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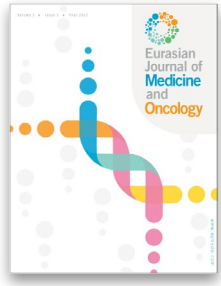

Research Article

Ki-67 Expression, Mast Cells Positive to Tryptase, and Angiogenesis in Gastric Cancer Patients Undergoing Radical Surgery

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Abstract

Objectives: Tumor proliferation is guided by neoangiogenesis; microvascular density (MVD) in the tumor micro-environment increases with the action of different immune system stromal cells such as the mast cells (MCs).

Methods: We evaluated Ki-67 proliferation rate, MCs positive to tryptase (MCPT), and MVD in a series of 85 gastric cancer (GC) tissue samples from patients undergoing radical surgery.

Results: In tumor tissue, a significant correlation between Ki-67 expression, MCPT, and MVD was found through Pearson t-test analysis (p ranged from 0.01 to 0.03).

Conclusion: Ki-67 expression and MVD may indicate the survival prognosis of patients and MCPT could repre-sent a biological marker of radical surgery and angiogenesis. Furthermore, MCPT could be consid-ered as a target of novel anti-angiogenic therapies in GC patients.

Keywords: Angiogenesis, gastric cancer, mast cells, surgical oncology, tumor microenvironment, tumor pro-liferation

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Gastric cancer (GC) is the fifth most common tumor in the world and the third cause of tumor-related death. It accounts for nearly 1 million cases annually, with East Asia accounting for more than half of those cases. In addition to incidence, the clinicopathologic characteristics of GC also differ among re-gions, especially Asia and the West. Compelling evidence indicates that GC is a heterogeneous dis-ease on the basis of anatomic site, histopathology, gene

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expression, gene amplification, DNA meth-ylation, relevant genetic aberrations, and oncogenic pathways.[1]

Cancer development is a multistep process characterized by genomic instability, gene expression dysregulation, and epigenetic abnormality that drive tumor progression. Gene mutations and mutant cells are constantly generated, but the immunosurveillance system detects and eliminates these cells. However, immune-resistant cells evolve sophisticated strategies to evade the immune system and go on to generate tumors. Various strategies are applied both in the prevention, and in the surgical and medical treatment of GC. In this regard, tumor markers (TMs) play an important role, especially in de-fining the prognosis of GC. CA 19.9, CEA, CA 72.4, CA 125, AFP, and CA 242 are some TMs, whose value correlates with the overall survival (OS) in GC.[2]

Moreover, TMs can represent the biological and histopathological characteristics of tumor cells; their levels can be associated with patient survival and can lead to a specific treatment option. The analysis of TMs can help the surgeon or oncologist predict the prognosis of GC.

A lot of published studies indicate the strong link between tumor microenvironment and epithelial tu-moral cells.[3, 4] To this regard, several reports in animal and human tumors indicate that mast cells (MCs) positive to tryptase (MCPT) stimulate microvessel density and increased of microvessel densi-ty paralleled with Ki-67 nuclear proliferation protein and these markers have been correlated with bio-logical aggressiveness of tumor and prognosis.[5-8] With special reference to GC, no studies have been published taken together all the three above parameters.

To be specific, Ki-67 is a TM used for its prognostic value in different tumors such as pancreatic duc-tal adenocarcinoma.[9]

Ki-67 is a nuclear protein, well-characterized, and encoded by the MKI-67 gene, which plays a role in the transcription of ribosomal RNA and cell proliferation; moreover, it is involved in proliferation and the development of tumors.[10, 11]

Some studies have shown that Ki-67 is activated in the G1, S, and G2 stages of the cell cycle during mitosis, but not in the G0 stage; thus, Ki-67 can be considered a biomarker of cell growth and prolif-eration.[12, 13]

MCs can also play a role in the prognosis of GC. Indeed, MCs are involved in tumor angiogenesis and have been studied in human tumor and in spontaneous animal tumor models.[14-16]

