

Inducible nitric oxide synthase, nitrotyrosine and apoptosis in gastric adenocarcinomas and their correlation with a poor survival

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Abstract

AIM: To detect the presence of inducible nitric oxide synthase (iNOS), nitrotyrosine (NT) and apoptosis in gastric adenocarcinomas and their possible correlations with the clinicopathological characteristics and prognosis of gastric adenocarcinoma.

METHODS: Sixty-six specimens of gastric adenocarcinoma and corresponding adjacent normal gastric tissues were studied. Immunohistochemistry was employed to localize iNOS and NT protein and an immunohistochemical scoring system was used. The occurrence of apoptotic cell death (apoptotic index [AI]) was analyzed by the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate biotin nick-end labeling (TUNEL) method.

RESULTS: Results showed that iNOS expression was detected at an intermediate or high level in 41 of 66 (62%) specimens of gastric adenocarcinoma. NT expression was 58%. Neither of them was found in the normal gastric tissues; there were significant positive correlations among iNOS expression, NT expression and AI. Many clinicopathologic characteristics of gastric adenocarcinoma, such as tumor size, depth of invasion, lymph node metastasis and TNM staging, were related to iNOS and NT expressions ($P < 0.05$). In 66 surviving patients, the 5-year survival rate of 41 patients who had tumors with intermediate or high iNOS expressions and high AIs (4.09%; 19.96%) was significantly lower than that of 25 patients who had tumors with negative or low iNOS expressions and low AIs (0.79%; 47.14%) ($P = 0.001$). COX's multivariate analysis revealed that the iNOS expression was identified as one of the significant independent prognostic factors predictive of a poor survival (relative risk [RR] = 2.69).

CONCLUSION: NO produced by iNOS may play a stronger role in promoting gastric adenocarcinoma growth than in suppressing its growth. iNOS and NT expressions by gastric adenocarcinoma may correlate with a poor survival.

INTRODUCTION

Gastric carcinoma is one of the most common malignant diseases in the world, and the first cause of gastrointestinal cancer-related mortality. The study conducted by Lala and Orucevic^[1] showed that inducible nitric oxide synthase (iNOS) might play an important role in tumor growth, invasion, and metastasis. Thus, iNOS has been proposed to affect the clinical features of tumors^[2-4]. Some tumors were reported to express iNOS, and the product of this enzymatic action (high output of NO), may induce peroxynitrite formation, resulting in a series of extensive oxidation reactions^[5,6]. Peroxynitrite can cause damage to proteins, lipids, and nucleic acids^[7], which may lead to cellular apoptosis and necrosis^[8]. Peroxynitrite nitrates tyrosine residues of proteins, resulting in the production of nitrotyrosine (NT)^[9]. Since this product is stable, the occurrence of NT in tissues has been measured as a marker indicative of formation and activity of the NO-derived oxidant peroxynitrite^[10].

Previous studies indicate that effects of NO synthesized by iNOS can be either tumor suppressing or tumor promoting. The tumor suppressing role has been identified by the typical findings in NO-mediated apoptosis, including accumulation of tumor suppressor protein p53, caspase activation, chromatin condensation, and DNA fragmentation^[11-13]. The tumor promoting role has been demonstrated by the evidence that NO produced by iNOS may promote tumor angiogenesis and blood flow in tumor neovasculature, and enhance tumor growth, invasion, and metastasis^[1]. The conflicting dual roles of iNOS might work together to influence the clinical pathology and pathophysiology of tumors. However, when there is iNOS expression in gastric adenocarcinoma, the direction and condition in which the tumor may develop (promoted or suppressed) remains to be defined.

Our present study was designed to determine whether gastric adenocarcinoma cells expressed iNOS and NT, whether the distribution of NT in gastric adenocarcinoma was related to the apoptosis, and whether the presence of iNOS, NT and apoptosis correlated with clinical features of gastric adenocarcinoma and influenced patient's prognosis.

MATERIALS AND METHODS

Specimens

Specimens of gastric adenocarcinoma and corresponding adjacent normal gastric tissues (at least 5 cm beyond the border of tumors, as control) were obtained from 66 surgically treated patients with gastric adenocarcinoma at the Department of Surgical Oncology, First Affiliated Hospital, China Medical University. Of them, 36 were males and 30 were females. The mean age was 57 years (range, 29-78 years). All the selected adjacent control gastric tissues were identified as normal by gross and microscope study. As to the grade of tumors, 36 cases were classified as poorly differentiated adenocarcinoma, of which 30 cases were well and moderately differentiated ones. None of the 66 patients received preoperative radiotherapy or chemotherapy, and all underwent curative surgical resection of the tumor along with regional lymph node dissection at least 5 years ago. Clinicopathologic characteristics of these patients were investigated based on the TNM classification of malignant tumors^[14].

