at 37°C.

**Western blot analysis.** Protein (40  $\mu$ g) extracted from cells (Ges-1, SGC7901, MKN45 and AGS) was resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a PVDF membrane. All target proteins were immunoblotted by the appropriate primary antibody: anti-p16 (Santa Cruz Biotechnology, CA, USA). Anti-actin antibody (Sigma, St. Louis, MO, USA) was used as an internal loading control. Antigen-antibody complexes were visualized using the ECL reagent (Pierce).

**Statistical analysis.** Statistical evaluation was performed using the  $\chi^2$  test to analyze the rank data. Differences of P<0.05 were considered statistically significant. SPSS 11.0 software (Chicago, IL) was used to analyze the data.

## Results

In this study, we detected the expression of p16 and AE1 protein by immunohistochemistry in gastric cancer tissues obtained from surgical resection and endoscopic biopsy. Four different expression patterns of p16 were observed in gastric cancer and its para-cancer cells. Fig. 1a shows a gastric cancer at an early stage (x10). The cancer and para-cancer cells are shown in Fig. 1b and c, respectively (x40). Expression of p16 was demonstrated in the cytoplasm of the cancer cells (Fig. 1b) and in the nucleus of para-cancer cells (Fig. 1c). In addition, p16 expression was particularly pronounced in either the cytoplasm (Fig. 1d) or the nucleus (Fig. 1e) of the cancer cells, and the p16 protein was upregulated in primary gastric cancer tissue and cell lines (MKN45 and AGS) compared with para-cancer tissue (Fig. 1a) or the normal gastric mucosa cell line Ges-1 (Fig. 1f).

The distribution of p16 expression in early and advanced stage gastric cancer is shown in Table I. In cells of early stage gastric cancer, p16 was found in the nucleus in 17.2% of cells, in the cytoplasm in 25%, and in both the nucleus and cytoplasm in 57.8%. In advanced stage gastric cancer, cytoplasmic p16 was significantly greater (54.5%), whereas nuclear p16 expression was less (1.8%). Both nuclear and cytoplasmic p16 expression was demonstrated in 43.9% of cancer cells in advanced stage gastric cancer.

We also observed that 57 (58.2%) of 98 cases of advanced gastric carcinoma had detectable expression of p16. In early stage gastric carcinoma, p16 expression was demonstrated in 64 (65.3%) of 98 cases. Thus, advanced gastric cancer had a lower frequency of expression of p16. These results suggest that cytoplasmic p16 is associated with the progression of gastric cancer.

These findings raise questions regarding the significance of cytoplasmic p16, and whether cytoplasmic p16 is related to clinical outcome. Therefore, we next analyzed the relationship between cytoplasmic p16 expression and clinicopathological

