

Deep inside of gastric signet-ring cell carcinoma

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The histology of signet-ring cell carcinoma (SRC) of the stomach has been revisited with the support of current immunohistochemical techniques in order to explain particular features of this tumor; its great capacity of local diffusion and lymph node metastasis, also through a neo-lymphoangiogenesis. An observational retrospective study on 50 cases of SRC in stage II and III has been performed with the addition of histochemical (Alcian Blue, DDD-Fast Blue B, Mercury Orange) and immunohistochemical (cytocheratin, CD3, CD4, CD8, CD10, CD56, CD68, perforin, granzyme B, podoplanin, collagen type IV) investigations for each case. The signet ring cells, typical for this tumor, show abundant content of electro-negative sialomucins and demonstrate a great capacity of diffusion through the gastric wall. They evoke production and deposition of collagen type IV in the sub-mucosa layer through the local action of fibroblasts. The immunological response to this tumor in the gastric wall and in the metastatic lymph nodes is represented by an increase of B and T-helper lymphocytes, but not of T-killers or natural killers. The neoplastic cells are curiously able to avoid these newly formed 'lymph nodules'. An extended neo-lymphangiogenesis has been observed around the primary tumor and in metastatic lymph nodes. A careful immunohistochemical characterization has allowed a better knowledge of SRC, regarding especially the peculiar behavior of local diffusion of its cells, the associated neo-lymph angiogenesis, and poor immunological reaction.

Key words: undifferentiated gastric carcinoma, signet-ring cell carcinoma, lymphangiogenesis, tumor-infiltrating lymphocytes, cancer, histology

Stomach cancers are overwhelmingly composed of adenocarcinomas (90%), followed by lymphomas (5%), carcinoids and stromal tumors [1]. Histologically, according to the Lauren's classification there are two major types of gastric adenocarcinomas: intestinal type or diffuse type. Intestinal type adenocarcinoma tends to form glandular structures with multiple lumens and reduced stroma with a 'back-to-back' appearance. Often, it associates with intestinal metaplasia in neighboring mucosa. Depending on glandular architecture, cellular pleomorphism and muco-secretion, it can present three degrees of differentiation: well differentiated (G1), moderate differentiated (G2) and poorly differentiated/undifferentiated (G3) [1]. Diffuse type adenocarcinoma (linitis plastica, leather-bottle stomach) tumor cells are discohesive and secrete mucus, which can be delivered into the interstitium and produce large pools, or can remain inside the tumor cell, pushing the nucleus to the periphery in a 'signet-ring' fashion. By definition, diffuse type adenocarcinoma is poorly differentiated/undifferentiated [1]. The great

majority of gastric adenocarcinomas develops histologically differentiated tumors and a significant proportion of these progress until they become undifferentiated. However, a subset of gastric adenocarcinomas develops as undifferentiated carcinomas, including signet-ring cell carcinoma (SRC) [1]. Our attention has been precisely focused on SRC for its particular histological features and clinical behavior. Today, the immunohistochemical techniques allow to better understand local and distant diffusion. Cancer is in fact characterized by some major hallmarks: sustaining proliferative signaling, evading growth suppressors, activating invasion and metastasis, enabling replicative immortality, inducing angiogenesis and resisting cell death [2]. From the beginning of XXI century, several evidences support the importance of immunity against cancer and, therefore, the ability to escape immune-mediated destruction was introduced as a further hallmark of cancer [3]. The most commonly used system for classifying the tumor extent and spread is TNM (Tumor Node Metastasis). However, it is well-known that the system

fails to consider the effects of host immune response against cancer cells and that clinical outcome can significantly vary among patients within the same TNM stage [4]. To overcome this limit, the concept of immunoscore was proposed, which incorporates immunity into tumor classification, improving the prognostic accuracy [5]. It is based on the evaluation and quantification of tumor-infiltrating lymphocytes and *in situ* immune response [6]. By exploiting specific immunohistochemical antibodies, we have also investigated in SRC all of the main lymphocytic sub-populations, the related most common molecules of cytotoxicity, and the presence of histiocytes, fibroblasts and collagen type IV, and correlated them with the above-mentioned cancer hallmarks of lymphangiogenesis and disruptive growth.

