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ORIGINAL ARTICLE

Clinical significance of neoangiogenesis and index of proliferation in the signet ring type of gastric adenocarcinoma

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Summary

Purpose: *The purpose of this study was to examine whether microvascular density and the level of proliferation in gastric signet ring cell carcinoma (SRCC) are important factors in the locoregional control of the disease.*

Methods: *Over a period of eight years, gastric resection specimens from 37 patients were examined. The proliferative index (labelled by Ki67) and microvascular density (MVD) index (mvdIDX) (labelled by CD105) were determined for each case of SRCC.*

Results: *Gastric SRCC was diagnosed more often in female than in male patients (21 females, 16 males ; p≤0.05) . The average age of female patients was 63 years, while the male patients were 62 years old on average (p=0.702). Immunohistochemical analysis showed that the median numbers of Ki67 positive cells and CD105 positive blood vessels were higher in tumors compared to surrounding non-tumor tissue. Higher proliferative index and higher mvdIDX were also established relative to tumor stage. Correlation analysis showed a high positive correlation between proliferation index and microvascular density (MVD) index (mvdIDX) (correlation coefficient=0.784). Receiver operating characteristics (ROC) analysis showed progression of both indices examined.*

Conclusion: *Our results showed that, although both proliferative and mvdIDXs are reliable, the former had better performance in identifying of disease progression (AUC=0.970).*

Key words: *gastric signet ring cell carcinoma, microvascular density index, proliferative index*

Introduction

Despite the fact that, in the last few decades, carcinoma of the stomach has declined in incidence, it is still the third commonest cause of death and, with 723,000 cases in 2012, represents an alarming problem worldwide [1]. In central Serbia, in the period from 2002 until 2011, a growing trend in mortality has been noted in both sexes, with the mortality rate being 1.3 times higher in the male population [2].

At a global level, it has been noted that the incidence of SRCC, differing from the other variants, is rising constantly, so that it makes up 8-30% of gastric malignancies [3,4]. It appeared for the first time as a specific histological entity in the 1990 World Health Organization (WHO) classification [5], but from 2010, the revised WHO classification classified this tumour as a poorly cohesive carcinoma in the adenocarcinoma group [6]. Signet ring cell adenocarcinoma is defined as a tumor composed primarily (more that 50% of tumor) or exclusively of signet ring cells that are characterized by a mucin-rich cytoplasm that pushes the nucelus to

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the cell periphery (the so called, "signet ring" appearance) [4]. There are several differences in the histological pattern of tumor growth and in biological behaviour between SRCC and the other types of gastric adenocarcinoma. First, a precancerous lesion has not been defined. Second, it is composed of poorly cohesive cells that diffusely infiltrate the gastric wall with a few or no glandular structures. Third, it is diagnosed in a younger group of predominantly female patients. And fourth, unlike the other variants of gastric adenocarcinoma, which have a tendency to metastasize to the liver, SRCC spreads directly to the peritoneum and adjacent organs [4,6,7].

Tumors require angiogenesis to grow beyond a certain volume; and angiogenesis is also necessary for the growth of metastases. Moreover, angiogenesis plays a significant role in unrestrained tumor cell proliferation, helps local and distant processes of tumor invasion and increases the risk of recurrence [8,9]. Tumor angiogenesis is also an important prognostic factor, and tumor neoangiogenesis is an interesting target for chemotherapy [9,10].

The purpose of this work was to examine the MVD and the level of proliferation in SRCC as two important factors in the locoregional control of the disease.

Methods

Patients and tissue samples

This study included 37 patients (16 men, mean age 61.7 years, range 41-84, and 21women, mean age 63.2 years, range 32-82) with SRCC who had been operated on in the Surgical Clinic of the Clinical Hospital Center Zemun (CHCZ) from 2009 to 2017. In the Department of Clinical Pathology of the CHCZ, according to standard protocols, 5-15 tissue samples were taken from the tumor, as well as 2-3 tissue samples of the surrounding non-tumor gastric tissue, from each operative specimen, depending on tumor size. After fixation in 10% neutral buffered formalin solution, the tissue samples were processed by routine histopathologic methods. The gastric SRCC tissue samples constituted the study (test) group, while the samples from the surrounding non-tumor tissue were the control group. The study protocol was approved by the CHCZ Ethics Committee.

