

Research article

Expression of p53, inducible nitric oxide synthase and vascular endothelial growth factor in gastric precancerous and cancerous lesions: correlation with clinical features

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Abstract

Background: The growth and metastasis of tumors depend on the development of an adequate blood supply via angiogenesis. Recent studies indicate that the inducible nitric oxide synthase (iNOS), vascular endothelial growth factor (VEGF) and the tumor suppressor p53 are fundamental play-markers of the angiogenic process. Overexpression of iNOS and VEGF has been shown to induce angiogenesis in tumors. P53 suppresses angiogenesis by down-regulating VEGF and iNOS. The correlation of expression of p53, VEGF and iNOS and clinical features in gastric carcinogenesis, however, has not been well characterized.

Methods: The expression of p53, iNOS and VEGF in gastric precancerous and cancerous lesions and its relation with the clinical features was determined with immunohistochemistry (avidin-biotin-peroxidase complex method) on 55 randomly selected GC patients and 60 symptom-free subjects from the mass survey in the high-incidence area for GC in Henan, northern China.

Results: The positive immunostaining rates for p53, iNOS and VEGF in gastric carcinomas were 51%, 44% and 51%, respectively, and correlated well with TNM stages, but did not show significant difference among the groups with different degrees of gastric wall invasion depth by GC. A positive immunostaining reaction for the iNOS protein was significantly correlated with lymph node metastasis ($p = 0.019$; Spearman correlation coefficient). P53 protein accumulation was higher in the poorly-differentiated gastric carcinoma than in well-differentiated one. In gastric biopsies, no positive immunostaining was observed for p53, iNOS and VEGF in the histologically normal tissue and chronic superficial gastritis (CSG). However, p53, iNOS and VEGF positive immunostaining was observed in the tissues with different severities of lesions of chronic atrophic gastritis (CAG), intestinal metaplasia (IM) and dysplasia (DYS), and the positive rates increased with the lesion progression from CAG to IM to DYS. A high coincidental positive and negative immunostaining rate for p53, iNOS and VEGF was observed both in biopsy samples with CAG, IM and DYS from the symptom-free subjects and in gastric cancer tissue from the GC patients.

Conclusions: The present results indicated that p53 protein accumulation and increased expression of iNOS and VEGF might be responsible for gastric carcinogenesis and tumor aggressiveness of gastric cancer.

Gastric adenocarcinoma (GC) is one of the most common malignant diseases in China. Although the GC has been declined in its mortality rate, it remains the leading cause of cancer-related deaths in this country (Parkin et al., 1992; World Health Organization, 1994; Nomura A, 1996). Gastric carcinogenesis is considered as a multistage progressive process. The early indicator for the subject predisposed to GC is abnormal hyperproliferation of gastric epithelial cells, such as chronic atrophic gastritis (CAG), DYS and intestinal metaplasia (IM), which have been considered as precancerous lesions of GC (You et al., 1993; Correa P, 1992). However, the information for the mechanism of gastric carcinogenesis is very limited.