Resected specimens from the patients were fixed in 40 g/L formaldehyde and embedded in paraffin. Before immunohistochemical examination, hematoxylin-eosin staining was used to verify the pathology.

Immunohistochemical detections of iNOS and NT expressions

Immunohistochemical staining was performed by using the avidin-biotin-peroxidase complex (ABC) method. Mouse monoclonal antibodies against iNOS (diluted 1:100; Santa Cruz, Heidelberg, Germany) and mouse monoclonal anti-NT antibody (diluted 1:50; Zymed, Berlin, Germany) were used for immunostaining. The immunohistochemical staining steps were the same for both iNOS and NT. In brief, sections (4 μ m) were fixed to the slides pretreated with polylysine (Sigma) and dried overnight at 50 °C. For immunohistochemistry the sections were deparaffinized, rehydrated in sequential alcohol baths, and washed with phosphate-buffered saline solution (PBS). The sections were boiled in 0.01 mol/L of citric acid buffer (pH 6.0) for 10 min, then cooled to room temperature and washed thrice with PBS for 9 min. The endogenous peroxidase activity was inactivated with 3% hydrogen peroxide in ethanol, and specific antibody binding was suppressed with 1.5% normal blocking serum in PBS. Sections were incubated in a humidified chamber for 2 h at room temperature. After intervening washes in PBS, sections were then incubated for 30 min with a biotin-conjugated secondary antibody. Goat anti-mouse immunoglobulin G (IgG; Santa Cruz) was used for anti-iNOS, and anti-NT antibodies. The avidin-biotin-immunoperoxidase complex (ABC staining system, Santa Cruz) was then applied. Peroxidase activity was visualized with diaminobenzidine (DAB; Boster, Wuhan, China). As controls, sections from all the samples were stained with the procedures described above, omitting the primary or secondary antibodies.

For iNOS and NT, weighted scores were computed, representing the percentage of positivity and intensity of gastric adenocarcinoma cells, as described previously^[15]. The percentage of tumor cell positivity was categorized as follows: 0, <5%; 1, 5-25%; 2, 26-50%; 3, 51-75%, and 4, >75%. Staining intensity was graded as absent (0), weak (1+), medium (2+), or strong (3+). On the basis of weighted

scores, expression was categorized as negative or low (0-4) and intermediate (5-8) or high (9-12). Before the study, specimens were scored, and representative sections displaying each of the four staining intensities were reviewed by two independent examiners.

Detection of apoptotic cells and bodies

Apoptotic gastric adenocarcinoma cells were detected by the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate biotin nick-end labeling (TUNEL) method. The ApoTag *in situ* detection kit (Boster, Wuhan, China) was used in this study. Briefly, paraffin sections were deparaffinized in xylene and rehydrated in graded ethanol. After being washed in Tri-buffer saline (TBS, pH 7.4), the sections were put into 3% H₂O₂ for 10 min, the tissues were then digested with proteinase K (20 μ g/mL in TBS) at 37 °C for 10 min to enhance nuclear staining of apoptotic cells. The digestion was stopped with the sections washed in TBS. The sections were then treated with terminal transferase enzyme and digoxigenin-labeled nucleotides, and antidigoxigenin-peroxidase solution was applied afterwards. The color was developed with DAB, after which the sections were lightly counterstained with hematoxylin. To confirm the staining specificity of the TUNEL method, a positive control section was prepared. The substitution of equilibration buffer for TdT was used as a negative control.

The apoptotic index (AI) was expressed as the ratio of positively stained tumor cells and bodies to all tumor cells given as a percentage for each case. Positively stained tumor cells with morphological characteristics of apoptosis were identified by using standard criteria, including chromatin condensation, nucleolar disintegration, and formation of crescentic caps of condensed chromatin at the nuclear periphery^[9,16]. To determine the AI, 10 representative areas without inflammation and necrosis but consisting of at least 1 000 gastric adenocarcinoma cells were counted for each sample with a light microscope (\times 400 magnification). The results were assessed by two independent observers.