Patients and methods

Retrospectively, we have in-depth evaluated the histology of 50 SRC cases, 25 females and 25 males, aged between 53 and 76 years, diagnosed from 2000 to 2010. All these patients were submitted to a curative subtotal distal gastrectomy with D2-lymphadenectomy and the gastric tumor was confirmed to be SRC in stage II (27 cases) or III (23 cases).

An unfavorable course of the neoplastic disease followed in all cases, with decease within 36 months. From a representative paraffin block of each neoplastic case, new tissue sections were obtained and submitted to histochemistry (Alcian Blue, DDD-Fast Blue B, Mercury Orange) and immunohistochemical characterization. More in detail, after deparaffinization, hydration, endogenous peroxidase blocking and heat-induced antigen retrieval, the tissue sections were incubated for 30 minutes at room temperature with anti-cytokeratin (clone MNF116; Dako, Glostrup, Denmark), anti-CD3 (clone 2GV6; Ventana, Tucson, AZ, USA), anti-CD4 (clone SP35; Ventana), anti-CD8 (clone SP57; Ventana), anti-CD10 (clone SP67; Ventana), anti-CD56 (clone SP35; Ventana), anti-CD68 (clone KP1; Ventana), anti-perforin (clone MRQ-23; Ventana), anti-granzyme B (polyclonal; Ventana), anti-podoplanin (clone D2-40; Ventana), anti-collagen type IV (CIV22; Ventana). Biotinylated secondary antibody was applied and the staining product was detected with avidin-biotin complex against a hematoxylin counterstain. Detection of the staining reaction was achieved by an enzyme conjugated polymer complex adapted for automatic stainers from Roche Ventana Medical Systems, with 3-3' diaminobenzidine tetrahydrochloride as chromogen.

