

Neutrophil-rich Gastric Carcinoma in the Integrated Cancer Registry of Eastern Sicily, Italy

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Abstract. *Background/Aim: Neutrophil-rich carcinoma is a variant of gastric carcinoma that has not been well-studied or characterized. The purpose of the present study was to reveal the incidence and clinicopathological findings compared to ordinary gastric carcinoma. Patients and Methods: A population-based series of 430 gastric cancers, identified between 2003 and 2006 from the province of Messina (insular Italy; population, 662,450), was used. The number of tumor-infiltrating neutrophils was assessed in a semi-quantitative manner using the mean value of 20 non-overlapping high-power fields (magnification, 400; 0.08 mm²). Tumors with >10 neutrophils per 20 high-power fields were arbitrarily considered as neutrophil-rich gastric carcinomas. Moreover, MUC1 immunohistochemical expression was investigated to show possible correlation with neutrophil infiltration in gastric carcinomas. Results: Among 193 gastric cancers resected for curative purposes, 30 (15.54%) were represented by neutrophil-rich gastric carcinomas. These tumors occurred more frequently in patients aged more than 72 years ($p < 0.05$), showing an inverse correlation with mucinous subtype according to the WHO classification ($p < 0.001$) and expressed MUC1. However, intensity and distribution of MUC1 was heterogeneous, and independent of neutrophil infiltration within the tumor stroma. Conclusion: Neutrophil-rich carcinoma seems to represent a distinctive morphological variant of gastric carcinoma, although the true mechanism for the infiltration of neutrophils is still unclear.*

Inflammatory cells and mediators are a key component of the tumor microenvironment and cancer-related inflammation

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has been proposed to represent the seventh hallmark of cancer (1). Neutrophils are believed to be rare in the tumor microenvironment (1). Recent data suggest that tumor-infiltrating neutrophils are characterized by a bi-polar pattern of activation similar to that observed in macrophages (M1/M2) and T-cell (Th1/Th2) polarization (2). Fridlender *et al.* showed the existence of N1 (antitumoral) and N2 (protumoral) tumor-infiltrating neutrophils (3). N1 neutrophils exert antitumor activities through tumor cytotoxicity and enhancement of antitumoral immune memory, whereas N2 neutrophils favor tumor growth, invasion and metastasis, *e.g.* through proteolysis of extracellular matrix components, promotion of angiogenesis and mediation of immunosuppression (3-6). Therefore, tumor-infiltrating neutrophils may play an important role in tumor pathology, either by inhibiting and/or promoting tumor cell growth.

There are a few studies regarding tumor neutrophilia and its association with gastric carcinomas (7-13). This present work was undertaken to determine the incidence and clinicopathological features of neutrophil-rich gastric carcinoma in a well-defined population of Southern Italy. As MUC1 (episialin, epithelial membrane antigen, CA15-3 antigen) on cancer cells is involved in chemotaxis of the cells of the innate immune system (14-19), the expression pattern of MUC1 in neutrophil-rich gastric carcinomas was also analyzed and the results discussed relevant to current literature.

Patients and Methods

Patient data. A population-based cancer registry was set up in 2005 to cover the resident population of Messina province (662,450 according to 2001 census). The cancer registry of Messina is part of Integrated Cancer Registry of Eastern Sicily, including the Siracusa, Enna, Catania and Messina and has checked data of patients diagnosed between January 2003 and December 2006. The registry is defined by the national committee of registries and operates in compliance with the recommendations of the IARC (International Agency for Research on Cancer). Using cancer registry data, we identified those patients diagnosed between January 2003 and

December 2006 with adenocarcinoma of the stomach. All patients had not received preoperative anticancer treatment.

The clinical and pathological parameters analyzed for all patients were gender, age, site and histologic type. In addition, depth of invasion into the gastric wall, nodal status (the number of regional lymph nodes examined and the number of those invaded) were analyzed for the resected cases. The tumors were restaged according to the 7th TNM version (20).