MCs enable the production of typical pro-angiogenic factors, such as vascular endothelial growth fac-tor (VEGF), fibroblast growth factor-2, and thymidine phosphorylase and can also secrete atypical pro-angiogenetic factors.[17, 18] Among these, tryptase is the most common factor accumulated in MCs secretory granules; it can stimulate endothelial cells (ECs) proliferation and induce microvascular pro-liferation in tumor cells by binding with protease-activated receptor-2 (PAR-2) expressed on ECs;[19, 20] moreover, tryptase can alter the composition of extracellular matrix increasing neo-angiogenesis.[21]

It is pivotal to underline how Ki-67 and MCPT increase in the tumor microenvironment (TME) of GC, contributing to enhance the neoangiogenesis process, immune evasion, and metastases niche formation.[22]

In this retrospective study, we evaluated the immunohistochemical expression of specific immune stromal cells in tumor microenvironment and angiogenesis. In particular, have been correlated Ki-67 proliferation rate, MCPT and tumor neo-angiogenesis in the transition from adjacent normal tissue (ANT) to tumor tissue (TT). We also speculate the possible role of three analyzed parameters as pos-sible panel, to predict surgical radicality and survival. We believe in fact that, part of these cells or one of their release factors could stay in the stroma, but another part could pour into the lumen of the blood and lymphatic vessels with metastatic effect. Finally, we believe that it is possible to slow tumor growth adopting anti-angiogenic tailored treatment and inhibiting the specific cells studied.

Methods

Study Population

A series of 85 GC patients diagnosed by means of preoperative gastric endoscopy were selected for radical surgery. The surgical techniques were open total and subtotal gastrostomy with D2 lymph node dissection. The selected cases were staged as T2-3N2-3M0 (by AJCC for GC 8th Edition) accord-ing to the American Joint Committee on Cancer 8th edition (AJCC-TNM) classification.[23-26] Clinical staging was performed through Full-Body CT Scan. All enrolled patients had adenocarcinomas. The main clinical-pathological characteristics of the patients are reported in Table 1. The research was de-veloped according to the Declaration of Helsinki, and the study was approved by the Ethics Commit-tee of the "Mater Domini" Hospital, "Magna Graecia" University, Catanzaro ((N° 242; 22 December 2016). Signed consent from each patient was obtained.

Immunohistochemistry

Ki-67 expression, MCPT, and microvascular density (MVD) were detected through immunohisto-chemistry using a three-layer biotin-avidin-peroxidase system.[27] 6 µm-thick

Table 1. Clinico-pathological features of patients (n=85)

	n
Age	
≤65	34 (40%)
≥65	51 (60%)
Gender	
Male	48 (56%)
Female	37 (44%)
Tumour site	
Cardia	13 (15%)
Lesser curvature	8 (9%)
Greater curvature	10 (12%)
Body and fundus	25 (30%)
Pyloric area	29 (34%)
TNM by AJCC Stage and type by Lauren Classification	
$T_{2\text{-}3}N_2M_0$	52 (61%)
$T_{2\text{-}3}N_3M_0$	33 (39%)
Intestinal type	50 (59%)
Diffuse type	35 (41%)
Histologic grade	
G1-G2	64 (75%)
G3	21 (25%)

serial sections of forma-lin-fixed and paraffin-embedded TT and ANT were cut. Obtained slides were processed with a mi-crowave oven at 500W for 10 min, and then, the endogenous peroxidase enzyme was inhibited with 3% hydrogen peroxide solution. Subsequently, slides were stained with the following primary anti-bodies: anti-tryptase (clone AA1; Dako, Glostrup, Denmark) diluted 1:100 for 1 h at room tempera-ture (for MCs identification), anti-Ki-67 (MIB-1; Immunotech, Inc., Marseilles, France) diluted 1:100 for 1 h at room temperature (for proliferation index), and anti-CD31 antibody (QB-END 10; Bio-Optica Milan, Milan, Italy) diluted 1:50 for 1 h at room temperature as a pan-endothelial marker. The immunoreactivity was highlighted employing a biotinylated secondary antibody, an avidin-biotin pe-roxidase complex red chromogen (LPS, K0640, Dako, Glostrup, Denmark). Cell nuclei were stained utilizing Gill's hematoxylin no.2 (Polysciences, Warrington, PA, USA). No primary antibody was employed in negative controls.

