



Differences in oxidative stress dependence between gastric adenocarcinoma subtypes

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CONCLUSION: The iNOS-oxidative pathway may play an important role in TC, but moderately in PolyC and DC. DNA oxidation and protein nitration occur in the three subtypes. Based on the significant differences of NTYR levels, TC and PolyC appear as two distinct subtypes.

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Key words: Gastric neoplasms; Nitric oxide synthase; *H pylori*; Nitrotyrosine; 8-hydroxydeoxyguanosine; Oxidative stress

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Abstract

AIM: To investigate the extent of oxidative stress in pre-neoplastic and neoplastic gastric mucosa in relation to their pathological criteria and histological subtypes.

METHODS: A total of 104 gastric adenocarcinomas from 98 patients (88 infiltrative and 16 intraepithelial tumors) were assessed immunohistochemically for expression of iNOS and occurrence of nitrotyrosine (NTYR)-containing proteins and 8-hydroxy-2'-deoxyguanosine (8-OH-dG)-containing DNA, as markers of NO production and damages to protein and DNA.

RESULTS: Tumor cells staining for iNOS, NTYR and 8-OH-dG were detected in 41%, 62% and 50% of infiltrative carcinoma, respectively. The three markers were shown for the first time in intraepithelial carcinoma. The expression of iNOS was significantly more frequent in tubular carcinoma (TC) compared to diffuse carcinoma (DC) (54% vs 18%; $P=0.008$) or in polymorphous carcinoma (PolyC) (54% vs 21%; $P=0.04$). NTYR staining was obviously more often found in TC than that in PolyC (72% vs 30%; $P=0.03$). There was a tendency towards a higher rate of iNOS staining when distant metastasis (pM) was present. In infiltrative TC, the presence of oxidative stress markers was not significantly correlated with histological grade, density of inflammation, the depth of infiltration (pT), lymph nodes dissemination (pN) and pathological stages (pTNM).

INTRODUCTION

Gastric carcinoma is one of the most common neoplasms in the world, and most are adenocarcinomas. According to the Lauren classification^[5], two epidemiologically distinct types^[1-4] exist. Intestinal-type carcinoma (tubular carcinoma, TC) is associated with *H pylori* infection, chronic gastritis, atrophy, intestinal metaplasia, and dysplasia that evolve as a multi-step process into this type of cancer. Diffuse-type carcinoma (DC), which occurs more often in younger patients, is also associated with *H pylori* infection but not with atrophy and intestinal metaplasia, and is associated with a worse prognosis. Mixed polymorphous carcinoma (PolyC) encompasses tumors showing both glandular and diffuse components.

Inducible nitric oxide synthase (iNOS), which is synthesized *de novo* in response to mucosal inflammation in *H pylori*-associated gastritis^[6-10], produces large amounts of nitric oxide (NO) over long periods of time. Inflammatory cells also produce reactive oxygen species such as superoxide anion which is subsequently converted into hydrogen peroxide and hydroxyl radical^[11]. The reaction of NO with oxygen and superoxide produces nitrogen oxides and peroxynitrite, respectively. Overproduction of such reactive species leads to oxidation, nitration and nitrosation, resulting in protein, DNA and tissue damage. Nitrotyrosine (NTYR), formed by nitration of tyrosine

residues in proteins, can be used as a marker of nitrate damage *in vivo*^[12,13], and we^[6,14] and others^[8,9] have shown that its levels are elevated in *H pylori*-infected gastric mucosa. 8-Hydroxy-2'-deoxyguanosine (8-OH-dG) is one of the most commonly used markers of oxidative DNA damage^[15]. We^[6] and others^[16-18] have demonstrated increased levels of 8-OH-dG in *H pylori*-infected gastric mucosa. Oxidative stress has been suggested to play a major role in carcinogenesis^[19,20], but the mechanisms involved still remain unclear. Expression of iNOS in various carcinomas has been reported^[21-25], but the studies of stomach cancer were performed with small numbers of cases^[26-29] and they considered only limited pathological data^[30-34]. The presence of NTYR in some carcinomas was previously reported^[35-37], but no data, to our knowledge, on gastric cancer are available. Elevated levels of 8-OH-dG were demonstrated in some carcinomas^[35,36,38,39], but in only two studies on gastric carcinoma^[30,40].

In this study, we therefore aimed to investigate the extent of oxidative stress in pre-neoplastic and neoplastic gastric mucosa in relation to the cell compartment concerned and pathological criteria, especially the cancer subtype, and to identify subsets of cancers with a distinctive profile for the oxidative stress markers.