Notes from surgical procedures and results of pathologic tests were the preferred sources of information for tumor site identification. Gastric carcinoma location included fundus, body, antrum and pylorus, as well as overlapping/unknown sites (overlapping sites or unknown primary site).

We excluded patients with ICD-O-3-defined cardiac location (C-16.0), which includes tumors of the gastric cardia and cardioesophageal/gastroesophageal junction. We also ruled out cases of leukemia, lymphoma, mesothelioma or Kaposi sarcoma (ICD for Oncology 3 histology codes 9050-9055, 9140, and 9590-9989) (21).

In all cases, multiple gastric neoplastic and non-neoplastic samples were available, taken from the macroscopic surgical specimens in compliance with standardized sampling protocols. All surgical specimens for light microscopy were fixed in 10% formalin and embedded in paraffin. Sections were stained with hematoxylin-eosin (H&E).

All patients gave informed consent prior to gastroscopy or operation and every procedure was according to the rules of good clinical practice. All samples were anonymized.

Gastric cancer classification. Each gastric carcinoma was classified according to each of the following histopathological classifications: WHO classification (22) and Laurèn (23) classification. The Laurèn classification (23) has the following categories: intestinal type and diffuse type. The WHO classification (22) distinguishes five major types of gastric carcinoma: papillary, tubular, mucinous, poorly cohesive (including signet-ring cells and other variants) and mixed carcinomas.

Definition of neutrophil-rich gastric carcinoma. Two pathologists (CRA, FF) independently analyzed the H&E-stained slides from each case and assessed tumor-infiltrating neutrophils according to our previous work (8). Briefly, H&E-stained sections were examined under low power (4×) to identify areas of neutrophilic aggregates within the tumor tissue. Ten non-overlapping fields were examined on two slides in representative areas of a given tumor for a total of 20 fields per patient. The number of stromal neutrophils was assessed in a semiquantitative manner using the mean value of 20 non-overlapping high-power fields (HPF; magnification of 400×; 0.08 mm²) by using a 40× objective and a square grid mounted in a 10× microscopic eyepiece. After the examination of histograms that revealed the distribution of tumor-infiltrating neutrophils, the neutrophil counts were divided into two categories based on numbers of neutrophils detected and expressed as minor, when <10 neutrophils were present, and extensive, when >10 neutrophils were found. The cut-off level of 10% was adopted, the same as used in the other studies (8, 15). Tumors with more than 10 neutrophils per 20 HPF were arbitrarily considered as neutrophil-rich gastric carcinomas.

Evaluation of background chronic gastritis in neutrophil-rich gastric carcinomas. The extensive sampling protocol adopted for surgical specimens allowed suitable characterization of the gastritis

phenotype, also ruling out the likelihood of false-negative *Helicobacter (H) pylori* cases. We then evaluated the following five items: (i) *H. pylori* density, (ii) neutrophil activity, (iii) degree of chronic inflammation, (iv) degree of glandular atrophy and (v) degree of intestinal metaplasia according to the updated Sydney system criteria (24). All items were graded as follows: 0, normal; 1, mild; 2, moderate and 3, severe or marked.

MUC1 immunohistochemistry. MUC1 mucin was evaluated immunohistochemically in neutrophil-rich gastric carcinomas. Immunohistochemical procedures were performed on 4 μm formalin fixed, paraffin embedded, tissue sections obtained from each representative paraffin block. Briefly, endogenous peroxidase activity was preliminarily blocked with 3% H₂O₂ in phosphate-buffered saline for 30 min at room temperature. Sections were successively incubated at 4°C overnight with the following primary monoclonal antibody: NCL-MUC1 (Ma695; 1:100, Novocastra, Newcastle, UK). Microwave pre-treatment using 0.01M sodium citrate buffer was employed for each immunoreaction. The bound primary antibody was visualized by the avidin-biotin-peroxidase detection complex (ABC) method with a commercial kit (Vectastain ABC Elite Kit, Vector Laboratories, Burlingame, CA, USA), according to the manufacturer's instructions. 3-3' diaminobenzidine (DAB) was used as the chromogen. Sections of mucinous breast tissue were used as positive controls for MUC1. Negative control slides were also made by omitting the primary antibody. The results of immunostaining were considered to be positive if at least 10% of the neoplastic cells were stained.