Statistical analysis

SPSS for Windows 10.0 (SPSS, Inc., Chicago, IL, USA) was used for statistical analysis. Statistical significance was determined by χ^2 test, Mann-Whitney *U*-test, and McNemar's test. The null hypothesis for the McNemar's test is that there is an agreement between the two parameters. Correlation between AI and NT expression was judged by the Spearman rank correlation test. Survival rates were calculated for the 66 patients by using the Kaplan-Meier method. Corrected survival rates were used, that is, only the deaths caused by gastric adenocarcinoma were taken as outcome events, and all the other deaths were considered as censored events. Log-rank test was used for univariate analysis to determine differences between curves. Multivariate analysis by Cox proportional hazards regression used only the variables that were significant in univariate analysis. *P* values less than 0.05 were considered statistically significant.

RESULTS

iNOS distribution

Positive iNOS staining was located in cytoplasm. iNOS was

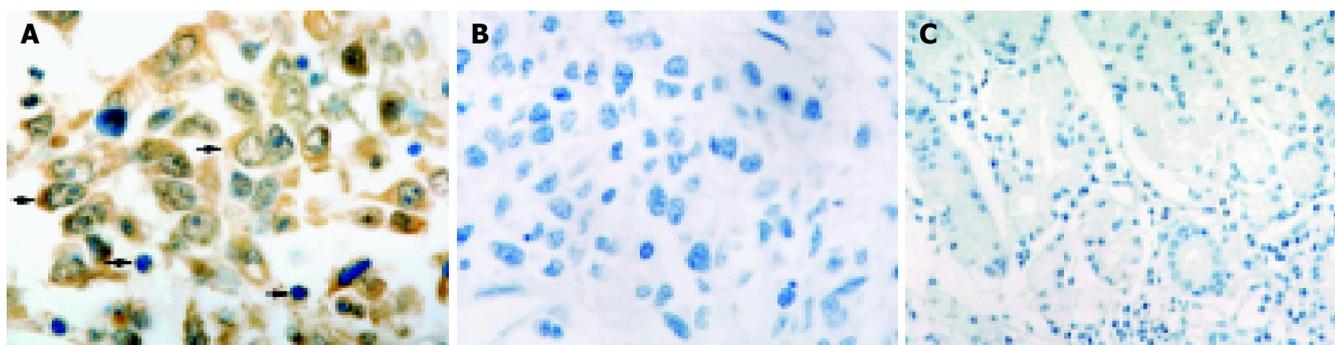


Figure 1 iNOS staining. **A:** iNOS staining detected in cytoplasm of tumor cells (thick arrow) and in inflammatory cells (thin arrows); **B:** Negative control; **C:** No

positive immunohistostaining for iNOS in normal gastric tissue sections. Magnification **A** and **B** $\times 1\,000$; **C** $\times 400$.

not only expressed in gastric adenocarcinoma cells, but also in inflammatory cells (Figure 1A). In contrast, in the normal gastric tissues, iNOS expression was barely detectable in epithelial and stromal cells (Figure 1C). Intermediate or high-level staining for iNOS was observed in 62.12% (41/66) of these specimens. The expression of iNOS was up-regulated with the advancement of the disease process. Gastric adenocarcinoma tissues, larger than 5 cm, expressed iNOS significantly stronger than smaller ones ($P = 0.014$). Intermediate or high iNOS expression was noted in 68% (28/41) of pT3/pT4 gastric cancers, but in only 32% (13/41) of pT1/pT2 gastric cancers ($P = 0.039$). Apart from that, TNM stages of the tumor affected iNOS expression, 75.61% (31/41) of stages III and IV tumors expressed an intermediate or high iNOS, whereas 24.39% (10/41) of stages I and II tumors showed an intermediate or high iNOS expression ($P = 0.048$). iNOS immunostaining rate in the patients with lymph node metastasis was higher than that in those without lymph node metastasis ($P = 0.024$). The other clinicopathologic characteristics, such as age, gender, degree of differentiation, Lauren's classification and Borrmann classification did not show any significant relation to the expression of iNOS (Table 1).

Nitrotyrosine

Cytoplasm and nuclei of gastric adenocarcinoma cells were positive for NT staining, which was also detected in stromal cells around the tumor cells (Figure 2A). No NT staining was observed in the normal gastric tissues (Figure 2C).