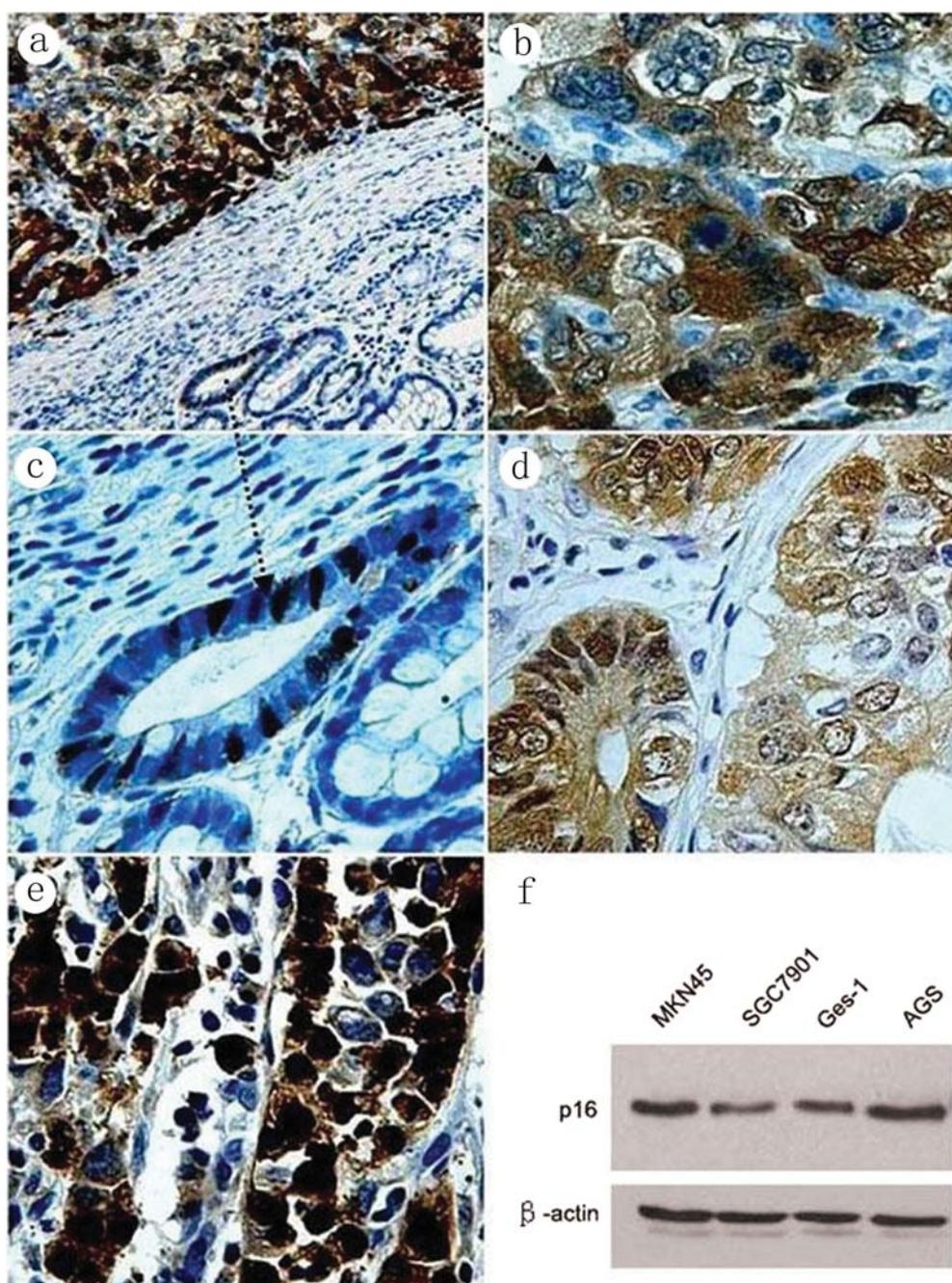


Figure 1. Expression patterns of p16 in gastric cancer. (a) Positive expression of p16 in gastric carcinoma and para-cancer tissue (original magnification x10). (b) Cytoplasmic expression of p16 in gastric cancer tissue (original magnification x40). (c) Nuclear expression of p16 in para-cancer tissue (original magnification x40). (d) Cytoplasmic and nuclear expression of p16 (cytoplasm expression higher) in gastric cancer tissue (original magnification x40). (e) Cytoplasmic and nuclear expression of p16 (nuclear expression higher) in gastric cancer tissue (original magnification x40). (f) Expression level of p16 in gastric cancer and normal gastric cell lines.

Table I. Distribution of location of p16 expression in early and advanced gastric cancer.

Location	Early stage		Advanced stage		P-value
	Positive number	Distribution (%)	Positive number	Distribution (%)	
Nucleus	64	17.2 (11 <sup>a</sup> )	57	1.8 (1)	0.000
Cytoplasm	64	25.0 (16)	57	54.4 (31)	
Nucleus and cytoplasm	64	57.8 (37)	57	43.9 (25)	0.008

<sup>a</sup>Positive number.

Table II. Association of cytoplasmic p16 expression and clinicopathological features of advanced gastric carcinomas.