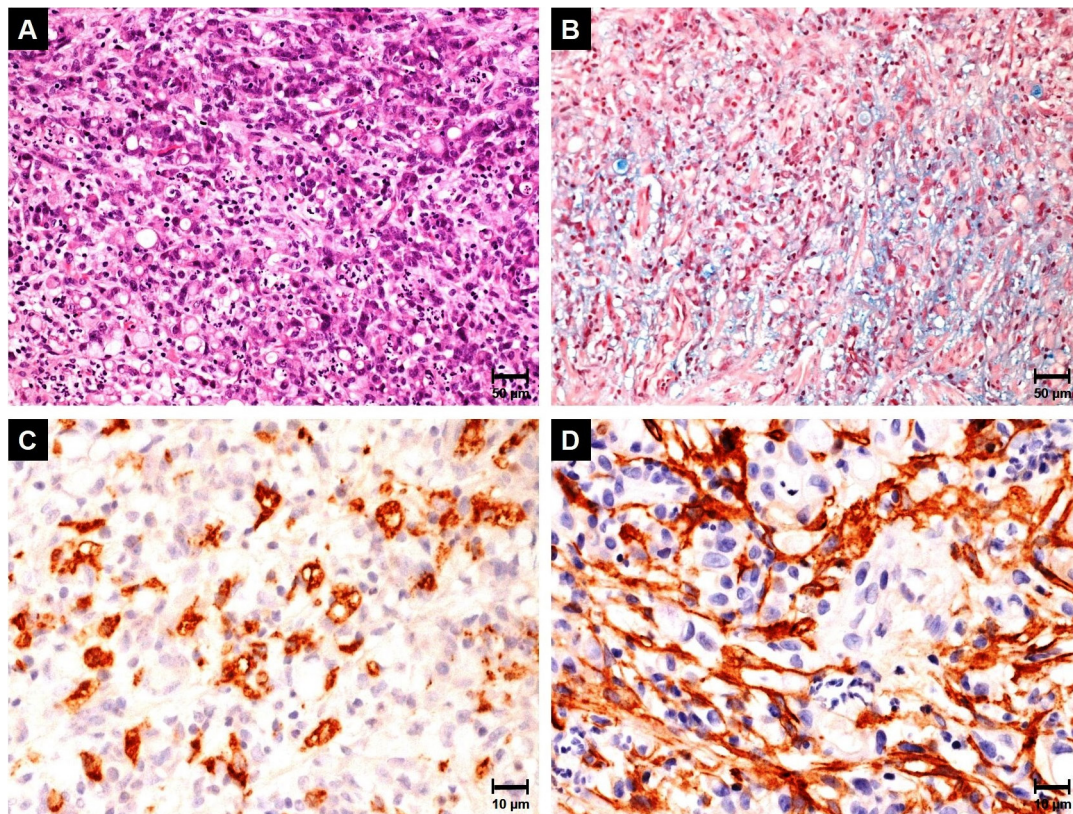


Figure 1. Gastric signet-ring cell carcinoma characterized by the presence of classical signet-ring cells (A, H&E, 20 \times): inside and outside of the neoplastic cells, accumulations of blue-stained sialomucins are observable (B, Alcian Blue, pH 2.5, 20 \times). Several histiocytes are intermingled with the cancer cells (C, CD68, 50 \times), and a diffuse deposition of collagen type IV is noticeable (D, collagen type IV, 50 \times).

Results

Inside the core of all primary tumors, only scanty CD68-positive macrophages (Figure 1) without neutrophils was observed. The apoptosis-derived fragments of neutrophil cells, usually present in other more differentiated carcinomas were absent. Around the primitive tumors, a neo-lymphangiogenesis was observed, represented by fine lymphatic podoplanin-positive capillaries, provided with a single layer of endothelial cells devoid of valves or other structures. Deposits of neoplastic cells were found inside in all cases. Interesting was the behavior of the signet-ring cells, typical constituents of this cancer. The abundant content of sialomucins present inside their cytoplasm was demonstrated by Alcian Blue staining (Figure 1). The accumulation of glycoconjugates rich in sialyl groups was further confirmed by both DDD-Fast Blue B and Mercury Orange staining methods for thiols. These cells did not aggregate in glandular-like structures, neither in the primary tumor, nor

in the metastatic lymph nodes. True budding of compact structures was therefore absent. On the contrary, in all the examined cases we observed a migration of these elements through the entire gastric wall in a procession-like fashion, coordinated to get away from the 'lymph nodules' in order to escape them. This surprising attitude was well highlighted by immunohistochemistry for cytokeratin (Figure 2). These nodules, containing different types of lymphocytes, were mainly situated in the gastric submucosa (Figure 2), but also in the muscularis and sub-serosa layer, where their number increased passing from stage II to stage III. They did not further evolve toward true lymph nodes, but originated active germinative follicles. The immunohistochemical characterization of the cell population demonstrated an increase of CD20-positive B lymphocytes and CD4-positive T-helper, but not of CD8-positive T-killer or CD56-positive natural (N) killer lymphocytes in all cases (Figure 2). Moreover, we noted the absence of perforin and granzyme B, molecules with a notorious cytotoxic activity. Besides, in