Statistical analyses. The categorical data were summarized using frequencies and percentages. Distribution of the continuous variable, such as age, was assessed but it was found not normally distributed. Therefore, this datum was summarized with median and interquartile range. Nominal variables were divided into two or three subgroups: gender (male *versus* female), location (fundus and corpus, distal third, overlapping/unknown), histological classification according to Laurèn (23) (intestinal *versus* diffuse) and according to WHO (22) (tubulopapillary, mucinous, non-cohesive), depth of invasion (T1-T2 *versus* T3-T4), lymph node metastasis (negative *versus* positive), stage of disease (I-II *versus* III-IV). The Chi-square test was used to compare characteristics between groups. *p*<0.05 was considered to indicate a statistically significant difference. Statistical analysis was performed using the STATA software (version 8.0; Stata Corporation, College Station, TX, USA).

Results

Between 2003 and 2006, a series of gastric carcinomas from 430 patients were identified from the Messina province cancer registry, corresponding to a mean annual age-standardized incidence rate of 18.52 per 100,000 in males and 13.96 per 100,000 in females, according to the world population. Among the 430 gastric carcinoma cases, 193 (44.88%) had a total or sub-total gastrectomy with curative intent, 65 (15.11%) had a laparotomy or palliative surgery and 172 (39.76%) did not receive any surgical treatment. In patients who did not undergo resection of the tumor, some were found to have unresectable disease because of

Table I. *Clinicopathological findings of patients.*

Patients	Neutrophil-rich carcinomas N=30	Neutrophil-poor carcinomas N=163	χ^2 (<i>p</i> -Value)
Age, median			
<72	9	82	0.04
≥73	21	81	
Gender			
Male	16	110	0.13
Female	14	53	
Location			
Fundus and corpus	8	46	0.98
Distal third	21	112	
Overlapping/unknown	1	5	
Laurèn classification			
Intestinal	22	105	0.34
Diffuse	8	58	
WHO classification			
Tubulopapillary	22	38	<0.001
Mucinous	0	67	
Non-cohesive	8	58	
Depth of invasion			
T1-T2	19	100	0.83
T3-T4	11	63	
Lymph node metastasis			
Negative	8	55	0.44
Positive	22	108	
Stage of disease			
I-II	21	111	0.83
III-IV	9	52	

metastasis. These metastases included liver metastasis, peritoneal dissemination and distant lymph node involvement. Other patients who did not undergo resection had severe complications, such as chronic heart failure, myocardial infarction, diabetes and aspiration pneumonia.

General features of neutrophil-rich gastric carcinoma. Among the 193 gastric cancers resected for cure, 30 (15.54%) were represented by neutrophil-rich gastric carcinomas. The mean patient age at diagnosis was 70 years, whereas median age was 72 years (25th -75th percentile range=64-80 years). Patients aged more than 72 years were more likely than younger patients to present with neutrophil-rich carcinomas ($p < 0.05$). There were 16 men and 14 women (male to female ratio, 1:1 as opposed to 2:1 in conventional neutrophil-poor carcinomas). Among the 30 neutrophil-rich gastric carcinoma, 22 were classified as intestinal type by the Laurèn classification or classified as tubulopapillary according to WHO classification. Eight neutrophil-rich tumors were classified as diffuse type in the Laurèn classification or classified as non-cohesive in the WHO classification (Table I).