Morphometrical Assay

Light microscopy integrated with an image analysis system (AXIO, Scope A1, ZEISS, Germany) was utilized.[27] For each serial section of TT and ANT, five most immunostained areas (hot spots) were selected at low magnification. Subsequently, Ki-67 expression, MCPT, and MVD were as-sessed at x40 magnification (0.19 mm^2 area) in the five identified hot spots for each serial section, re-spectively (Figs. 1-3a-b).

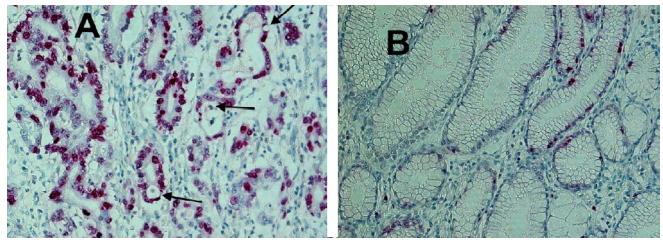

Figure 1. (a) Gastric cancer tissue and high rates of proliferation stained with anti-Ki-67 antibodies. Single ar-rows red-stained proliferating nuclei (x40 magnification). **(b)** Case control in normal tissue.

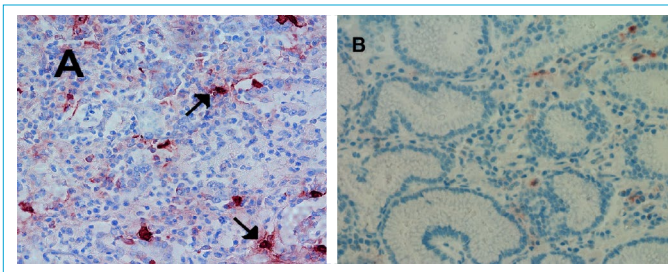

Figure 2. (a) Gastric cancer tissue and red-stained mast cells positive to the anti-tryptase anti-body. Arrows in-dicates single mast cells near microvessels (x40 magnification). **(b)** Case control in normal tissue.

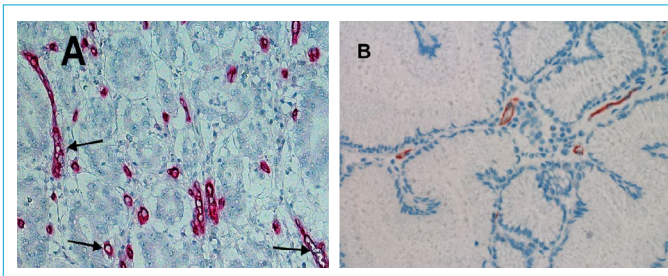

Figure 3. (a) Gastric cancer tissue and red-stained microvessels positive to anti-CD31 antibody. Arrows indicates single microvessels with a visible lumen (x40 magnification). **(b)** Case control in normal tissue.

Statistical Analysis

Mean value for each section and in the global series was obtained for all the analyzed parameters in both TT and ANT groups. Difference between groups was measured by Student's t-test. Mean values ± 1 Standard Deviation (SD) of all the evaluated tissue parameters is reported in Table 2.

Correlations between Ki-67 expression, MCPT, and MVD were calculated using Pearson's (r) analy-sis (Fig. 4). Correlations among all the analyzed parameters and the main clinical-pathological features listed in Table 1 were performed by the Chi-square test (χ^2). All analyses were considered statistically significant with $p<0.05$. Statistical analyses elaboration was performed with the SPSS statistical soft-ware package (SPSS, Inc., Chicago, IL).

Table 2. Ki-67 (MIB-1), MCPT and MVD means±SDs as a function of tumor tissue (TT), adjacent normal tissue (ANT) and statistical significance of their differences by t Student-test

Tissue type	MVD x400 magnification (0.19 mm² area)	MCPT x400 magnification (0.19 mm² area)	Ki-67 positive fraction in terms of MIB-1 positive nuclei, x400 magnification (0.19 mm² area)
ANT	13±5[a]	5[a]±3	20±5
TT	31±7[a]	14[a]±4	68±15
p (t-test)	p<0.05	p<0.05	p<0.05

[a]Mean±1 standard deviation.

