

## Case Control Study

**Recurrence in node-negative advanced gastric cancer:  
Novel findings from an in-depth pathological analysis of  
prognostic factors from a multicentric series**

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**Abstract****AIM**

To analyze the clinicopathological characteristics of

patients with both node-negative gastric carcinoma and diagnosis of recurrence during follow-up.

### METHODS

We enrolled 41 patients treated with curative gastrectomy for pT2-4aN0 gastric carcinoma between 1992 and 2010, who developed recurrence (Group 1). We retrospectively selected this group from the prospectively collected database of 4 centers belonging to the Italian Research Group for Gastric Cancer, and compared them with 437 pT2-4aN0 patients without recurrence (Group 2). We analyzed lymphatic embolization, microvascular infiltration, perineural infiltration, and immunohistochemical determination of p53, Ki67, and HER2 in Group 1 and in a subgroup of Group 2 (Group 2bis) of 41 cases matched with Group 1 according to demographic and pathological characteristics.

### RESULTS

T4a stage and diffuse histotype were associated with recurrence in the group of pN0 patients. In-depth pathological analysis of two homogenous groups of pN0 patients, with and without recurrence during long-term follow-up (groups 1 and 2bis), revealed two striking patterns: lymphatic embolization and perineural infiltration (two parameters that pathologists can easily report), and p53 and Ki67, represent significant factors for recurrence.

### CONCLUSION

The reported pathological features should be considered predictive factors for recurrence and could be useful to stratify node-negative gastric cancer patients for adjuvant treatment and tailored follow-up.

**Key words:** N0 gastric cancer; Recurrence; Prognostic factors; Pathological analysis; Lymphatic embolization; Perineural infiltration; p53; Ki67

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**Core tip:** We analyze the clinicopathological characteristics of patients with both node-negative gastric carcinoma and diagnosis of recurrence during follow-up in 4 Italian centers belonging to the Italian Research Group on Gastric Cancer between 1992 and 2010. Lymph node metastasis is the most important prognostic factor in patients undergoing radical surgery for gastric carcinoma. In-depth pathological analysis of two homogenous groups of pN0 patients, with and without recurrence during long-term follow-up, revealed two striking patterns: lymphatic embolization and perineural infiltration (two parameters that pathologists can easily report), and p53 and Ki67, represent significant factors for recurrence. The reported pathological features should be considered predictive factors for recurrence and could be useful to stratify node-negative gastric cancer patients for adjuvant treatment and tailored

follow-up.

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## INTRODUCTION

Nodal metastases are a well-known prognostic factor after radical treatment of gastric cancer. Although patients with adequately staged pN0 cancer have a good prognosis and usually do not undergo adjuvant chemotherapy, nevertheless a subset of them has a recurrence and, later, dies of this disease. Therefore, the identification of prognostic factors for cancer recurrence becomes extremely important for the identification of the small proportion of cases at risk for recurrence.

In some published studies focusing on the clinical-pathological features and prognostic factors of node-negative gastric cancer patients, surgical approaches were not homogenous, particularly in terms of lymph node dissection<sup>[1-10]</sup>. Other limitations were a too short follow-up, as well as the inclusion of T1 cases and of patients with less than 15 retrieved nodes. A previous multicentric series, which we published as members of the Italian Research Group for Gastric Cancer (GIRCG), studied 301 patients from 7 Italian centers<sup>[11]</sup>. These patients were treated during a 10-year period from 1992 to 2002, underwent almost 5 years of regular follow-up, and about 10% developed tumor recurrence. The much valuable feedback and insightful suggestions of peer-reviewers, who have refereed our earlier article, inspired our next project and current paper. Those peer-reviewers strongly encouraged us to carry out an in-depth pathological analysis of the subgroup of patients experiencing recurrence despite having negative nodes on e-e staining; this group clearly represents a very interesting sample for a biological study.