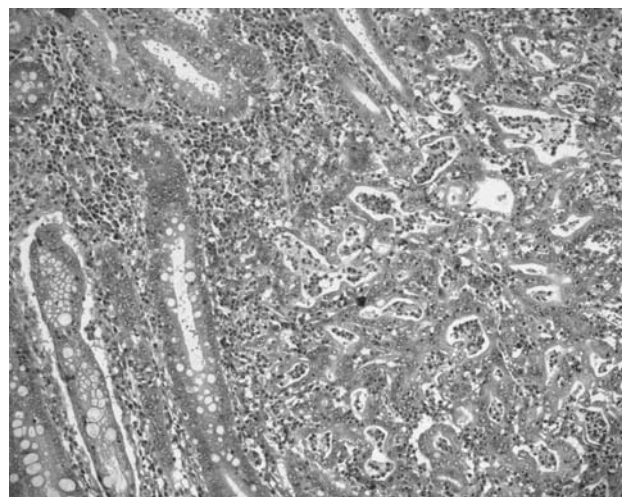


Figure 1. *Neutrophil-rich gastric carcinoma. The adjacent non-neoplastic mucosa shows intestinal metaplasia. Note tumor-centric infiltration of neutrophils. H & E, ×100.*

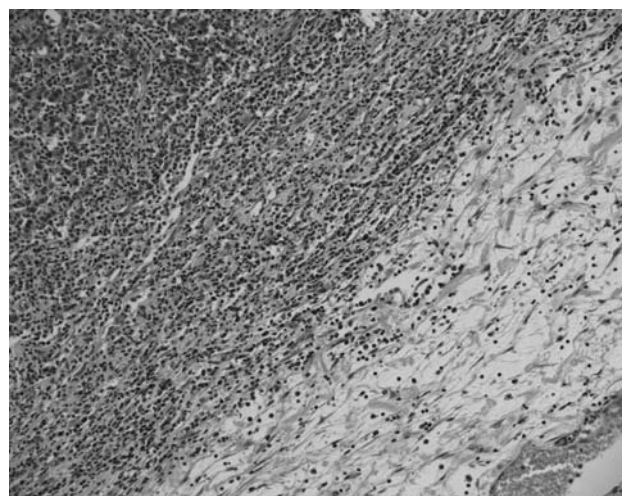


Figure 2. *Tumor-centric distribution of neutrophils at the deep invasive front of gastric carcinoma. H & E, ×100.*

In the 22 cases of intestinal-type gastric carcinomas, neutrophils formed multi-focal abscess formation scattered throughout the tumor stroma (Figure 1). Some neutrophils were seen passing through the neoplastic epithelium and others were found lying within neoplastic glands similar to “crypt abscesses” (Figure 1). Distinct focal gland disruptions and neutrophil infiltration were detected in tumor tissue. The size of disruption varied substantially among glands, ranging from a few cells to over 80% of the neoplastic glands (Figure 1). In the 8 cases of diffuse-type gastric carcinomas, there was a single massive infiltrate in the tumor stroma (Figure 2).

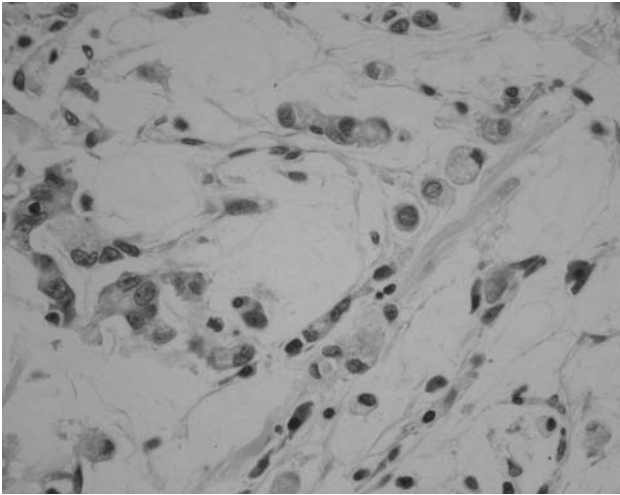


Figure 3. Mucinous adenocarcinoma of the stomach. Note the absence of neutrophil infiltration. H & E $\times 200$.