MATERIALS AND METHODS

Subjects

Our retrospective study included 98 consecutive French patients with gastric adenocarcinoma and 26 non-tumorous patients. All subjects were endoscopically biopsied for diagnostic purposes, between 1989 and 2000. Demographic information and medical history were obtained in all patients from the hospital records. The 104 carcinomas from the 98 patients were either infiltrative ($n=88$; 58 men and 30 women; mean age 70 years, range 30-98 years), or intraepithelial (*i.e.*, high-grade dysplasia; $n=16$), of which 6 had an adjacent infiltrative tumor (4 men and 2 women; mean age 64 years, range 54 to 75 years) and 10 had no coexisting infiltrative cancer (6 men and 4 women; mean age 70 years, range 44 to 85 years). Of the 88 infiltrative cancers, 57 were classified as tubular/intestinal (TC) (38 men and 19 women; mean age 64 years, range 44 to 76 years), 17 as diffuse (DC) (12 men and 5 women; mean age 68 years, range 49 to 92 years), and 14 as polymorphous (PolyC) (8 men and 6 women; mean age 70 years, range 57 - 98 years). *H pylori* was detected in 21% (19/92) of patients with carcinoma, including 10% (1/10) with intraepithelial carcinoma, 19% (11/54) with TC, 33% (4/12) with PolyC, and 19% (3/16) with DC.

Diagnostic biopsy samples with precursor lesions ($n=94$) were obtained from 57 of the 98 patients with gastric adenocarcinoma and from 26 randomly selected non-tumorous subjects. They were classified as low-grade dysplasia ($n=19$), of which 12 had no coexisting carcinoma (9 men and 3 women; mean age 68 years, range 43 to 88 years), and 7 were associated with invasive carcinoma, either adjacent ($n=3$) or distant ($n=4$). Of the 75 atrophic gastritis lesions, all but seven in association with intestinal metaplasia, 51 cases were in patients with intraepithelial or infiltrative cancer, either adjacent ($n=36$) or distant ($n=15$),

and 24 cases had no coexisting cancer. Among the latter 24 cases, 10 also showed low-grade dysplasia, and of the remaining 14, all but one had intestinal metaplasia (11 men and 3 women; mean age 64 years, range 24 to 90 years). *H pylori* infection was found in 32% (6/19) of patients with low-grade dysplasia and in 33% (25/75) of those with atrophic gastritis.

Diagnostic biopsy samples without precursor lesions ($n=48$) were obtained from non-tumorous mucosa of 38 of the 98 patients with gastric carcinoma and from 5 of the 12 non-tumorous patients with isolated low-grade dysplasia. The 43 biopsies from 38 gastric cancer patients were taken from mucosa surrounding ($n=36$) or distant from ($n=7$) the tumor. Mucosa was classified as normal ($n=8$, including 3 from non-cancerous subjects), or non-metaplastic gastritis ($n=40$, including 2 from non-cancerous subjects). *H pylori* infection was found in 23% (10/43) of subjects.

Histopathology and assessment of *H pylori* infection

We examined Bouin-fixed, paraffin-embedded archival biopsies from gastric mucosa. Sections (5- μ m thick) were stained using hematoxylin-eosin-saffron for histological diagnosis and with a modified Giemsa stain for identification of *H pylori*. Additional serial sections were used for immunohistochemistry. *H pylori* infection was graded as absent, mild, moderate or severe^[41]. In addition, a rapid urease test (CLO test) was performed in one third of cases.

The histological criteria were based on previous descriptions. According to the Lauren's classification^[5], infiltrative gastric cancers were classified as intestinal-type or tubular (TC), diffuse-type (DC), or mixed polymorphous (PolyC), the latter encompassing all tumors showing 5% or more glandular as well as 5% or more diffuse components^[42]. The DC consisted of dispersed cells or of minute cellular aggregates. Tumor grade was classified into three categories (well, moderately, or poorly differentiated). Dysplasia was categorized as low-grade or high-grade based on the severity of neoplastic features^[43,44]. High-grade dysplasia, as an intra-epithelial non-invasive neoplasia, was grouped with intraepithelial carcinoma according to the consensus terminology^[43,44].

Gastritis was classified and intestinal metaplasia (IM) was noted according to the guidelines of the updated Sydney system^[41]. In view of the small number of cases with severe gastritis ($n=4$), they were combined with the moderate gastritis cases ($n=36$) to form one group.

Pathologic stage (pTNM)

The pathologic stage (pTNM) was determined according to the newly revised classification of the American Joint Committee on Cancer (AJCC) and the International Union Against Cancer [UICC]^[45] for 53 patients who benefited from a curative resection either by gastrectomy ($n=42$), or endoscopy ($n=11$). Staging laparoscopy was performed for four subjects because of peritoneal metastasis. No surgery was performed for some cases because of either distant metastasis ($n=8$) or associated neoplasm ($n=5$). No staging data were available for 28 patients who underwent surgery in another centre.