## MATERIALS AND METHODS

The 4 Italian centers participating in this study were the University of Brescia, the Forlì Morgagni Pierantoni Hospital, the University of Siena, and the University of Verona. Inclusion criteria were patients with pN0 gastric carcinoma, treated during the period 1992-2010, who developed cancer recurrence during the 5-year follow-up. All patients had more than 15 retrieved nodes analyzed after surgery, all resulting negative

for metastases at routine hematoxylin-eosin staining. Exclusion criteria were (1) patients with gastric stump cancer; (2) patients with T1 or T4b tumors or peritoneal dissemination; (3) patients with fewer than 15 retrieved nodes; and (4) patients who underwent pre- or post-operative chemotherapy. As a result, out of 1725 patients who underwent radical gastrectomy for T2-T4a gastric cancer, 478 patients without lymph node metastases were studied, and among those 41 developed tumor recurrence (8.57%, Group 1) after a mean period of 17 months (range 9-89). Control group comprises the remaining 437 pN0 patients who did not develop cancer recurrence (Group 2).

We then performed a retrospective review of prospectively collected data, taking into account patient's age, sex, tumor markers CEA and CA 19.9 when available, tumor size, tumor location (upper/medium/lower third), depth of tumor invasion (T2/T3/T4a), histological grading (G1/G2/G3), Lauren histotype (intestinal/diffuse/mixed or other), the type of operation (subtotal, GST/total gastrectomy, GT), the number of retrieved nodes, associated resections, post-operative major morbidity, and post-operative mortality. Survival rate was reported as mean + SD. We adopted the 2012 American Joint Committee on Cancer (AJCC) TNM staging system<sup>[12]</sup>, whereas histological evaluation was performed according to the Japanese General Rules for Gastric Cancer Study in Surgery and Pathology<sup>[13]</sup>.

We discontinued the enrollment of patients on the 31st of December, 2010, whereas we conducted follow-up until the 31st of December, 2015 or until the patient's death. The median follow-up interval for 313 patients, who were alive at the cut-off date, was 113.4 months (range, 61-178 mo). Twenty-one patients (4.39%) had been lost to follow-up. There were 9 (1.99%) in-hospital deaths. Lost cases and operative mortality cases were censored for the analysis of survival.

For in-depth histological analysis, we selected a subgroup (Group 2bis) of 41 controls from Group 2 from one of the four centers participating to our study (the University of Brescia), matched with Group 1 according to age, sex, tumor size, tumor location, tumor invasion (T), histological grading (G), Lauren histotype, and the number of retrieved nodes. We selected cases from the database of the University of Brescia. The same pathologist (CB) retrieved and analyzed all the specimens of tumors from Group 1 and Group 2bis, and recorded lymphatic embolization, as well as microvascular and perineural infiltration, expressed as present/absent. Also, p53, Ki67, and HER2 were studied using immunohistochemical analysis, performed on formalin-fixed, paraffin-embedded sections of surgical specimens with commercially available primary antibodies Abcam anti-ErbB2 (CB11)ab8054, MIB-5 (DAKO) for ki67 and Clone BP-53-12 (Genemed

Biotechnologies, United States, 1:150) for p53. For HER2 evaluation, the following classification was used: 0, 1+, 2+, 3+ for < 10%, > 10% partial staining, > 10% moderately complete staining, and > 10% intensely complete staining, respectively; 0 and 1+ were considered negative testing, 2+ and 3+ positive testing. The presence of p53 and ki67 was expressed by nuclear staining: the rate was calculated as the number of positive nuclear reactions over 100 cells, and for every case a final expression rate was estimated.

### Statistical analysis

We classified variables as discrete (gender, tumor markers, location, T, histotype, grading, associated resections, post-operative morbidity and mortality, lymphatic embolization, vascular infiltration, perineural infiltration, HER2), or continuous (age, size, the number of retrieved nodes, p53, Ki67). We used  $\chi^2$  and the Student *t* test to evaluate the statistical significance of differences for discrete and continuous variables, respectively. We then computed survival rates using the Kaplan-Meier method. *P* values less than 0.05 were considered statistically significant. The statistical methods of this study were reviewed by Professor Giovanni Parrinello (Department of Biotechnologies, Section of Medical Statistics, University of Brescia, Italy).