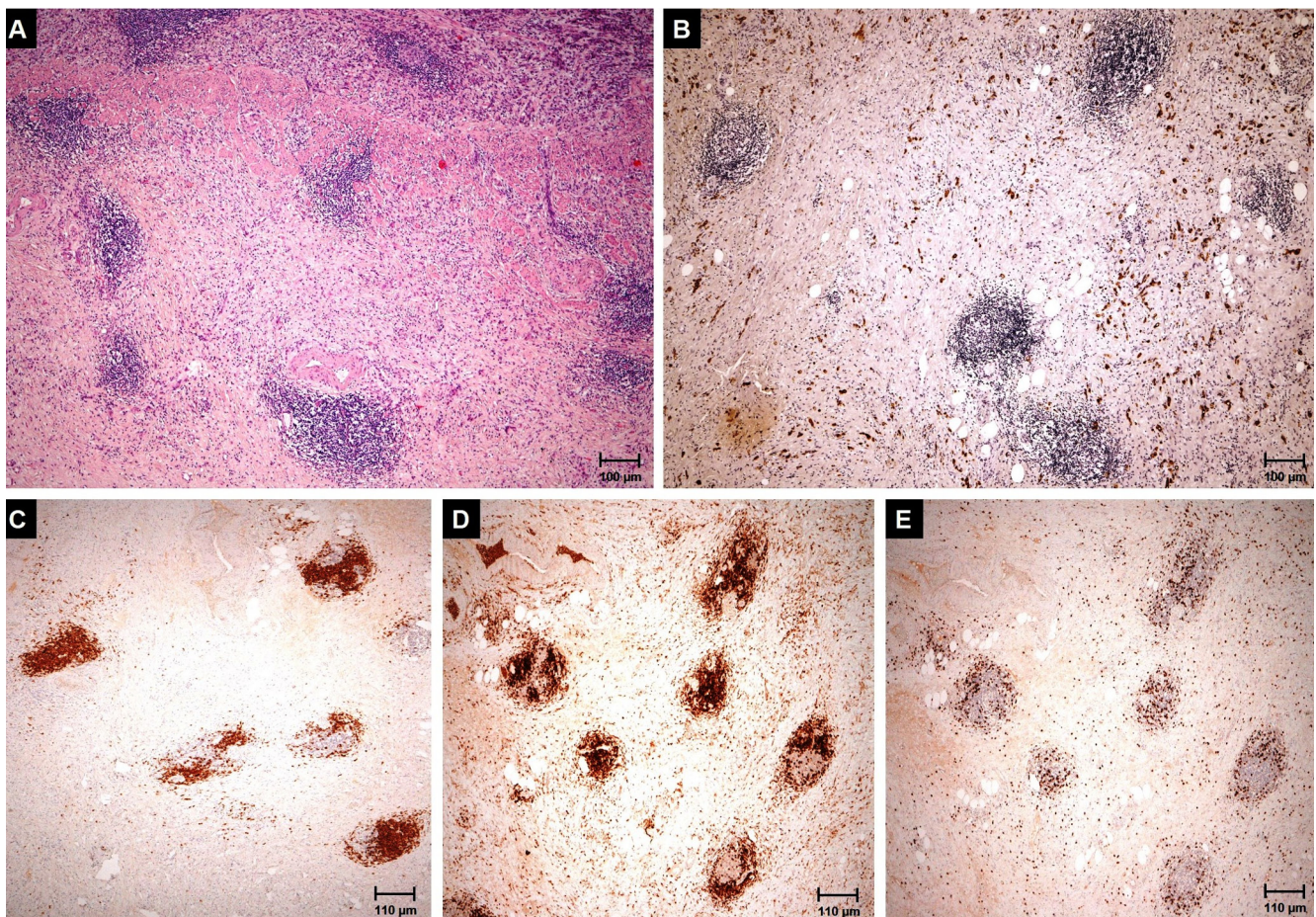


Figure 2. Numerous 'lymph nodules' in the submucosa of a stomach involved in signet-ring cell carcinoma (A, H&E, 5 \times); neoplastic cells, brown-stained by anti-cytokeratin, escape the 'lymph nodules' (B, cytokeratin, 5 \times). They consist of B lymphocytes (C, CD20, 5 \times), T-helper lymphocytes (D, CD4, 5 \times) and only scant T-killer lymphocytes (E, CD8, 5 \times).