We defined early gastric carcinoma as a tumor restricted to the mucosa or to the mucosa and submucosa (pT1) regardless of nodal status.

Immunohistochemistry

Bouin-fixed paraffin-embedded sections (4- μ m thick) were routinely deparaffinized in xylene and rehydrated through a series of ethanol solutions. The immunohistochemical procedures were performed as previously described^[6,7]. The avidin-biotin complex method was applied to the serial sections mounted on poly-L-lysine-coated plates. Briefly, after deparaffinization and inactivation of endogenous peroxidases, non-specific immunolabelling was blocked and overnight incubation was carried out with the primary antibody at 4°C. Primary antibodies used were as follows: (1) Polyclonal antibodies raised in the rabbit against iNOS (dilution 1:100), purified from rat livers with acute liver necrosis induced by iv administration of *Propionibacterium acnes* and lipopolysaccharide provided by one of us (HO)^[7,46,47]; (2) mouse monoclonal antinitrotyrosine antibody (dilution 1:250) supplied by Upstate Biotechnology (Lake Placid, NY); and (3) purified mouse monoclonal antibody against 8-hydroxy-2'-deoxyguanosine (N45.1, 3 μ g/mL)^[48,49]. A secondary biotinyl-labeled goat antirabbit or horse antimouse IgG serum and avidin-biotinylated horseradish peroxidase macromolecular complex (ABC Kit, Elite Vectastain, Vector Lab, Burlingame, CA) were sequentially used as previously described^[7]. Colorimetric reaction was performed with 3,3'-diaminobenzidine tetrachloride and counterstaining was carried out with hematoxylin. The degree of immunopositivity was expressed according to the percentage of positive cells as follows: <2%, negative (score 0); 2-10%, slightly positive (score 1); 10-50%, positive (score 2); and >50%, strongly positive (score 3). In addition, the intensity of staining was rated from weak to intense. Because the scoring system and the rating of intensities (data not shown) brought a great complexity that was useless for interpretation of the study, the results were simplified by collapsing in positive or negative groups. Sections in which the primary antibody was omitted or those treated with normal mouse serum instead of the primary antibody showed no staining. Furthermore, immunostaining of iNOS and NTYR was completely removed by absorption tests with iNOS purified from rat liver or with free NTYR (data not shown).

Statistical analysis

Statistical analysis was performed using the Fisher and Chi-square tests for contingency tables. $P < 0.05$ was considered statistically significant.

RESULTS

Correlations of the three markers with clinicopathological data and with pTNM pathologic classification and stage grouping are shown in Table 1, Table 2 and Table 3, respectively. The co-occurrence of staining for all the three markers is shown in Table 4.

iNOS immunostaining

Immunostaining for iNOS was located in the cytoplasm of

tumor cells (Figure 1A). It was more frequently observed in men than in women (Table 1), but the correlation was borderline ($P = 0.06$). Of the 85 infiltrative carcinoma cases, 41% showed weak to strong immunoreactivity. The staining was significantly correlated with infiltrative cancer histological subtype (Table 1), being markedly higher in TC than in DC ($P = 0.008$) and than in PolyC ($P = 0.04$). Comparing TC with DC + PolyC, the correlation remained significant ($P = 0.04$). However, in infiltrative TC, the iNOS immunoreactivity was not significantly correlated with either the depth of invasion (pT) or lymph node dissemination (pN) (Table 2). A higher rate of iNOS staining was observed when distant metastasis (pM) was present (Table 2), but this correlation did not reach statistical significance, presumably because of the small number of patients. The importance of iNOS in TC metastasis was reflected by its higher staining rate in stage IV than that in other stages (Table 3), although the difference was not statistically significant. The frequency of iNOS in intraepithelial carcinoma (not coexisting with infiltrative cancer - pTis) was lower than that in infiltrative TC (Table 1), but this was not statistically significant ($p = 0.10$). Similarly, staining was less common in early cancer than in other stages (Table 3), but not statistically significant ($p = 0.48$). The small number of DC and PolyC samples did not permit the study of any correlation of iNOS with pTNM and stage grouping criteria. There was no significant association of iNOS expression with histological grade for infiltrative TC [well ($n = 32$), moderately ($n = 3$) and poorly ($n = 9$) differentiated; iNOS positivity 56%, 46%, and 56%, respectively], *H pylori* infection (Table 1) or density of inflammation [weak ($n = 36$) vs strong ($n = 56$); iNOS positivity 42% and 38%]. In addition, iNOS expression was not found to depend on the cancer site (Table 1), such as body, cardia, gastric stump, and antrum with angulus and pylorus.