## RESULTS

Table 1 summarizes the clinical and pathological features of pN0 patients. They represent a typical Western series, with a significant rate of diffuse or mixed (34.0%) and proximal (35.8%) cancers. At the same time, N0 patients had most frequently T2 (60.2%) and G1-2 (72.2%) neoplasms, compared to the general gastric cancer population. The standard lymphadenectomy was D2, with a mean number of retrieved nodes equal to 26.2. However, this data should be interpreted taking into account the exclusion from the analysis of patients with less than 15 retrieved nodes. Less than 10% of the patients underwent associated splenectomy, whereas pancreas tail resection was done in only 7 cases. Major morbidity rate was 12.7% and included 9 cases of anastomotic and 3 cases of duodenal leak (3 of these complications required re-intervention), 8 subphrenic abscesses (all treated by percutaneous drainage), 4 pancreatic fistula, 2 splenic necroses requiring splenectomy, 3 postoperative hemorrhages, and 7 mechanical occlusions requiring re-intervention. Nine patients (1.88% of the sample) died before hospital discharge (8 because of surgical complications and 1 because of myocardial infarction).

Of the whole series of N0 patients, 41 developed cancer recurrence during the follow-up. Table 2

**Table 1 Clinical, pathological, and surgical features of 478 N0 gastric cancer patients *n* (%)**

	Values
Male	291 (60.9)
Female	187 (39.1)
mean age (range)	67.1 (31-90)
Tumor markers <sup>1</sup> (positive)	33/282
Tumor location	
Upper	67 (14.1)
Middle	104 (21.7)
Lower	261 (55.6)
Multiple	46 (9.6)
Tumor mean size (range), cm	4.311 (0.8-14)
Tumor invasion (T)	
T2	278 (58.2)
T3	181 (37.8)
T4a	19 (4.0)
Lauren histotype	
Intestinal	315 (65.9)
Diffuse	81 (16.9)
Mixed / Unavailable	82 (17.1)
Histological grading (G)	
G1	87 (18.2)
G2	287 (60.0)
G3	104 (21.8)
Type of surgery	
Subtotal gastrectomy	266 (55.7)
Total gastrectomy	212 (44.3)
Mean retrieved node number <sup>2</sup>	26.2
(range)	(15-85)
Associated resections	53 (11.1)
(splenectomies)	[41 (8.5)]
30-d mortality	9 (1.88)
Major morbidity	61 (12.7)

<sup>1</sup>CEA and/or CA19.9 above the normal values of 7 ng/mL and 36 UI/mL, respectively; <sup>2</sup>This value reflects the exclusion from our analysis of patients with less than 15 retrieved nodes.

shows the comparison between Group 1 (N0 patients with recurrence) and Group 2 (N0 patients with no recurrence). Significant prognostic factors for recurrence in N0 patients were T4a and diffuse histotype, while gender, age, tumor markers, location, size, grading, the number of retrieved nodes, associated resections, and major complications were not. Of the 41 patients with cancer recurrence, 11 developed their recurrence during the first year after surgery, 16 during the second year, 8 during the third, and 6 in the following years. The sites of recurrence were liver in 21 cases, peritoneum in 27 cases, loco-regional/lymphatic in 7 cases, and other sites in 5 cases. In only 2 cases, cancer recurrence was treated by surgery with potentially radical intent; other 23 patients underwent palliative chemotherapy.