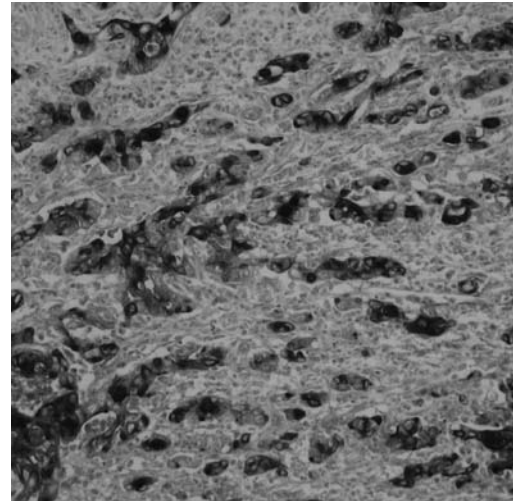


Figure 4. Neutrophil-rich diffuse-type gastric carcinoma. Strong MUC1 immunoreactivity in the cytoplasm of the tumor cells. $\times 100$.

In all 30 cases, neutrophils infiltrated selectively the tumor tissue, being few or absent away from the neoplasm (Figures 1-2). The adjacent non-neoplastic mucosa revealed chronic inactive gastritis, characterized by mononuclear cell infiltration consisting of various amounts of lymphocytes, plasma cells, mast cells and macrophages in the lamina propria without the presence of neutrophils. There was no histological evidence of *H. pylori* infection, whereas various degrees of multifocal intestinal metaplasia were present in the background mucosa (Figure 1).

Among 163 neutrophil-poor gastric carcinomas, 105 were classified as intestinal-type in the Laurén classification; these tumors were mainly classified as tubulopapillary or mucinous in the WHO classification. The remaining 58 tumors were classified as diffuse type in the Laurén classification, with these tumors being mainly classified as non-cohesive in the WHO classification. Mucinous histological type (WHO classification) was not detected in patients with neutrophil-rich carcinomas ($p < 0.001$) (Figure 3). Moreover, neutrophil-rich carcinomas were not correlated with location, depth of invasion, lymph node metastasis and pTNM stage (Table I).

Immunohistochemical findings. Staining for MUC1 mucin was evidenced in normal gastric epithelium present in the analyzed samples. All 30 cases of neutrophil-rich gastric carcinomas evaluated for MUC1 expression were positive (Figure 4). MUC1 immunopositivity was heterogeneous, with immunoreactive cells dispersed singly or clustered in focal areas (Figure 5). Stained cells exhibited apical membrane or diffuse cytoplasmic immunoreactivity. Intra-luminal expression of MUC1 was also found. Although

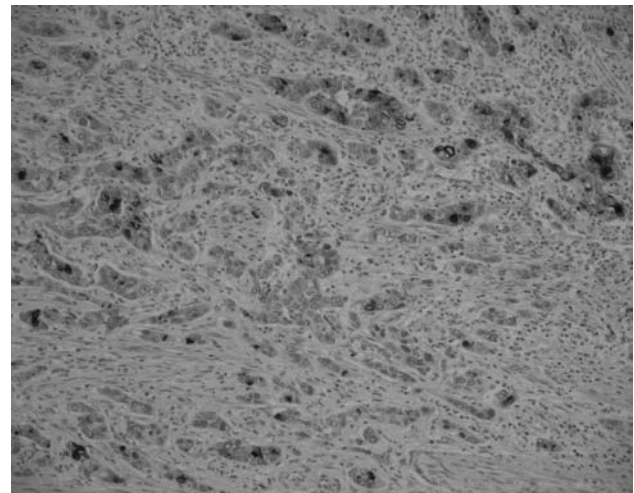


Figure 5. Heterogeneous MUC1 immunoreactivity. Many neoplastic glands, although markedly infiltrated by neutrophils, did not exhibit MUC1 positivity. $\times 100$.

infiltrated by neutrophils, many glands were characterized by MUC1 immunonegativity (Figure 5).