Non-metaplastic mucosa in the same patient, either adjacent to or distant from the tumor, showed lower epithelial reactivity than atrophic metaplastic gastritis mucosa from cancer and non-cancer patients (Table 1), but the tendency was not statistically significant ($P = 0.13$). A similar observation was found when comparing non-metaplastic mucosa and low-grade dysplasia.

Moreover, we detected iNOS expression in 44% (39/89) of infiltrating lymphocytes, regardless of their density and the carcinoma subtype.

NTYR immunostaining

NTYR immunostaining was also observed in the cytoplasm of tumor cells (Figure 1B). Of the 65 infiltrative carcinoma cases, 62% showed weak to strong immunoreactivity. The staining was significantly correlated with the histological subtype of infiltrative cancer, being significantly higher in TC than in PolyC ($P = 0.03$) (Table 1). However, when comparing TC with DC or PolyC + DC, the correlation was not statistically significant. In infiltrative TC, NTYR was not significantly correlated with the depth of invasion (pT), lymph node dissemination (pN) or distant metastasis (pM) (Table 2), nor was any relationship observed with pathological stages in TC (Table 3). The level of NTYR in intraepithelial carcinoma (not coexisting with infiltrative

Table 1 Correlation of staining with clinicopathologic data

Clinicopathologic features ^a	iNOS ^b			NTYR ^b			8OH-dG ^b		
	No. of cases			No. of cases			No. of cases		
	Total	positive (%)		Total	positive (%)		Total	positive (%)	
Infiltrative cancer subtypes	85	35 (41)		65	40 (62)		82	41 (50)	
TC	54	29 ¹ (54 ¹)		43	31 ² (72 ²)		54	28 (52)	
DC	17	3 ¹ (18 ¹)		12	6 (50)		14	7 (50)	
PolyC	14	3 ¹ (21 ¹)		10	3 ² (30 ²)		14	6 (43)	
Intraepithelial cancer									
Peri-infiltrative cancer	5	3 (60)		6	4 (67)		6	4 (67)	
Without infiltrative cancer	7	1 (14)		7	6 (86)		10	5 (50)	
Topography of infiltrative cancer									
Cardia	10	4 (40)		7	3 (43)		11	5 (45)	
Gastric stump	12	6 (50)		9	5 (56)		8	3 (38)	
Antrum with angulus+pylorus	45	18 (40)		35	22 (63)		41	22 (54)	
Body	14	7 (50)		11	9 (82)		15	11 (73)	
Peritumorous mucosa									
Adjacent: N + no IM G	15	5 (33)		23	9 (39)		30	10 (33)	
Adjacent: atrophy IM	23	14 (61)		21	10 (48)		26	18 (69)	
At distance: N + no IM G	3	1 (33)		6	1 (17)		5	2 (40)	
At distance: atrophy IM	7	4 (57)		7	5 (71)		13	9 (69)	
Adjacent+distant: N + no IM G	18	6 (33)		29	10 (34)		35	12 ³ (34 ³)	
Adjacent+distant: atrophy IM	30	18 (60)		28	15 (54)		39	27 (69)	
Precancerous lesions									
Low grade dysplasia	13	9 (69)		14	11 (79)		17	14 ³ (82 ³)	
Atrophy IM without cancer	10	8 (80)		7	7 (100)		11	6 (55)	
Atrophy IM with + without cancer	40	26 (65)		35	22 (63)		50	33 ³ (66 ³)	
<i>H. pylori</i> infection									
Negative	68	27 (40)		54	35 (65)		77	41 (53)	
Positive	18	7 (39)		13	7 (54)		16	9 (56)	
Sex									
Male	60	28 (47)		50	32 (64)		66	36 (55)	
Female	30	8 (27)		29	20 (69)		35	18 (51)	

(a) TC: Intestinal or tubular carcinoma; DC: Diffuse carcinoma; PolyC: Mixed polymorphous carcinoma; N: Normal; IM: Intestinal metaplasia; G: Gastritis. (b) iNOS: Inducible nitric oxide synthase; NTYR: Nitrotyrosine; 8OH-dG: 8-hydroxy-2'-deoxyguanosine. ¹TC vs DC $P = 0.008$; TC vs PolyC, $P = 0.04$; TC vs DC+PolyC, $P = 0.04$; ²TC vs PolyC, $P = 0.03$; ³non-metaplastic mucosa vs atrophic metaplastic mucosa low-grade dysplasia, $P = 0.005$.

cancer - pTis) was not significantly different from that in infiltrative TC (Table 1). Similarly, the level of NTYR in early gastric cancer was not different from that of other stages (Table 3). There was no significant association between NTYR expression and sex, cancer site (Table 1), histological grade in TC [well ($n = 28$), moderately ($n = 10$) and poorly ($n = 5$) differentiated; NTYR positivity 75%, 60%, and 80%, respectively], *H. pylori* infection (Table 1) or density of inflammation [weak ($n = 27$) vs strong ($n = 45$); NTYR staining 63% and 64%].