Thirty-eight of these sixty-six cases (57.58%) under study showed an intermediate or high expression for NT. The correlation between iNOS and NT expression within the same tumor specimen ($P = 0.25$) was found by McNemar's test, suggesting that there might be an agreement between the two markers. All negative or low iNOS expression specimens were also negative or low for NT by immunohistochemistry. Only 3 of 41 intermediate or high iNOS expression specimens did not express NT. NT expression was related with the clinicopathologic characteristics of gastric adenocarcinoma. So was iNOS expression.

Apoptotic index (AI)

Almost all the positively stained cells and bodies were considered to be apoptotic cells corresponding morphologically to the standard criteria of apoptotic cells as defined previously. Nonspecific staining in necrotic foci showed a faint and diffuse staining and could be distinguished from the apoptotic nuclei by simple morphological examination (Figure 3A). The incidence of AI varied from 0% to 8.9%. The mean AI was 2.69% (s , 2.61) with a median of 2.09%. The mean AI in intermediate or high NT expression group was 4.09% (s , 2.57%), while it was significantly higher than the mean AI of 0.79% (s , 0.95%) observed in negative or low NT expression group by using the Mann-Whitney U test ($P < 0.001$).

Correlations between AI and NT expressions

To examine whether NT expression corresponded to AI or

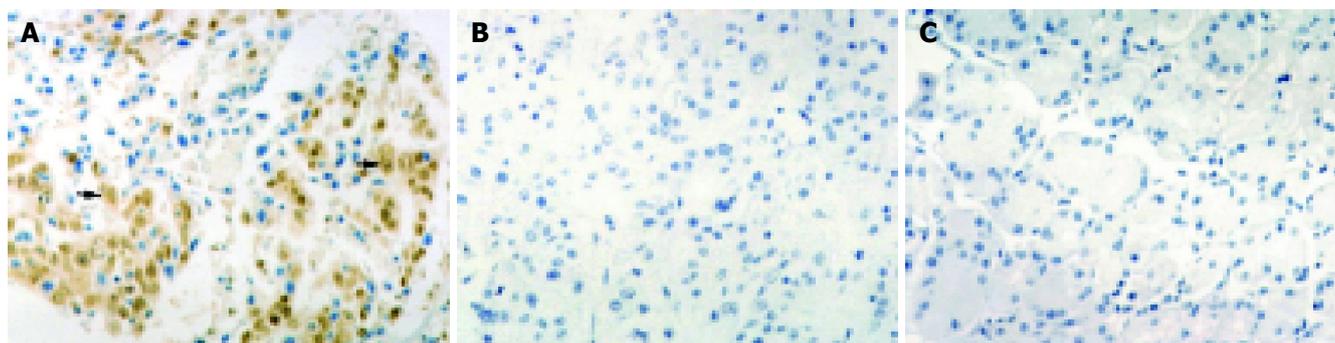


Figure 2 NT staining. **A:** NT staining detected in cytoplasm and nuclei of tumor cells (arrow); **B:** Negative control; **C:** No positive immunoreactivity in

normal gastric tissue sections. Magnification $\times 400$.

Table 1 iNOS expression and clinicopathologic features in gastric adenocarcinoma

Variable	Definition	Intermediate or high n (%)	Negative or low n (%)	P
Age (yr)	≤59 yr	22 (53.66)	14 (56)	0.853
	≥60 yr	19 (46.34)	11 (44)	
Gender	Female	20 (48.78)	10 (40)	0.487
	Male	21 (51.22)	15 (60)	
Tumor size	<5 cm	12 (29.27)	15 (60)	0.014
	≥5 cm	29 (70.73)	10 (40)	
Borrmann classification	0, 1, 2	20 (48.78)	12 (48)	0.951
	3, 4	21 (51.22)	13 (52)	
Depth of invasion	pT1, 2	11 (26.83)	13 (52)	0.039
	pT3, 4	30 (73.17)	12 (48)	
Lymph node metastasis	Negative	13 (31.71)	15 (60)	0.024
	Positive	28 (68.29)	10 (40)	
Lauren's classification	Intestinal	15 (36.59)	12 (48)	0.360
	Diffuse	26 (63.41)	13 (52)	
Degree of differentiation	Well and moderate	19 (46.34)	11 (44)	0.853
	Poor	22 (53.66)	14 (56)	
TNM staging	III, IV	10 (24.39)	12 (48)	0.048
	I, II	31 (75.61)	13 (52)	

not, the specimens were further stratified into different groups according to a comprehensive scoring system as described above. As AI was considered as a continuous variable, regression analysis with the Spearman rank correlation coefficient showed that AI had a significant direct correlation with NT expression scores ($r = 0.718$, $P < 0.001$) (Figure 4).