Table 3 reports the comparison between Group 1 (41 N0 patients with recurrence) and Group 2bis (41 N0 patients without recurrence, matched to those of Group 1 according to demographic and pathological characteristics), for which we carried out in-depth histological analysis. Lymphatic embolization and perineural infiltration, which are histological parameters usually available with e-e staining, resulted highly

significant in predicting recurrence; microvascular infiltration was not. Moreover, immunohistochemical study of p53, Ki67, and HER2 revealed that p53 and Ki67 were expressed in a significantly greater rate of cancer cells of patients who subsequently developed cancer recurrence, while this was not true for HER2.

At the time of the analysis, 313 (65.5%) patients were alive and free of disease, including 2 patients having a recurrence that was surgically treated with success (hepatic metastasis resection and total gastrectomy for gastric stump recurrence), 39 patients (8.15%) had died because of cancer recurrence, whereas 96 patients (20.0%) had died of other diseases. The remaining patients had died after surgery (9) or lost at follow-up (21). Due to the long period of follow-up after ending the recruitment of patients, no patient with recurrence is currently alive. Mean and median survival times were 93.4 and 81.1 months, respectively. The 3, 5, and 10-year overall survival and cancer-related survival rates of the whole group were 79.2%, 72.6%, 41.4% and 91.7%, 87.2%, 76.8%, respectively.

## DISCUSSION

As mentioned earlier, the helpful feedback and suggestions of peer-reviewers, who have refereed an earlier article of ours<sup>[11]</sup> based on an Italian multicentric series of patients with pN0 gastric cancer, inspired our next project and current paper. Those peer-reviewers encouraged us to carry out an in-depth pathological analysis of the subgroup of patients experiencing recurrence despite having negative nodes on e-e staining; this group clearly represents a very interesting sample for a biological study. Meanwhile, the increased number of available pN0 cases also enabled us to perform a confirmation analysis of the significant clinico-pathological basic factors found in our earlier study.

In our current study we present a large, multicentric series of pN0 patients with gastric cancer, who have undergone radical surgery with reliable nodal harvesting (more than 15 analyzed nodes). Our main goal is to investigate which factors, easily available after standard pathological examination, are related to a higher likelihood of recurrence; this information is clearly very valuable to both the oncologists when deciding whether to use adjuvant chemotherapy, and the referring physicians when planning the targeted follow-up.

The secondary but potentially equally relevant purpose of our study is to discover if an in-depth pathological analysis, including three ancillary ematoxilin-eosin data (lymphatic embolization, microvascular infiltration, and perineural infiltration) and three immunohistochemical examinations (p53, Ki67, and HER2) may add valuable information for such clinical

**Table 2** Prognostic factors for recurrence in N0 gastric cancer patients

	Group 1 (with recurrence) (n = 41)	Group 2 (no recurrence) (n = 437)	P value <sup>3</sup>
Gender (male)	28	263	0.308
Age (> 65)	19	201	0.966
Tumor markers <sup>1</sup>	6/25	27/207	0.138
Tumor location (upper)	7	60	0.555
Tumor size (> 5 cm)	16	132	0.242
Tumor invasion (T)			
T2	19	259	0.108
T3	13	168	0.395
T4a	9	10	0.001
Lauren histotype			
Intestinal	24	291	0.768
Diffuse	12	69	0.012
Mixed / Unavailable	5	77	0.509
Histological grading (G)			
G1	7	80	0.844
G2	22	265	0.382
G3	12	92	0.222
Retrieved nodes <sup>2</sup> (mean) > 25	26	273	0.905
Associated resections - yes	8	45	0.772
Major morbidity - yes	8	53	0.175

<sup>1</sup>Almost 1 tumor marker positive, including CEA, CA 19.9, or other tumor marker. Only 282 cases were available for analysis; <sup>2</sup>This value reflects the exclusion from our analysis of patients with less than 15 retrieved nodes; <sup>3</sup>P values of  $\chi^2$  test for gender, tumor markers, tumor location, tumor invasion (T), Lauren histotype, histological grading (G), associated resections, post-operative morbidity. P values of Student t-test for age, tumor size, and the number of retrieved nodes.