Non-metaplastic mucosa in the same patient, either adjacent to or distant from the tumor, showed lower epithelial staining for NTYR than atrophic metaplastic mucosa from cancer and non-cancer patients (Table 1), but the tendency was not statistically significant ($P = 0.18$). A similar observation was found when comparing non-metaplastic mucosa and low-grade dysplasia (Figure 1F).

In addition, immunostaining of NTYR was detected in the cytosol of inflammatory cells at a higher rate in intraepithelial carcinoma ($n = 7$, 71%) than that in inflammatory cells in all subtypes of infiltrative cancer ($n = 67$, mean 33%).

8-OH-dG immunostaining

Staining was predominantly confined to the nuclei of tumor cells (Figures 1C and 1D). Because the lymphoid follicles were strongly reactive, they could be used as internal positive controls (Figure 1C). Of the 82 cases of infiltrative cancer, 50% showed strong immunoreactivity.

In contrast with iNOS and NTYR, no significant difference in the percentage of positive staining was observed among different histological subtypes of infiltrative cancer (Table 1). The presence of 8-OH-dG was not significantly associated with sex, cancer site (Table 1), histological grade in TC [well ($n = 32$), moderately ($n = 12$) and poorly ($n = 10$) differentiated; positivity 47%, 67%, and 50%, respectively], *H. pylori* infection (Table 1), depth of invasion (pT), lymph node dissemination (pN), distant metastasis (pM) (Table 2), or pathological stage in TC (Table 3). The levels of 8-OH-dG in intraepithelial carcinoma (not coexisting with infiltrative cancer - pTis) (Figure 1E) and in infiltrative TC were not significantly different (Table 1). Immunostaining of 8-OH-dG in early cancer was similar to that in all other stages (Table 3).

Staining of 8-OH-dG in non-metaplastic mucosa from cancer patients was significantly lower than that in atrophic metaplastic mucosa from cancer and non-cancer patients and than that in low-grade dysplasia ($P = 0.005$) (Table 1). Immunoreactivity of 8-OH-dG was significantly higher in the nuclei of inflammatory cells in cases with mild inflammatory stroma (38%, 12/32) than that with dense inflammatory stroma (24%, 12/49).

Co-staining for iNOS, NTYR and 8-OH-dG

The expressions of all three markers were determined simultaneously in 62 cases (Table 4). The absence of all three markers was more frequent in PolyC (50%), but less frequent in TC (12%) and DC (16%). When iNOS was the

Table 2 Correlation of staining with pTNM pathological classification^a

Pathological classification ^b	iNOS ^c			NTYR ^c			8OH-dG ^c		
	No. of cases			No. of cases			No. of cases		
	Total positive (%)			Total positive (%)			Total positive (%)		
pTis	7	1	(14)	7	6	(86)	10	5	(50)
pT1	18	7	(39)	16	12	(75)	16	10	(63)
TC	13	7	(54)	12	9	(75)	12	8	(67)
DC	3	0	(0)	2	2	(100)	2	2	(100)
PolyC	2	0	(0)	2	1	(50)	2	0	(0)
pT2	7	3	(43)	8	6	(75)	8	4	(50)
TC	5	2	(40)	6	5	(83)	6	3	(50)
DC	0	0	(0)	0	0	(0)	0	0	(0)
PolyC	2	1	(50)	2	1	(50)	2	1	(50)
pT3/T4	22	9	(41)	15	8	(53)	21	10	(48)
TC	12	7	(58)	8	6	(75)	12	6	(50)
DC	5	1	(20)	3	1	(33)	4	2	(50)
PolyC	5	1	(20)	4	1	(25)	5	2	(40)
pN0	20	10	(50)	20	14	(70)	20	11	(55)
TC	16	8	(50)	16	13	(81)	16	10	(63)
DC	1	1	(100)	1	0	(0)	1	1	(100)
PolyC	3	1	(33)	3	1	(33)	3	0	(0)
pN+	24	8	(33)	17	9	(53)	22	11	(50)
TC	10	6	(60)	7	4	(57)	10	4	(40)
DC	7	0	(0)	5	3	(60)	5	3	(60)
PolyC	7	2	(29)	5	2	(40)	7	4	(57)
pM0	38	14	(37)	3	21	(64)	36	20	(56)
TC	25	12	(48)	2	17	(77)	25	15	(60)
DC	6	1	(17)	4	2	(50)	4	4	(100)
PolyC	7	1	(14)	7	2	(29)	7	1	(14)
pM1	16	9	(56)	12	8	(67)	16	8	(50)
TC	9	7	(78)	8	6	(75)	9	5	(56)
DC	4	0	(0)	3	2	(67)	4	1	(25)
PolyC	3	2	(67)	1	0	(0)	3	2	(67)