Discussion

The aim of the current population-based study was to identify the incidence, as well as clinicopathological characteristics of neutrophil-rich gastric carcinomas. Out of 193 gastric carcinomas, resected in Messina province from 2003 to 2006, 30 fulfilled the criteria as neutrophil-rich

gastric carcinomas. The incidence (15.5%) was slightly higher to that reported in other studies (7).

The present study also showed that neutrophil-rich gastric carcinomas tended to occur in older patients (more than 72 years) than conventional neutrophil-poor gastric carcinomas ($p < 0.05$). Male predominance appears not to be as striking as it is in conventional gastric carcinomas (1:1 *versus* 2:1).

The mechanism whereby neutrophil infiltration involved 15.5% of patients is not clear. It can be argued that increasing bulk of carcinoma provides potential for greater tumor necrosis and secondary neutrophil infiltration. In the present study, however, tumor anatomical extent, described by pTNM, was not correlated with neutrophil infiltration. However, a statistically significant correlation between tumor-infiltrating neutrophils and certain histological types of gastric carcinomas was observed. Several histopathological classifications including Laurén (23) and WHO (22) classification have been proposed to identify the morphological heterogeneity in gastric carcinomas. The WHO classification is more precise than the Laurén classification concerning gastric carcinoma, individualizing the mucinous subtype (25). Mucinous cancers are characterized by extensive extracellular accumulation of mucus-forming gelatinous lakes into which more or less aggregated tumor cells are immersed (26). Mucin invades tissues, dispersing neoplastic cells. Furthermore, it inhibits reaction and the immunological recognition of tumor cells (26). These data explain the lack of neutrophil infiltration in mucinous carcinomas observed in our study. Finally, there was no significant relationship between neutrophil infiltration and tumor location.

MUC1 is a high-molecular weight transmembrane protein expressed at the apical surface of most glandular epithelial cells (27). MUC1 over-expression is found in almost 95% of cancer cells, a molecular pathological feature that is associated with carcinogenesis and poor prognosis (28). Moreover, gastric carcinomas are characterized by aberrant glycosylation and loss of apical expression of MUC1 (29-30). In the present study, there was a considerable variability in the incidence, intensity and the subcellular distribution of MUC1 immunopositivity in neutrophil-rich gastric carcinoma. Therefore, the MUC1 expression pattern is not constantly correlated with neutrophil infiltration within the tumor stroma.

There were several limitations to the present study. This was a retrospective study and surgical procedures were performed by different surgeons using non-standardized procedures. This resulted in inconsistent lymphadenectomy with consequent under-estimation of nodal staging. Furthermore, neutrophil count was based on H&E-stained sections and no specific immunohistochemistry for neutrophils was performed. After the draft of this manuscript, we have read a recently published meta-analysis that suggested the utility of CD66b immunohistochemistry as a

marker of activated neutrophils (31). Further studies are needed to reveal the role of activated N1 neutrophils in the tumor stroma of gastric carcinomas.

In conclusion, the present study revealed the experience of cancer registry regarding the incidence of neutrophil-rich gastric carcinoma. These tumors occurred more frequently in patients aged more than 72 years and showed an inverse correlation with the mucinous subtype according to the WHO classification ($p < 0.001$). Neutrophil-rich gastric carcinomas expressed heterogeneous MUC1 immunoreactivity that was not spatially correlated with neutrophil infiltration. Further studies are required to investigate the mechanisms responsible for marked neutrophil infiltration in gastric carcinomas.

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