Clinicopathological features	No.	p16 expression					P-value <sup>b</sup>
		Negative No. (%)	Positive			Cytoplasm blend (%)	
			Nucleus No. (%)	Cytoplasm No. (%)	Blend <sup>a</sup> No. (%)		
Age (years)							0.725
<75	55	24 (43.6)	2 (3.6)	16 (29.1)	13 (23.6)	56.4	
≥75	44	18 (40.9)	2 (4.5)	15 (34.1)	9 (20.5)	59.1	
Gender							0.305
Male	53	25 (47.2)	0 (0.0)	13 (24.5)	15 (28.3)	52.8	
Female	46	17 (37.0)	4 (8.7)	18 (39.1)	7 (15.2)	63.0	
Tumor size (cm <sup>3</sup> )							0.405
<4	7	2 (28.6)	0 (0.0)	1 (14.3)	4 (57.1)	71.4	
≥4	90	40 (44.4)	4 (4.4)	28 (31.1)	18 (20.0)	55.6	
Lymph metastasis							0.033
(-)	19	4 (21.1)	0 (0.0)	10 (52.6)	5 (26.3)	78.9	
(+)	78	37 (47.4)	4 (5.1)	20 (25.6)	17 (21.8)	52.6	
Lauren classification							0.067
Intestinal type	26	15 (57.7)	2 (7.7)	4 (15.4)	5 (19.2)	42.3	
Diffuse type	73	27 (37.0)	2 (2.7)	27 (37.0)	17 (23.3)	63.0	

<sup>a</sup>p16 was expressed in both the cytoplasm and nucleus. <sup>b</sup>Comparison of p16 expression (including cytoplasm and blend) with negative expression.

Table III. Correlation between the expression of AE1 and p16 in tumor cells of advanced gastric carcinoma.

Grade <sup>a</sup>	Late gastric carcinomas <sup>b</sup> (n=98)	
	AE1 (%)	p16 (%)
1	45.9 (45/98) <sup>c</sup>	41.8 (41/98)
2	21.4 (21/98)	23.5 (23/98)
3	18.4 (18/98)	21.4 (21/98)
4	14.3 (14/98)	13.3 (13/98)

<sup>a</sup>Grade 1, (-); grade 2, (+); grade 3, (++) ; grade 4, (+++) ; <sup>b</sup>r=0.984; P=0.016. <sup>c</sup>Positive number/total number.

features. No significant relationship was found by comparing the separate groups (negative, nuclear, cytoplasmic, or both nuclear and cytoplasmic); however, total expression of cytoplasmic p16 (including exclusively cytoplasmic expression, as well as nuclear and cytoplasmic expression) was significantly correlated with the absence of lymph node metastasis (Table II, P=0.033).

Since we previously found that p16 directly binds to the AE1 C-terminus, we next analyzed the association of cytoplasmic p16 with AE1 in terms of frequency of expression, and the relationship between the two proteins and lymph node

Table IV. Association of p16 and AE1 expression and the clinicopathological features of advanced gastric carcinomas.

	No.	Lymph node metastasis		P-value
		Negative	Positive	
AE1 (-) P16 (-)	22	1 (4.5 <sup>a</sup> )	21 (95.5)	0.046
AE1 (+) P16 (+)	33	10 (30.3)	23 (69.7)	

<sup>a</sup>Represents the percentage.

metastasis. We found a significant correlation between AE1 and p16 in both expression frequency (Table III, r=0.984, P=0.016) and absence of lymph node metastasis (Table IV, P=0.046).

## Discussion

Since p16 was identified as a protein that negatively regulates the cell cycle, it was generally believed that p16 functioned in the nucleus; however, to date, there has been no direct evidence to support this assumption. On the contrary, increasing reports have demonstrated the cytoplasmic localization of p16 in different primary tumor cells, such as breast, lung and prostate carcinoma cells, suggesting that cytoplasmic p16 is associated with carcinogenesis (20-23). However, results concerning the



ip between cytoplasmic p16 and clinical features are not. In primary breast carcinoma, reports indicate that cytoplasmic expression of p16 is associated with unfavorable prognosis; however, no association between cytoplasmic p16 and patient outcome has been described (24).