(a) Determination according to the newly revised classification of the AJCC and UICC. (b) TC: Intestinal or tubular carcinoma; DC: Diffuse carcinoma; PolyC: Mixed polymorphous carcinoma. (c) iNOS: Inducible nitric oxide synthase; NTYR: Nitrotyrosine; 8OH-dG: 8-hydroxy-2'-deoxyguanosine

only positive marker found, its frequency was similar in the three infiltrative cancer subtypes (5-10%). When the only positive marker found was either NTYR or 8-OH-dG, its frequency was similar for both of them in the three cancer subtypes, but higher in DC (17%) compared to PolyC (10%) and TC (5-10%). The couple NTYR + 8-OH-dG was the only one found in PolyC and occurred at a similar frequency in the three cancer subtypes (20-25%). It was also the most common one in DC (25%) among the three possible couples. In TC, the two couples iNOS + NTYR and NTYR + 8-OH-dG occurred at the same frequency (20%). The couple iNOS + 8-OH-dG occurred rarely in TC and DC (8% and 2.5%). All three markers were simultaneously observed only in TC (25%).

DISCUSSION

The current study investigated the expression of iNOS, NTYR, and 8-OH-dG, as markers of NO production, nitrative damage of proteins, and oxidative DNA damage respectively, in gastric adenocarcinoma and pre-neoplastic mucosa. The expression of these markers was studied in relation to clinicopathological characteristics, including histological subtype, metastasis and pathological stages.

Table 3 Correlation of staining with stage grouping^a

Stages ^b	iNOS ^c			NTYR ^c			8OH-dG ^c		
	No. of cases			No. of cases			No. of cases		
	Total positive (%)			Total positive (%)			Total positive (%)		
Stage 0	7	1	(14)	7	6	(86)	10	5	(50)
Stage I	22	9	(41)	21	15	(71)	21	12	(57)
TC	16	8	(50)	16	12	(75)	16	10	(63)
DC	3	0	(0)	2	2	(100)	2	2	(100)
PolyC	3	1	(33)	3	1	(33)	3	0	(0)
Stage II+III	13	5	(38)	10	4	(40)	12	5	(42)
TC	8	4	(50)	5	4	(80)	7	3	(43)
DC	2	1	(50)	2	0	(0)	2	2	(100)
PolyC	3	0	(0)	3	0	(0)	3	0	(0)
Stage IV	19	9	(47)	14	10	(71)	18	10	(56)
TC	9	7	(78)	8	6	(75)	9	5	(56)
DC	5	0	(0)	3	2	(67)	4	1	(25)
PolyC	5	2	(40)	3	2	(67)	5	4	(80)
All stages (early cancer excluded)	36	16	(44)	29	17	(59)	35	17	(49)
TC	20	12	(60)	17	13	(76)	20	10	(50)
DC	7	1	(14)	5	2	(40)	6	3	(50)
DC+PolyC	16	4	(25)	12	4	(33)	15	7	(47)
Early cancer (pTis + pT1)	25	8	(32)	23	18	(78)	26	15	(58)
TC	20	8	(40)	19	15	(79)	22	13	(59)
DC	3	0	(0)	2	2	(100)	2	2	(100)
DC+PolyC	5	0	(0)	4	3	(75)	4	2	(50)

(a) Determination according to the newly revised classification of the AJCC and UICC. (b) TC: Intestinal or tubular carcinoma; DC: Diffuse carcinoma; PolyC: Mixed polymorphous carcinoma. (c) iNOS: Inducible nitric oxide synthase; NTYR: Nitrotyrosine; 8OH-dG: 8-hydroxy-2'-deoxyguanosine.

We found, for the first time, a positive correlation of gastric cancer subtype with both iNOS expression and NTYR staining, but not with 8-OH-dG staining. Indeed, the prevalence of iNOS staining in TC (54%) greatly exceeded that in DC (18%) and PolyC (21%), suggesting that iNOS probably plays an important role in TC, but is weakly involved, if at all, in DC and PolyC subtypes. We also found for the first time, nitrated proteins present in all cancer subtypes. Similarly to iNOS expression, the prevalence of NTYR staining in TC (72%) greatly exceeded that in PolyC (30%), but the difference was not significantly different from that in DC (50%). Half of the infiltrative carcinoma cases showed 8-OH-dG staining in cancerous cells, regardless of the subtypes.