The growth and metastasis of tumors depend on the development of an adequate blood supply via angiogenesis. Recent studies indicate that the inducible nitric oxide synthase (iNOS), vascular endothelial growth factor (VEGF) and tumor suppressor p53 are fundamental play-makers of the angiogenic process (Ambs et al., 1994; Jenkins et al., 1995; Forrester et al., 1996; Chiarugi et al., 1998). iNOS is one of the isoforms of nitric oxide synthase that catalyzes the formation of nitric oxide, a regulator of vascular permeability. VEGF is a dimeric glycoprotein and has specific mitogenic activity for endothelial cells. Over-expression of iNOS and VEGF could induce angiogenesis in tumors. P53 suppresses angiogenesis by down-regulating VEGF and iNOS. Mutant p53 potentiates protein kinase C induction of VEGF expression (Zhang et al., 2000). In a tumorigenicity model using human fibroblast cells, the loss of wild-type p53 and transfection of activated ras led to increased VEGF expression (Devavrata et al., 1995). Transient transfection of wild-type p53 and of v-src has been found to exert opposing effects on human VEGF gene promoter activity in human glioblastoma and transformed human fetal kidney cells (Devavrata et al., 1995). However, because wild-type p53 did not repress hypoxia-induced transcription of the VEGF gene in stably transfected human colorectal carcinoma and human hepatoblastoma cells with an inducible p53-estrogen-receptor fusion protein expression system (Ambs et al., 1998), it has been proposed that there is a need for caution in interpreting p53 function on the basis of cells transiently overexpressing this protein. Nevertheless, mutant p53 has been correlated with increased VEGF expression in human lung cancer (Ambs et al., 1994), and colorectal cancer (Ambs et al., 1999) by immunohistochemical staining of surgical specimens. It has been reported that dysfunction of the p53/MDM-2 pathway can be found in more than two-thirds of angiosarcomas; thus, mutant p53 influences the development of angiosarcoma not only by affecting growth and apoptosis control but also by up-regulating VEGF (Chinrugi et al., 1998). We hypothesized that alterations of p53, iNOS and VEGF expression may contribute to gastric carcinogenesis and influence the clinical behav-

ior of GC via angiogenesis. The correlation of expression of p53, VEGF and iNOS and clinical features in gastric carcinogenesis, however, has not been well characterized.

To characterize the changes of p53, iNOS and VEGF expression in human gastric carcinogenesis, the present study was undertaken to determine the protein level of p53, iNOS and VEGF in normal tissues and the tissues with different severities of lesions of CSG, CAG, DYS, IM and GC with and without lymph node metastasis from surgically resected human GC and gastric biopsies from symptom-free subjects. The subjects were from Linzhou and nearby county Huixian in Henan province, the high-incidence area for GC in northern China.

Materials and Methods

Patients

The present investigation included 55 randomly selected GC patients and 60 symptom-free subjects from the mass survey in the high-incidence area for GC. Fifty-five GC patients were performed gastric cancer surgery at the Linzhou People's Hospital (n = 26) and The Second Affiliated Hospital of Henan Medical University (n = 29) from 1996 to 1998. Before surgery, none of the patients received any kinds of chemical or radiation therapy. The median age of the patients was 61 years (30 ~ 77 years). Cancer specimens were classified based on TNM as stage T1 (19 patients, 35%), T2 (14 patients, 25%), T3 (12 patients, 22 %) and T4 (10 patients, 18%). The tumors were histologically graded as well-differentiated (15 patients, 27%), moderate-differentiated (14 patients, 25%) and poorly-differentiated (26 patients, 47%). Of the 55 patients, 35 occurred local lymph node metastasis (64%). Gastric biopsies were taken from 60 symptom-free subjects who volunteered to participate in a routine endoscopic screening for esophageal and gastric cancer in Huixian, China. No selection process was involved. Of the 60 subjects examined, there were 35 males and 25 females. The median age of the subjects was 59 years (28 ~ 70 years). The biopsies were taken each from the pylorus of the stomach from each subject.

Tissue processing

All the surgically resected specimens and biopsy samples were fixed with 10% neutral formalin, embedded with paraffin, and serially sectioned at 5 μ m. The sections were mounted onto the histostick-coated slides. Four or five adjacent ribbons were collected for histopathological analysis (hematoxylin and eosin stain) and for immunohistochemical staining.

Histopathological Analysis

Histopathological diagnosis for gastric epithelia was made according to cellular morphological changes and tissue architecture using previously established criteria

(Wang, et al., 1994; You, et al., 1993). In brief, CSG: an inflammation manifested by mild lymphocyte and plasma-cell infiltration; CAG: glandular morphology disappeared partially or completely absent in the mucosa and replaced by connective tissue, inter-glandular space was infiltrated mainly by plasma cells and lymphocytes; DYS: characterized by nuclear atypia with or without architectural abnormalities in the gastric epithelium, but without invasion; IM: confirmed by the presence of goblet cells in gastric mucosa; adenocarcinoma (AC): invasion of neoplastic gastric cells through the basement membrane.