Survival analysis

After more than 5 years of follow up, 18 patients were alive at the time of this analysis, 4 patients died unrelated to gastric cancer, and the remaining 44 patients died of recurrent diseases. As a result, for the 66 subjects under study, the 5-year survival rate was 30.56%. The 5-year survival rate of the 41 patients in the intermediate or high iNOS expression group was 19.96%, which was significantly lower than 47.14%, the 5-year survival rate of the 25 patients in the negative or low iNOS expression group ($P = 0.001$). The 5-year survival curves in terms of iNOS expression are shown in Figure 5. Univariate analysis demonstrated that iNOS expression, depth of invasion, lymph node metastasis, TNM staging, tumor size, Lauren's classification, Borrmann classification, and degree of differentiation were significant prognostic variables for survival ($P = 0.001$, $P < 0.001$, $P < 0.001$, $P = 0.043$, $P = 0.032$, $P = 0.026$, and $P = 0.023$, respectively; Table 2), while age and gender

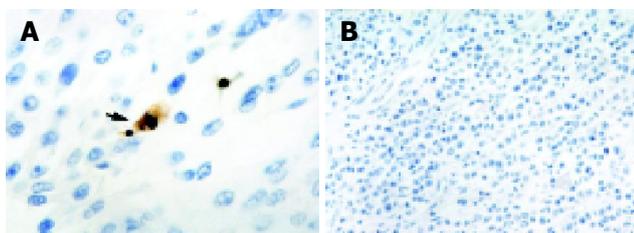


Figure 3 Apoptotic cells and bodies staining. **A:** An apoptotic body characterized by a pyknotic nucleus surrounded by shrunken cytoplasm and separated from the surrounding cells by a halo (arrow); **B:** Negative control. Magnification A $\times 1000$; B $\times 400$.

Table 2 Univariate and multivariate regression analyses of prognostic indicators for long-term survival

Factor	Categories	Univariate	Multivariate	
		P (Log-rank test)	P (Wald test)	RR (95% CI)
iNOS expression	≥5 scores	0.001	0.012	2.69 (1.5–4.8)
	≤4 scores			
Depth of invasion	pT3, 4	<0.001	0.020	4.11 (1.5–4.7)
	pT1, 2			
Lymph node metastasis	Positive	<0.001	<0.001	5.77 (2.0–6.6)
	Negative			
TNM staging	III, IV	<0.001	0.007	3.94 (1.4–7.2)
	I, II			
Tumor size	≥5 cm	0.043	NS	
	<5 cm			
Lauren's classification	Diffuse	0.032	NS	
	Intestinal			
Borrmann classification	3, 4	0.026	NS	
	0, 1, 2			
Degree of differentiation	Poor	0.023	NS	
	Well and moderate			
Age (yr)	≤59	0.178		
	≥60			
Gender	Female	0.201		
	Male			

≥5 scores: intermediate or high iNOS expression; ≤4 scores: negative or low iNOS expression; RR: relative risk; CI: confidence interval; NS, not significant.

were not. However, Cox's multivariate analysis revealed that iNOS expression, lymph node metastasis, TNM staging, and depth of invasion were significant independent prognostic factors ($P = 0.012$, $P < 0.001$, $P = 0.007$, and $P = 0.020$, respectively; Table 2). The group with an intermediate or high iNOS expression and a high AI showed a poorer prognosis.

DISCUSSION

This study was undertaken to investigate the expression of iNOS and NT in gastric adenocarcinoma. Because NO is known to influence apoptosis, we also studied the extent of apoptosis in gastric adenocarcinoma.

Our results showed that normal gastric tissues did not express iNOS or NT, while the rate of iNOS expression in stages III and IV gastric adenocarcinoma was higher than that in stages I and II, revealing that expression of iNOS of gastric adenocarcinoma increased with the staging of cancer, which is in accordance with a previous study^[17]. We also found that the expression of iNOS of gastric adenocarcinoma in patients with lymph node metastasis was higher than that in those without metastasis, suggesting that the significant increase of iNOS expression in gastric adenocarcinoma might contribute to lymph node metastasis of gastric cancer cells. Similarly, there were strong differences in iNOS immunostaining among the cases with different sizes of tumors or depths of invasion. But the degree of differentiation, Lauren's classification, and Borrmann classification did not show any significant relation to the expression of iNOS. These results suggest that there is an up-regulation of iNOS positivity along with the biological aggressiveness of gastric cancer. The higher the iNOS expression of gastric cancer was, the more advanced was