the gastric sub-mucosa, we observed an increased number of CD10-positive fibroblasts, accompanied by an abundant deposition of collagen type IV in 100% of cases (Figure 1). The metastatic lymph nodes appeared located not following a continuous array, but scattered in the whole tributary zone. Inside of them we noticed the same cells, just ascertained in the 'lymphatic nodules'. A neo-lymphangiogenesis, equivalent to that of the primary tumor was associated in all cases (Figure 3). The lymph nodes not involved in metastasis showed a normal compartmentalization with a standard lymphocytic distribution in absence of neo-lymphangiogenesis.

Discussion

The abundant content of sialomucins inside the cytoplasm of the tumor cells gives them the typical signet-ring appearance and determines a negative electric charge on their surface. This opposes their contiguous standing and generation of budding and gland-like structures [4]. A cross-talking

develops between SRC and the tissue immune-competent cells, and involves the 'lymphatic nodules', which show germinal centers, increase in number, and become present along the entire gastric wall, also where they are usually absent. Their immunological activation is expressed by newly produced B-lymphocytes and T-helper, but not T-killer or N-killer lymphocytes, which could express a cytotoxic action against the neoplastic cells. This correlates well with the absence of corresponding markers of cytotoxicity, such as perforin and granzyme B. We are prone to believe that this immunosuppressive activity is performed by SRC cells through the selective release of cytokines, which by a paracrine action inhibit the recruitment of T-killer or N-killer lymphocytes, potentially active against the neoplastic elements [7]. This equally takes place in the lymphatic structures of the gastric wall and of the metastatic lymph nodes. Similarly, the numerous fibroblasts present in the sub-mucosa generate collagen IV, an extracellular component that does not seem to influence *per se* the diffusion of the neoplastic cells. However, the same