Immunohistochemical staining

Anti-p53 antibody is a monoclonal mouse anti-serum against p53 of human origin, and recognizes both wild-type and mutant p53 (Ab-6, Oncogene Science, Manhasset, NY). Anti-iNOS antibody is a monoclonal mouse anti-serum against the C-terminal domain of iNOS of human origin (sc-7271, Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Anti-VEGF antibody is a polyclonal rabbit anti-serum against VEGF of human origin and recognizes an amino-terminal epitope found in VEGF^{121, 165, 189} and ²⁰⁶ (Ab-2, Oncogene Science, Manhasset, NY). The avidin-biotin-peroxidase complex method was used for the immunostaining of p53, iNOS and VEGF. In brief, after dewaxing, inactivating endogenous peroxidase activity and blocking cross-reactivity with normal serum (Vectastain Elite Kit; Vector, Burlingame, CA), the sections were incubated overnight at 4C with a diluted solution of the primary antibodies (1:1000 for p53, 1:200 for iNOS and 1:100 for VEGF). Location of the primary antibodies was achieved by subsequent application of a biotinylated anti-primary antibody, an avidin-biotin complex conjugated to horseradish peroxidase, and diaminobenzidine (Vectastain Elite Kit, Vector, Burlingame, CA). The slides were counter-stained by hematoxylin. Negative controls were established by replacing the primary antibody with PBS and normal mouse or rabbit serum. Known immunostaining-positive slides were used as positive controls.

Specific staining for each protein was categorized as either positive or negative based on the presence of brown-color staining. More than 10% cells positively stained were graded as positive. Clear staining for nuclei (p53), cytoplasm and cell membrane (VEGF and iNOS) was the criterion for a positive reaction (Wang et al., 1994). All the immunostaining slides were observed by two pathologists (LD Wang and LH Jiao). The slides with different diagnosis by two pathologists were read again (less than 5%), until the agreed diagnosis was made.

Statistical Analysis

The X^2 test was used for the percentage of samples with positive stain. Spearman correlation test and linear tendency test were used for the correlation between positive

rates and different severities of lesions ($p < 0.05$ was considered significant).

Results

Histopathologically, all the 55 surgically-resected gastric specimens were confirmed as adenocarcinoma. Of the 55 GC patients, 11 cases occurred the invasion within sub-mucosa, 44 with whole-muscle layer. Of the 60 biopsy-samples examined, 10 were diagnosed as normal, 15 as CSG, 9 as CAG, 16 as IM with CAG, and 10 as DYS (Table 1).

The positive immunostaining for p53, iNOS and VEGF was observed in the gastric epithelial cells and cancer cells with different rates in the lesions of CAG, DYS, IM and GC. But, the positive immunostaining for p53, iNOS and VEGF was not observed in normal gastric epithelial tissue and CSG. Figure 1, 2 and 3 shows the representative of p53, iNOS and VEGF immunostaining in GC. p53 immunoreactivity was located in the nuclei, VEGF and iNOS immunoreactivity were located mainly in the cytoplasm and cell membrane. iNOS immunostaining was confirmed by Western Blot on part of iNOS positive and negative slides (three slides for each), and consistent results were obtained for iNOS immunohistochemistry and Western Blot (data not shown). The positive immunostaining rate for p53 was very low in CAG, and slightly increased in IM and DYS, and significantly increased in GC (Table 1). Similar results were observed in iNOS and VEGF (Table 1). P53 positive immunostaining rate in well-differentiated GC was lower than that in poorly-differentiated GC ($p = 0.012$, Table 2). iNOS positive staining was similar among