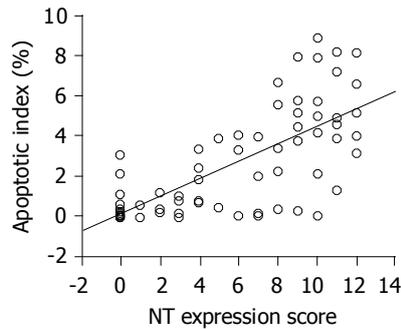


Figure 4 Significantly direct correlation between AI and NT expression observed by using the Spearman rank correlation coefficient ($r = 0.718$; $P < 0.001$).

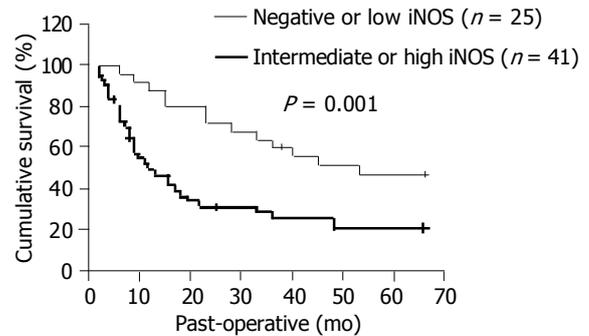


Figure 5 Survival of patients relative to iNOS status. Solid line: negative or low iNOS expression; dotted line: intermediate or high iNOS expression.

the gastric cancer, and the worse was the prognosis, suggesting the tumor-promoting role of iNOS, also shown in Koh's^[18] and Doi's studies^[19].

In our previous studies, frequent iNOS expression was also observed in many types of tumors such as colorectal, breast and bladder carcinomas and played an important role in tumor progression^[20-22]. There is mounting evidence that NO acts as a carcinogen^[23-26]. It was reported that elevated NO production could enhance the growth of some tumors through the suppression of antitumor immune responses^[27,28]. Nitric oxide as a mediator of tumor vascularization might enhance tumor growth^[29]. These observations suggest that NO may have some role in the growth, progression, maintenance, and/or metastasis of tumors.

Although this result is appealing, it has not been unanimously accepted. Some studies also revealed that iNOS activity was inversely correlated with tumor promotion, and some tumor cells lost their tumorigenic and metastatic abilities as a result of NO-mediated tumor-cell apoptosis^[30,31]. Our study showed that there was a gradual increase in AI with a coexistent up-regulation of total iNOS in gastric cancer. The result suggested that NO produced by iNOS in gastric cancer and stromal cells could be an additional factor participating in the enhancement of gastric cancer cell apoptosis. Apoptosis mediated by iNOS may be one of the body's defense mechanisms to restrict the growth of gastric adenocarcinoma, suggesting the tumor-suppressing role of iNOS.

It is thus obviously controversial as to the roles of iNOS in gastric clinicopathology as shown in our previous studies. However, the genetic constitution of cells may determine NO sensitivity or resistance. Lala *et al.*^[30] proposed that the genetic composition of tumor cells and the concentrations of NO in tumor-cell microenvironments were the main determinants of the role of NO. During clonal evolution of tumors in the presence of high NO concentrations, NO-sensitive cells might be deleted and NO-resistant cells might emerge owing to mutational events mediated by NO^[30].

High AI of tumor cells *in vivo*, taken in isolation, might not necessarily indicate a favorable outcome. Our study showed that gastric carcinomas with intermediate or high iNOS and NT expressions had a higher AI and much poorer prognosis in the 5-year survival analysis. iNOS expression might have a higher predictive value (RR = 2.69) for determining the recurrence of gastric carcinoma patients

following a curative resection. This indicates that in the process of gastric adenocarcinoma development the role of iNOS in enhancing tumor growth, invasiveness and metastasis may be stronger than that in promoting tumor cell apoptosis.

In conclusion, gastric adenocarcinomas can express iNOS and NT, and there is a correlation between NT and tumor apoptosis. Although tumors with a high AI may have a better prognosis, as noted by Ikeguchi^[32] and Sugamura^[33], tumor expression of iNOS and NT with a high AI is associated with a poor survival of gastric adenocarcinoma patients, which may result from the co-effects of a double-edged role of NO generated by iNOS. Further studies are necessary to investigate the precise mechanisms of this process.

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