Results

Immunostaining shows that an increase in Ki-67 proliferation index, MCPT, and MVD can be found in stromal microenvironment, predominantly in TT compared to ANT (Figs. 1-3a and b). For each se-rial section of TT and ANT, five most immunostained areas (hot spots) were selected and parameters were counted in each hot spot; the mean value for each section and in the global series was calculated (Table 2).

The mean values±SD for Ki-67 expression, MCPT, and MVD were, respectively: 68±15 in TT and 20±5 in ANT, 14±4 in TT and 5±3 in ANT, 31±7 in TT, and 13±5 in ANT. These differences were statistically significant (p<0.05; Table 2).

In TT, a higher Ki-67 expression was associated with an increase of MCPT and MVD supporting the hypothesized role of stromal cells in tumor proliferation and progression.

Statistically, a significant correlation between Ki-67 expression and MVD (r=0.63, p<0.05), MCPT and MVD (r=0.68, p<0.05), Ki-67 expression, and MCPT (r=0.66, p<0.05) is shown (Fig. 4).

In fact, a linear Pearson correlation tending to 1 makes us understand the strength of the relationships between the three variables.

Although this is a preliminary study, giving a look at the mean values reported above, the Ki-67 iden-tification in TT underlines how stromal cells, in particular MCPT, develop and sustains TME in GC. Our goal is to increase the recruitment of patients, thus, to evaluate the prognosis (as OS or disease-free survival) in relation with the three parameters and to define better molecular relationships between MCPT and GC.

Discussion

The surgical treatment of GC is technically complex and sometimes follows a late diagnosis; the 5-year survival rate in Europe oscillates from 10% to 30%.[28]

The aim of our study is to analyze the correlation between Ki-67, MPCT, and MVD and their changes in the transition from ANT to TT. The possible role of prognosis of these three parameters in TT is also discussed. Our data indicated a good correlation each to other between the three analyzed bi-omarkers in particular in TT. It is interesting underline that all studied TM increased in TT supporting the biological network among them and the interaction between tumor and stromal microenvironment. A panel including a higher ki-67 proliferation rate, a higher MCPT, and MVD suggested a more bio-logical aggressive disease and may suggest a prognostic role. To this regard, various studies have suggested that high Ki-67 expression was associated with poor OS in GC patients.[29-35]

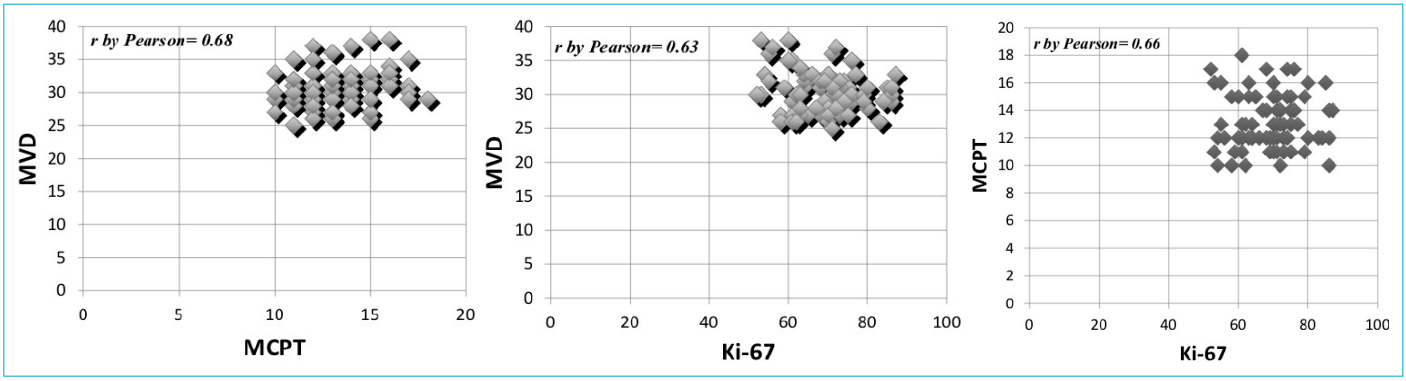

Figure 4. Correlation analysis between Ki-67 proliferation rate, MCPT and MVD in gastric cancer tissue. MCPT and MVD (r= 0.68; p<0.05); and Ki-67 and MVD (r=0.63; p<0.05); Ki-67 and MCPT (r=0.66; p<0.05).