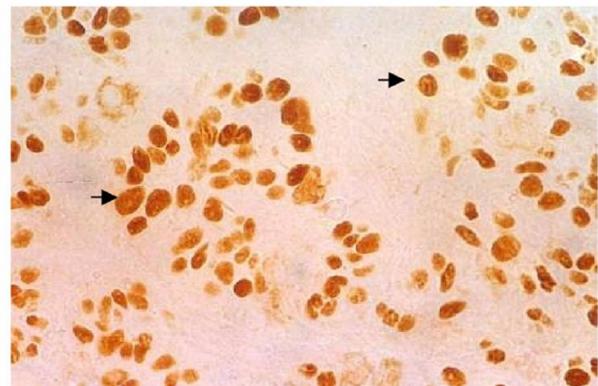


Figure 1
Immunostaining of p53 in gastric carcinoma. Immunoreactivity is located in cell nuclei (Arrows). 20X

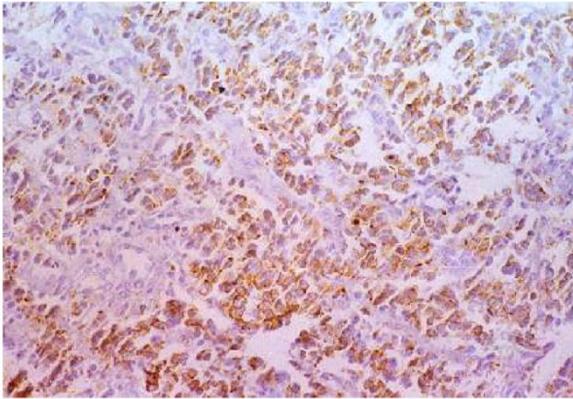


Figure 2
Immunostaining of iNOS in gastric carcinoma. Immunoreactivity is located mainly in cytoplasm and cell nuclear membrane. 10X

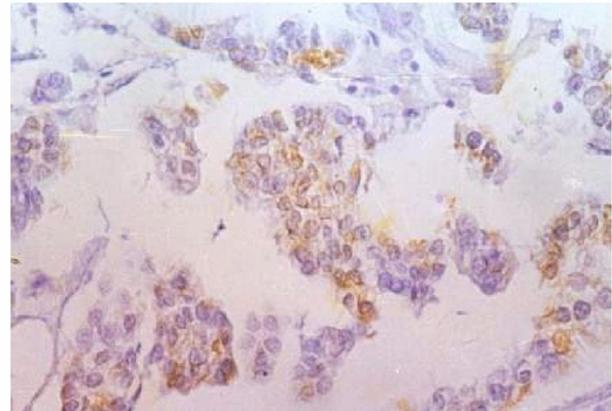


Figure 3
Immunostaining of VEGF in gastric carcinoma. Immunoreactivity is located in cytoplasm and cell membrane. 10X

the GC with different degree of differentiation. Although VEGF immunostaining rate in poorly-differentiated GC was higher than in well-differentiated GC, the difference was not significant (Table 2).

iNOS positive immunostaining rate in the cases with deep invasion of GC (through muscle layers) was higher than in those with superficial invasion (within submucosa), but, the difference was not significant (Table 3). P53 and VEGF immunostaining was similar in GC patients with superficial and deep invasion.

iNOS immunostaining rate in the cases with lymph node metastasis was higher than that in those without lymph node metastasis ($p = 0.014$, Table 4). However, there was no difference observed for p53 and VEGF between the cases with and without lymph node metastasis.

P53 immunostaining rate in the cases with TNM grade T1 was lower than that in those with TNM grade T2, T3 and T4 (T1:T3, $p = 0.023$; T1:T4, $p = 0.012$). Similar results were observed in VEGF (T1:T2 and T2:T3, $p = 0.015$; T1:T4, $p = 0.014$; T2:T4, $p = 0.01$, Table 5). However, there was no difference for iNOS immunostaining between the cases with different TNM stages.

A higher coincidental expression of p53, iNOS and VEGF in GC was observed. Of the 55 cases examined, there were 20 cases showing positive staining both for p53 and iNOS (36%), 23 cases showing negative staining for both p53 and iNOS (42%) ($p = 0.005$, Table 6), 19 cases showing positive staining both for p53 and VEGF (35%) and 18 cases showing negative for both p53 and VEGF (33%) ($p = 0.01$, Table 7). The correlations were significant.