**Table 3** Histological assessment of N0 patients with and without cancer recurrence, matched according to demographic and pathological characteristics

	Group 1 (41 N0 patients with recurrence)	Group 2bis (41 N0 patients without recurrence)	P value <sup>3</sup>
Lymphatic embolization	34	13	0.001
Microvascular infiltration	11	7	0.285
Perineural infiltration	19	6	0.001
p53 <sup>1</sup>	49.1% (0-100)	33.8% (0-100)	0.041
Ki67 <sup>1</sup>	52.7% (30-80)	33.1% (15-70)	0.038
HER2 <sup>2</sup>	7	8	0.785

<sup>1</sup>Our immunohistochemical analysis of p53 and Ki67 differ from some of those found in other studies because we expressed them as the number of cancer cells showing immunohistochemical staining over 100 cells, thus as a rate and not as a binomial variable (yes/no). Statistical analysis was retrospectively performed by Student t-test; <sup>2</sup>Immunohistochemical detection, both 2+ and 3+ were considered positive; <sup>3</sup>P values of  $\chi^2$  test for lymphatic embolization, microvascular infiltration, perineural infiltration, and HER2. P values of Student t-test for p53 and Ki67.

decisions. Pathologists do not typically analyze those 6 ancillary data; however, these additional data, which are very easy and relatively inexpensive to analyze, may provide extremely helpful information in addition to the one from the basic pathological items.

We defined the study group taking into account the shortcomings of previous Western studies about node-negative gastric cancer patients, and, hence, excluding T1, T4b, and Nx patients. T1N0 patients have a very good outcome, thus making adjuvant chemotherapy unlikely to be valuable. In contrast, T4b patients usually undergo a kind of adjuvant therapy (chemo- or radiotherapy) irrespective of other pathological parameters. Patients with less than 15 retrieved nodes are not adequately staged, so we cannot define them N0 patients.

Factors associated with recurrence in N0 patients

were only T4a and diffuse histotype. Nine out of 19 patients with a true, pathologically proven serosal and extragastric fat infiltration experienced recurrence (47.3%), mainly peritoneal (6/9 cases) and nodal (5/9 cases); this subgroup is thus suitable for adjuvant therapy. Therefore, T3 diffuse cases should also be considered for adjuvant therapy and intensive follow-up, considering that about a quarter of them will experience recurrence.

The most striking result of our study is that lymphatic embolization and perineural infiltration are significant parameters associated with recurrence in pN0 gastric cancer.<sup>[14]</sup> Out of 41 patients experiencing recurrence, 34 and 21 had lymphatic embolization and perineural infiltration, respectively. In Group 1 + Group2bis (82 cases), 15 patients had both parameters, and 10 of them subsequently developed

cancer recurrence (66.6%). In our earlier article including 301 patients from the same 4 Italian centers<sup>[11]</sup>, data on these parameters were available for only 144 patients (confirming that basic pathological analysis does not typically provide information on these parameters); however, the 3 parameters were considered all together, with a rate of positive cases (26.3%) similar to the previously reported rate of about 20% of cases for microvascular infiltration and about 30% for perineural infiltration; they were not significantly related to the recurrence rate both at univariate and multivariate analysis.

According to the Japanese classification of gastric carcinoma<sup>[15]</sup>, further research to classify lymphatic invasion and venous invasion into three more specific grades would help clarify the association between these anatomic-pathological characteristics of tumor and the rate of recurrence. Also, a further study in which also a subgroup among non-T4a and intestinal histotype are added, would provide a more in-depth analysis of prognostic factors of recurrence.