The prevalence of iNOS staining we found in gastric infiltrative carcinoma (41%) was very similar to that reported by Feng *et al.*^[32], but was slightly lower than that reported in two other studies^[26,31] that used immunohistochemistry as we did. However, two studies from the same laboratory reported different results. Using RT-PCR, Son *et al* found expression of iNOS in 100%^[27] and 65%^[34] of gastric cancer. Rajnakova *et al*^[50] were not able to detect iNOS staining in 80% of gastric adenocarcinomas (in their first paper), whereas the same authors found it in all cases in a more recent study^[33]. Chang *et al*^[30] reported higher iNOS expression in cancerous tissue than that in adjacent non-cancerous mucosa, for which the pathological status was not reported. Concerning iNOS, histological subtype was not considered in four studies^[27,29,30,34]. Two studies^[26,33] did not

Table 4 Co-occurrence of immunostaining for iNOS, NTYR and 8-OH-dG^a

Markers	No. of samples (%)		
	TC ^b n = 40	DC ^b n = 12	PolyC ^b n = 10
All negative	5 (12)	2 (16)	5 (50)
One positive			
iNOS	2 (5)	1 (8)	1 (10)
NTYR	4 (10)	2 (17)	1 (10)
8-OHdG	2 (5)	2 (17)	1 (10)
Two positive			
iNOS + NTYR	8 (20)	1 (8)	0 (0)
NTYR + 8-OHdG	8 (20)	3 (25)	2 (20)
iNOS + 8-OHdG	1 (2.5)	1 (8)	0 (0)
All positive			
iNOS + NTYR + 8-OHdG	10 (25)	0 (0)	0 (0)

(a) iNOS: Inducible nitric oxide synthase; NTYR: Nitrotyrosine; 8OH-dG: 8-hydroxy-2'-deoxyguanosine. (b) TC: Intestinal or tubular carcinoma; DC: Diffuse carcinoma; PolyC: Mixed polymorphous carcinoma.

report a difference in relation to Lauren's classification, but one of them included only 19 cases (53% positive)^[26] and all cases were stained in the other one^[33]. We^[6] and others^[8,9] have previously shown the occurrence of NTYR-containing proteins in *H pylori*-associated gastritis preceding TC, but no data are available on gastric cancer. The marker of oxidative DNA damage, 8-OH-dG, has been reported in only two studies^[30,40]. Our findings can not be compared with these published data because of a difference in methodologies. Lee *et al*^[40] and Chang *et al*^[30] found 8-OH-dG in DNA extracted from cancer tissue using HPLC-electrochemical detection. This technique does not allow localization of the cellular compartment concerned (tumor cells and/or inflammatory cells), unlike our immunohistochemical method. This is particularly important because of the strong reactivity of 8-OH-dG in lymphoid follicles. Our results also supported the previous observations concerning the absence of an association of iNOS with the degree of differentiation^[31-33,50]. In addition, we found an absence of relationship of NTYR and 8-OH-dG with the degree of differentiation.

In contrast with previous reports which studied relationships with respect to only some pTNM categories (*e.g.*, pM often missing), we investigated the presence of the three markers as a function of all components of pTNM and complete staging. We found a tendency towards higher levels of iNOS in TC when distant metastasis (pM) was present, suggesting that high levels of iNOS oxidative stress may stimulate tumor progression^[51]. However, the levels of NTYR and 8-OH-dG were not related to the presence of distant metastasis. In agreement with previous findings^[31], we found no correlation between iNOS staining and depth of invasion (pT). One study^[32] showed increased staining of iNOS with depth of invasion, but without statistical significance. Similarly, in our study, the levels of NTYR and 8-OH-dG were not related to the depth of invasion. To our best of knowledge, this is the first study to investigate the three markers in gastric intraepithelial (pTis) and early gastric cancers. There is a tendency for a lower level of iNOS in