Table 1: Immunoreactivity of p53, iNOS and VEGF in gastric normal epithelia and the epithelia with different severities of lesions

| | No. of samples examined | P53 No. of positive stain n (%) | iNOS No. of positive stain n (%) | VEGF No. of positive stain n (%) |
|---------------------|-------------------------|------------------------------------|-------------------------------------|-------------------------------------|
| Normal ^a | 10 | 0 (0) | 0 (0) | 0 (0) |
| CSG ^b | 15 | 0 (0) | 0 (0) | 0 (0) |
| CAG ^c | 9 | 1 (11) | 0 (0) | 2 (22) |
| IM ^d | 16 | 3 (19) | 2 (13) | 5 (31) |
| DYS ^e | 10 | 3 (30) | 4 (40) | 5 (50) |
| GC ^f | 55 | 28 (51) | 24 (44) | 28 (50) |

P53: ^{c:f} $p = 0.02$ ^{d:f} $p = 0.013$; iNOS: ^{c:f} $p = 0.03$ ^{d:f} $p = 0.015$; VEGF: ^{c:f} $p = 0.014$ ^{d:f} $p = 0.015$

Table 2: Immunoreactivity of iNOS, p53 and VEGF in GC and its correlation with different degrees of differentiation

| Differentiation degree of GC | No. of samples examined | P53* No. of positive stain n (%) | iNOS No. of positive stain n (%) | VEGF No. of positive stain n (%) |
|--------------------------------------|-------------------------|-------------------------------------|-------------------------------------|-------------------------------------|
| Well-differentiated ^a | 15 | 5 (33) | 7 (47) | 6 (40) |
| Moderate-differentiated ^b | 14 | 6 (43) | 5 (36) | 6 (43) |
| Poorly-differentiated ^c | 26 | 17 (66) | 12 (46) | 16 (61) |

* a:c p = 0.012

Table 3: Immunoreactivity of p53, iNOS and VEGF and its correlation with invasion depth of gastric carcinoma

| Invasion depth of GC | No. of samples Examined | P53 No. of positive stain n (%) | iNOS No. of positive stain n (%) | VEGF No. of positive stain n (%) |
|----------------------|-------------------------|------------------------------------|-------------------------------------|-------------------------------------|
| Within mucosa | 11 | 5 (46) | 3 (27) | 6 (55) |
| Muscle layer | 44 | 23 (52) | 21 (48) | 22 (50) |

Table 4: Immunoreactivity of p53, iNOS and VEGF and its correlation with lymph node metastasis of gastric carcinoma

| Lymph node metastasis | No. of samples Examined | P53 No. of positive stain n (%) | iNOS* No. of positive stain n (%) | VEGF No. of positive stain n (%) |
|-----------------------|-------------------------|------------------------------------|--------------------------------------|-------------------------------------|
| Yes | 35 | 18 (51) | 19 (55) | 21 (60) |
| No | 20 | 10 (50) | 5 (25) | 7 (35) |

* p = 0.014

Table 5: Immunoreactivity of p53, iNOS and VEGF and its correlation with Clinical TNM classification

| TNM stages | No. of samples examined | P53* No. of positive stain n (%) | iNOS No. of positive stain n (%) | VEGF** No. of positive stain n (%) |
|------------|-------------------------|-------------------------------------|-------------------------------------|---------------------------------------|
| T1 | 19 | 7 (37) ^a | 7 (37) | 8 (42) ^a |
| T2 | 14 | 6 (43) ^b | 4 (29) | 4 (29) ^b |
| T3 | 12 | 8 (67) ^c | 7 (58) | 8 (67) ^c |
| T4 | 10 | 7 (70) ^d | 6 (60) | 8 (80) ^d |

*a:c p = 0.023, a:d p = 0.012; **a:b and b:c p = 0.015, a:d p = 0.014, b:d p = 0.01