In a meta-analysis, Liu et al. have demonstrated already that Ki-67 levels in 3,825 patients with GC, who underwent radical surgery, were dramatically increased especially in patients with poor progno-sis. This aspect can facilitate the use of Ki-67 as a predictive TM in patients with GC.[29]

Histopathological studies conducted on 85 patients show a high Ki-67 proliferative rate, perhaps relat-ed to the specific cell cycle stages, in which Ki-67 is expressed.[12 ,13] Tumor cells have a strong repli-cative rate, and Ki-67 can be used to detect the cell proliferation rate.

Even though there are few studies linking Ki-67 and tumor neoangiogenesis, we suppose that the role of Ki-67 as TM can be reinforced by the relevant impact that MPCT plays in angiogenesis. In tumor progression, both high replicative cellular rate and neoangiogenesis are the potential targets for novel tailored therapies and surgery.

The angiogenesis in GC is supported by various biological events; in particular, we have focused on MCPTs.[36-38] Tumor angiogenesis is defined by the formation of new blood vessels from the pre-existing vascular network, determined by an increase of vessel permeability, degradation of extracellu-lar matrix, promoted proliferation, differentiation, migration, and motility of ECs.[39-42]

Stromal cells in TME, especially tumor associated macrophages (TAM) and MCs, induce microvessel formation by means of proangiogenic factors. For example, the stem cell factor has an influence on the C-kit receptor; VEGFs stimulate VEGFR1 and VEGFR2; angiopoietin-1 acts on TIE-2 receptor; and CXCL-8 acts on CXCR-1 and CXCR-2. Some chemokines (CCL-2, CCL-5, CXCL-1, CXCL-10, and CXCL-12) produced by tumor and stromal cells activate their specific receptors (CCR-2, CCR-3, CXCR-2, CXCR-3, and CXCR-4), which are important for TAM localization in TME.[43-46, 19]

Alongside, MCPT produces tryptase that facilitates neovascularitation; moreover, tryptase is the ago-nist ligand of PAR-2 on vascular ECs, which induces their proliferation: following PAR-2 stimula-tion, the intracellular signal activates the MAPK phosphorylation pathway leading to EC proliferation and neoangiogenesis.[47-74, 20]

Immune stromal cells density is increased in cancer and there is a correlation with angiogenesis. These cells exert a pro-tumorigenic role in cancer through the release of classical and non-classical angiogen-ic factors, so high proliferation index associated with a neoangiogenic process and a greater presence of MCs in TT is related to tumor progression and therefore a worse prognosis. Thus, immunohistochemical analysis of endoscopic diagnostic biopsies or serum levels of novel biomarkers could guide us on the state of the disease before surgical treatment.

Conclusion

We propose that the MCPT and Ki-67 pair may be considered as a marker of angiogenesis, tumor pa-rameters growth, and proliferation in GC.[32-33, 44] Taken together these three biomarkers may suggest a novel panel of cancerogenesis and tumor malignancy due to the increasing value from ANT to TT. From a prognostic point, we speculate that the panel may be evaluated to correlate it with survival in future studies. Intriguingly, a higher ki-67 proliferation rate, MCPT count, and MVD may be targeted by means of several agents (e.g., trabectedin, peptide M2, PLX-3397, STI-571, and AB-1010) and available tryptase inhibitors such as Gabexate or Nafamostat mesilate.[75, 76]

Disclosures

Ethics Committee Approval: The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Ethics Committee Regione Calabria Section Area Center A.O.U. Mater Domini Catanzaro (protocol code no 242, date of approval 22 December 2016).

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Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: All relevant data are included in the article.

Conflicts of Interest: The authors declare that they have no competing interests.

Authorship Contributions: Conceptualization – M.A., G.A., G.R.; Methodology – F.V., R.P., G.C.; Validation – M.L.; Formal analysis – G.N.; Investigation – S.C.; Resources; R.M.; Data curation – V.Z., C.L.; Writing original draft preparation – G.C., G.R.; Writing review and editing – M.L.; Visualization – G.C., G.N., V.Z.; Supervision – R.P., G.R.; Project administration – M.A., G.R.

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