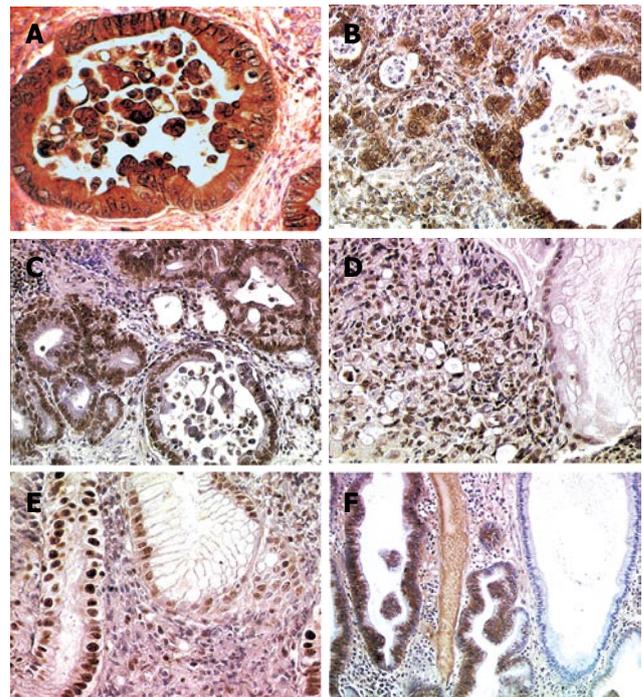


Figure 1 Immunohistochemical staining in human gastric adenocarcinoma and precancerous mucosa. **A:** Intense cytoplasmic immunostaining of iNOS in tumor cells of tubular infiltrative carcinoma (TC), well differentiated; **B:** Intense cytoplasmic immunostaining of NTYR in tumor cells of TC, moderately differentiated; **C:** Intense nuclear immunostaining of 8-OH-dG in tumor cells of TC, well differentiated, note the reactivity of the lymphoid follicle (upper left); **D:** Intense nuclear immunostaining of 8-OH-dG in tumor cells of diffuse carcinoma, note the weak reactivity of some cells of a normal crypt (right); **E:** Intense nuclear immunostaining of 8-OH-dG in high-grade dysplastic epithelium, note the weak reactivity of a normal crypt (right); and **F:** Intense cytoplasmic immunostaining of NTYR in low-grade dysplastic epithelium, note the absence of reactivity of a normal crypt (right).

intraepithelial carcinomas and early gastric cancer than that in infiltrative carcinomas, but further studies with larger number of patients would be required to judge the statistical significance. In contrast to a previous report^[32], we neither found any relationship of iNOS with lymph node dissemination (pN), nor any relationship of NTYR or 8-OH-dG with pN.

The levels of the three markers did not vary significantly according to cancer site (especially gastric stump, at high risk for cancer), or with the density of inflammation or *H pylori* infection. During the sequential steps from gastritis to TC, we^[6,7] and others^[8,16] have shown that iNOS produced continuously by *H pylori*-induced inflammatory cells plays a crucial role in the initiation of the carcinogenic process. In cancer, the absence of any relationship between iNOS and the two other markers with inflammation suggests that inflammation-induced damage does not contribute markedly to the final stages of the neoplastic disease. Only iNOS was more often expressed in males as compared with females, consistent with the higher frequency of gastric cancer in males as compared with females, but this reached only borderline significance.

Sequential steps (atrophic gastritis, intestinal metaplasia, and dysplasia) of precancerous changes precede the intestinal-type of gastric carcinoma^[2]. We found an increase

in epithelial levels of iNOS, NTYR, and 8-OH-dG from non-metaplastic non-atrophic gastritis to atrophic metaplastic gastritis and low-grade dysplasia, but reached statistical significance only for 8-OH-dG, thus supporting our previous results^[6]. Therefore, epithelial over-expression of these three markers is an early event in the neoplastic process leading to the intestinal cancer which occurs at the precancerous stage, in which certain genetic modifications are also observed frequently^[11].

Our study suggests that the iNOS-oxidative pathway plays a minimal role in PolyC, as none of the three markers measured were found in half of the patients, and NTYR was detected less frequently in PolyC compared to the other cancer subtypes. In DC, one or two markers were present in 83% of subjects, suggesting a higher level of oxidative processes, but DC was not dependent mainly on the iNOS-oxidative pathway. In contrast, in TC, the strong oxidative process mediated through iNOS may be important in gastric carcinogenesis, as only 12% of subjects had none of the three markers measured, and 25% of subjects showed all these markers simultaneously. In addition, our study suggests that iNOS-associated oxidative stress is an early event in intestinal-type carcinogenesis. These findings support the traditional identification of TC and DC as two distinct tumor entities. Our study is also in accordance with the identification of PolyC as a distinct histotype of gastric carcinoma.

In conclusion, our study shows (1) the occurrence of iNOS, NTYR and 8-OH-dG in tumor cells of infiltrative gastric adenocarcinoma and epithelial cells of precancerous mucosa, as well as in intraepithelial carcinoma, (2) a correlation of both iNOS and NTYR with cancer subtype, (3) no association of the three markers with the degree of inflammation or with *Hi pylori* infection, and (4) that oxidative stress occurs in the three cancer subtypes, and the iNOS-oxidative pathway may be important in gastric carcinogenesis in TC, but its involvement is minimal in PolyC and moderate in DC.

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