Table 6: Coincidental expression of p53 and iNOS protein in the gastric carcinoma

| | p53 (+) n (%) | p53 (-) n (%) |
|----------|------------------|------------------|
| iNOS (+) | 20 (36) | 4 (7) |
| iNOS (-) | 8 (15) | 23 (42) |

$$\chi^2 = 15.68, p = 0.005$$

Table 7: Coincidental expression of p53 and VEGF protein in the gastric carcinoma

| | p53 (+) n (%) | p53 (-) n (%) |
|---------|------------------|------------------|
| VEGF(+) | 19 (35) | 9 (16) |
| VEGF(-) | 9 (16) | 18 (33) |

$$\chi^2 = 6.56, p = 0.01$$

Discussion

An interesting observation in this study was that the immunoreactivity of p53, iNOS and VEGF occurred in the early stage of gastric carcinogenesis. The positive immunostaining cells were invariably associated with cell proliferate activity. With the lesions progressed from normal to CSG to CAG to IM to DYS, and to GC, the positive immunostaining rates for p53, iNOS and VEGF increased significantly, the increasing tendency for p53, iNOS and VEGF had a good linear correlation with lesions progression. The present results indicated that p53 protein accumulation and increased expression of iNOS and VEGF may be important molecular events involved in the early stage of gastric carcinogenesis.

Another interesting observation was that, a high coincidental positive immunostaining rates for p53 and iNOS (36%) and p53 and VEGF (35%) was observed (Table 6 and 7). This result was consistent with the findings in human lung and colon carcinomas (Ambs et al., 1999). Although p53 antibody used in this study could not differentiate wild-type p53 from mutant p53, previous studies have indicated that p53 protein detected by immunohistochemistry seems to representative most mutant p53. Accumulation of p53 is an indicator for a loss of p53 tumor suppressor function. Both mutations and protein-protein interactions can cause a p53 accumulation (Wang, et al., 1994). Considering that p53 protein accumulation was correlated with poorly-differentiation and T2, T3 and T4 TNM stage of GC, we concluded that p53

protein accumulation in this study may lead to the loss of cell proliferation control and inhibition for iNOS and VEGF, possibly because of p53 gene mutation. Thus, the high coincidental expression of iNOS and VEGF and p53 protein accumulation may be important events to enhance gastric carcinogenesis and poorly clinical features and may be useful biomarkers to assess risk for the development of GC. Recent studies on Japanese GC patients have showed that increased expression of VEGF and p53 protein accumulation are associated with intra-tumoral angiogenesis (Takeuchi, et al., 2000; Saito, et al., 1999) and poor prognosis (Skatani, et al., 2000; Ikeguchi, et al., 2000; Maeda, et al., 1998). Rajnkova reported that increased expression of iNOS may promote gastric cancer progression by providing a selective growth advantage to tumor cells with non-functioning p53. (Rajnkova, et al., 2001)

The third observation in this study was that increased expression of iNOS and VEGF and p53 protein accumulation occurred with a high frequency in gastric biopsy samples with IM. IM, which may be defined as the replacement of gastric mucosa by glands which have the histological, histochemical, and physiological characteristics of the small intestine, has been strongly associated with stomach cancer. The present results demonstrated that IM was a unstable stage with active molecular changes, and further analysis to compare these changes with normal small intestine will provides more information for the significance of IM in gastric carcinogenesis.

Finally, the present study demonstrated that the positive immunostaining rates of iNOS was correlated well with GC lymph node metastasis, increased expression of VEGF and p53 protein accumulation was related with TNM stages and GC differentiation. These findings indicate that p53, iNOS and VEGF may be useful biomarkers for GC aggressiveness. The molecular basis for the observed expression of iNOS and VEGF and p53 protein accumulation and their roles in the progression of gastric pre-cancerous lesions and angiogenesis requires to be carefully investigated in the laboratory and follow-up studies.

Abbreviations

The abbreviations used are: CSG, chronic superficial gastritis; CAG, chronic atrophic gastritis; DYS, dysplasia; GC, gastric adenocarcinoma; iNOS, inducible nitric oxide synthase; IM, intestinal metaplasia; VEGF, vascular endothelial growth factor.

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