In the present study, p16 was expressed in the nucleus and cytoplasm of cancer cells in the early stages of gastric cancer, but was mainly expressed in the cytoplasm in advanced stages of the disease. As shown in Table I, in the series of 121 gastric cancer specimens with positive p16 expression, cytoplasmic p16 protein was found in 25% of early gastric carcinomas, but was found with greater intensity in advanced gastric carcinoma, and in a higher percentage of cases (54.4%). This suggests that cytoplasmic p16 staining increases along with the progression of gastric cancer. Cytoplasmic p16 is therefore associated with the depth of invasion of gastric cancer, by virtue of the fact that the distinction between early and advanced gastric cancer is based on the criterion of the depth of invasion of cancer cells. This finding is in agreement with that of another study, in which the authors concluded that the invasion of basal cell carcinoma was induced by p16 up-regulation (in both the nucleus and cytoplasm) with the requirement of a functional p16/cyclin D/Rb pathway (25).

Although the details of the mechanisms involved in the cytoplasmic accumulation of p16 remain unclear, one piece of solid evidence addressing this issue was previously reported by our group. We found that AE1 was unexpectedly expressed in gastric cancer cells, and that it interacted with p16. This resulted in the sequestration of p16 protein in the cytoplasm. Expression of AE1 was also correlated with deeper invasion (data not shown). This observation is consistent with our observations in the present study. Other hypotheses have been presented in several reports, including the mutation of nuclear localization signals and the deregulation of p16 chaperone proteins (22,26). In addition, we found p16 over-expression in advanced gastric carcinoma. Studies have shown that p16 is expressed at very low levels in normal gastric epithelium, and that the overexpression of p16 is associated with tumor progression and poor prognosis (27,28). These findings suggest that p16, acting as a negative cell cycle regulator, may also take part in carcinogenesis.

In contrast to its association with cancer invasion, cytoplasmic p16 is correlated with the absence of lymph node metastasis. This observation raises a bewildering question regarding the role of cytoplasmic p16 in the progression of gastric cancer. Invasion and metastasis are two properties which contribute to the serious consequences of cancer (29,30). Invasion is associated with increased cellular motility, increased proteolytic activity of enzymes, and alterations in cell adhesion. Metastasis is the process whereby malignant tumor cells spread from their site of origin to distant sites in the body. In a general way, invasion and metastasis are likely to be manifestations of the same properties of tumor cells; however, experimental results and clinical observations have shown that different signal pathways mediate the processes of invasion and metastasis. For example, some early stage carcinomas metastasize to distant organs, whereas other carcinomas of advanced stages do not metastasize until a later time. The results suggest that cytoplasmic p16 expression can influence the phenotype of a tumor cell to be invasive with

low potential for metastasis, suggesting that the tendency of tumor cells to invade or to metastasize could be predicted by the observation of p16 activity, and that invasion and metastasis represent two distinct aspects of malignant behavior.

As we demonstrated previously, transient expression of AE1 upregulates the expression of endogenous p16 protein, and the enhanced expression of p16 facilitates the movement of AE1 to the plasma membrane in the human embryo kidney cell line HEK293 (18). By contrast, in gastric cancer cells, AE1 was unconventionally expressed and failed to move to the plasma membrane, thus resulting in the sequestration of p16 in the cytoplasm (19). In addition, expression of AE1 was correlated with the absence of lymph node metastasis (data not shown). This observation is consistent with the relationship between cytoplasmic p16 and clinical outcome in gastric cancer. The expression of p16 in the cytoplasm is at least in part due to the sequestration of AE1. Co-expression of AE1 could affect the construction and the function of cytoskeletal protein, because the AE1 protein interacts with several cytoskeletal proteins that play a crucial role in the metastasis of cancer (31,32).

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