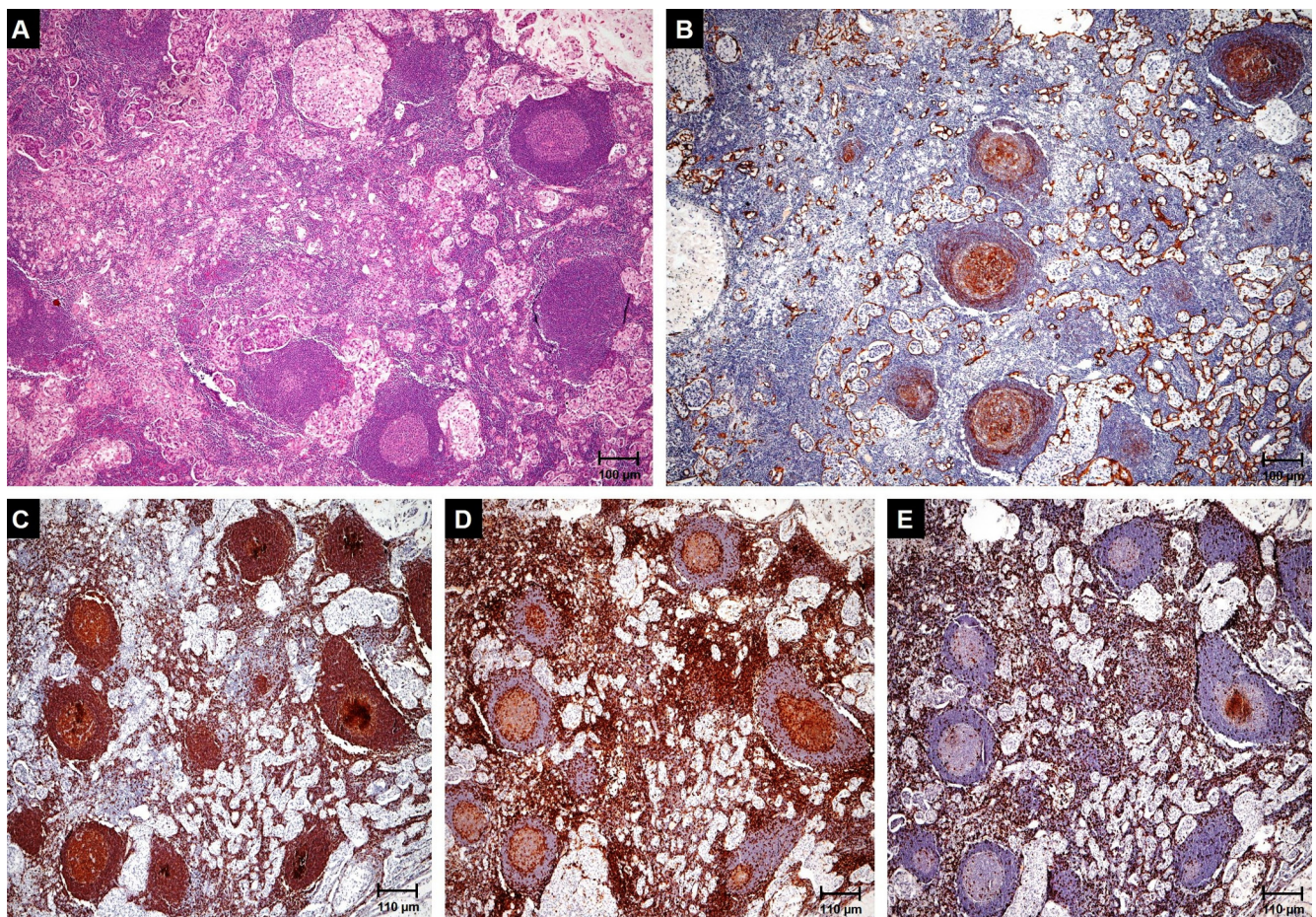


Figure 3. Nodal metastasis from gastric signet-ring cell carcinoma (A, H&E, 5 \times); the neoplastic nests evoke a conspicuous neo-lymphangiogenesis, invading the newly formed lymphatic capillaries (B, podoplanin, 5 \times). The residual lymph node shows a prevalence of B (C, CD20, 5 \times) and T-helper (D, CD4, 5 \times) lymphocytes, with only scant T-killer lymphocytes (E, CD8, 5 \times).

activated fibroblasts are considered as agents potentially favoring the growth and diffusion of the neoplastic cells [8–11]. The presence of macrophages inside the tumor and in the metastatic lymph nodes testifies their role in promoting the tumor growth and diffusion through a neo-lymphoangiogenesis stimulated by the release of humoral vascular growth factors. The absence of any other type of inflammatory circulating cells in the primary tumor or in the metastatic lymph nodes supports this interpretation, suggesting a particular nature and role of these macrophages [12–15]. The evident neo-lymphangiogenesis that accompanies the development of SRC, correlates with the great invasiveness of this cancer. It can be favored by various humoral factors released by different cellular components, directly or indirectly correlated with the tumor growth. Here, paracrine action can be considered [16–21]. The methodology of our study is rather not complete. It would be interesting to research the same histological aspects in the hepatic or pulmonary metastases from SRC, but the rapid and unfavorable course of the neoplastic disease made this impossible. We acknowledge that our results could be better and more appropriately interpreted, if disposing of equivalent complete bio-humoral correlations. This could not be performed by our observational retrospective methodology.

Our observations confirm SRC as a highly malignant tumor with a great local invasiveness and power of metastasizing. It is not accompanied by an effective immunological response. The contemporary process of neo-lymphoangiogenesis represents a direct consequence [22–25]. We hope that in a near future, the classification and staging of each gastric tumor case will include its immunological characterization, a complete study of its lymphatic apparatus, and of the immune response [26–33]. Our research represents a first approach to this problem, even if limited to a single particular gastric cancer.

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