From an immunohistochemical point of view, the p53 protein, encoded by a tumor suppressor gene located on the short arm of chromosome 17, is involved in different cell functions, including apoptosis and cell cycle regulation<sup>[16]</sup>. This protein is usually poorly expressed in normal cells, but overexpressed and/or mutated in a number of human malignancies<sup>[17]</sup>; overexpression of p53 generally reflects an underlying mutation(s) in the *p53* gene<sup>[16]</sup>. A correlation among high protein levels, older age, advanced stage, and poor prognosis has been shown for colorectal, breast, and lung carcinoma<sup>[18]</sup>. Studies that have evaluated p53 expression and/or mutation in gastric cancer, have confirmed enhanced expression in cancer cells in comparison with normal cells (33.8% vs 4% in the series published by Zhou and Coll), but the benchmark value is different, ranging from 19%-29%<sup>[19]</sup> to 34%-65%<sup>[20,21]</sup>; however, correlation of p53 expression and worse prognosis was shown in some<sup>[16,22]</sup>, but not in other series<sup>[23]</sup>.

Antigen KI-67 (Ki67) is a nuclear proliferation associated antigen, involved in cycle regulation and cell proliferation. It is considered a reliable marker of tumor biology, having a clear prognostic value in several types of cancer, such as GIST<sup>[24]</sup>; in two recent retrospective analyses of patients with gastric cancer, Ki67 expression was associated with distant metastasis and survival<sup>[25]</sup>, T stage and 3-year disease free survival<sup>[26]</sup>.

Human epidermal growth factor receptor 2 (HER2) is a transmembrane tyrosine kinase receptor, a 185 kDa glycoprotein encoded by a gene located in the long arm of chromosome 17. HER2 is involved in various cancerogenesis steps, including de-differentiation, angiogenesis, and metastasis<sup>[27]</sup>. As noticeably shown in the case of breast cancer, expression of HER2 is

a marker of more aggressive behavior and may be related to a worse prognosis compared with HER2 negative tumors. In gastric cancer, an overexpression of HER2 has been reported in approximately 10%-30% of cases; however, a clear relationship with histopathological parameters or survival has not been established<sup>[28,29]</sup>. A comprehensive literature review published in 2011<sup>[30]</sup> confirmed the lack of association between HER2 overexpression and patient-related, as well as cancer-related, main parameters. Other small series, however, suggested an association with older age, larger tumors, and advanced stage<sup>[31,32]</sup>. Our results clearly confirm that HER2 overexpression, determined only by immunohistochemical analysis and not by FISH method, is not related to a greater risk of cancer recurrence in pN0 cases. The overall expression of HER2 in the whole series is similar to the one previously reported, with a marginal proportion of cases having a clear 3+ overexpression; none of the 41 patients with recurrence had 3+ HER2 expression, and the sum of 3+ and 2+ was also greater in group 2bis (*i.e.*, patients without recurrence).

In conclusion, despite a good overall prognosis, about 10% of pN0, R0 gastric cancer patients actually have cancer recurrence and die of this disease. The prognostic factors analyzed and reported in our study may help identify node-negative patients who would significantly benefit from existing or future adjuvant strategies. Moreover, for these patients an appropriate surveillance protocol in terms of follow-up should be proposed.

Further research with extra pathological examinations could be useful to define an algorithm for the management of pN0 gastric cancer patients.

## COMMENTS

### Background

Lymph node metastasis is the most important prognostic factor in patients undergoing radical surgery for gastric carcinoma. Even if node-negative patients have better outcomes, a subgroup of them develops recurrence. Hence, this is a key group to study for adjuvant treatment and follow-up.

### Research frontiers

Clinicopathological characteristics of patients with both node-negative gastric carcinoma and diagnosis of recurrence during follow-up are key factors in the management of gastric cancer patients.

### Innovations and breakthroughs

In-depth pathological analysis of two homogenous groups of pN0 patients, with and without recurrence during long-term follow-up, revealed two striking patterns: lymphatic embolization and perineural infiltration (two parameters that pathologists can easily report), and p53 and Ki67, represent significant factors for recurrence.

### Applications

The reported pathological features should be considered predictive factors for recurrence and could be useful to stratify node-negative gastric cancer patients for adjuvant treatment and tailored follow-up.

**Peer-review**

It is very important in-depth pathological analysis added in this study.

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