Histopathology

Serial sections, 3-5 μm in thickness, were cut from the paraffin blocks of all the tissue samples of tumor and regional lymph nodes. Routine hematoxylin-eosin staining was performed for histopathological analysis of the lesions and the following features were recorded: histopathological type of tumor, histological tumor grade, depth of invasion, invasion of lymph vessels, lymph nodes' histology, angioinvasion, perineural invasion, inflammatory response, desmoplasia and necrosis.

Immunohistochemical examination

The nuclear antigen Ki67 was used to assess proliferation. For the assessment of angiogenesis CD105 (Endoglin) was used and, based on its expression, the MVD in the tumor and in the peritumoral stroma were evaluated.

Representative tissues 3μm thick were heated at 55°C to melt the paraffin, deparaffinised in xylene (3-5 min) and then rehydrated through graded ethanol. Antigen retrieval was enhanced by autoclaving slides in sodium citrate buffer (pH 6.0) for 30min. Endogenous peroxidase activity was blocked by 0.3% hydrogen peroxide-methanol buffer for 25 min. To reduce nonspecific background staining, the section was incubated with 10% bovine serum albumin (BSA) for 30 min at room temperature. Rabbit monoclonal Ki67 antibody (Abcam, Cambridge, UK 1:100) and monoclonal mouse-anti human CD105 antibody (Dako, Copenhagen, Denmark; Clone SNGh, 1:10) were incubated at 4°C overnight. Immunostaining was performed using the avidin-biotin-peroxidase complex (ABC) method (Vectastain ABC-*Elite* kit, Vector Laboratories, Burlingame, CA). Staining was visualized with 3,3-diaminobenzidine tetrachloride (DAB). The slides were counterstained with Mayer's hematoxylin and mounted in Canada balsam. Negative controls were done by replacing the primary antibody with phosphate buffered saline (PBS).

Quantification of immunohistochemical staining

For the purpose of evaluating the expression of Ki67, only stained nuclei were taken into account. The multipurpose test system M42 by Weibel [11] was used for the designation of density of Ki67-positive cells per mm^2. An objective micrometer (Leica, Biosystems, Nussloch, Germany) 2mm/200 was used to determine the measuring area of 0.016 mm^2. Ki67 positive cells/mm^2 were counted successively in 10 "hot spots" and the absolute value of the density of positive cells in each "hot spot" was determined stereometrically [12]. The arithmetic mean of the values of the "hot spots" gave the final number of Ki67 positive cells per mm^2 for each case. The median was subsequently determined and the absolute values of the density of positive cells were divided into two groups: those with low expression level (value ≤ the median value) and those with high level of expression (value> the median value). These values represented the proliferative activity (proIDX).

MVD was calculated by counting microvascular CD105 positive structures at a total microscopic magnification of 400 x. First, areas with the highest MVD ("hot spots") were selected. Every single cell or field marker was counted as a microvascular structure. The multitasking test system M42 according to Weibel [11] was also used for the determination of MVD, using an Olympus BH-2 microscope with a field size of 0.016 mm^2. For the investigation of MVD per mm^2, 10 "hotspots" were counted successively, and the absolute value of positive vascular structure density in each "hotspot" was determined stereometrically [11]. The final result was from the study of 10 consecutive fields on average. After obtaining data regarding the number of microvascular

structures for each patient, the median was determined, according to which the patients were divided into two groups: those with low grade of angiogenesis (MVD in tumor ≤ than median value) and those with high grade of angiogenesis (MVD> than median value). The mvdIDX was obtained from the absolute values of MVD regarding deviation from the median.

Statistics

Median levels of nuclear proliferation antigen Ki67 and hematopoietic progenitor cell antigen CD105 were compared between the groups of the investigated tissues from gastric SRCC and healthy tissues (control group) by means of the non-parametric Mann-Whitney U test. Other clinicopathological variables between the groups of low and high index of nuclear proliferative antigen and neoangiogenesis index were compared using Student's t-test for continuous variables. Statistical analysis was performed using x^2 or Fisher's exact tests and Mann-Whitney U test for nominal or ordinal variables. Spearman's rank correlation coefficient was used to analyse correlation between variables. Statistical analysis was carried out using SSPS 20.0 software. P values less than 0.05 were considered statistically significant.

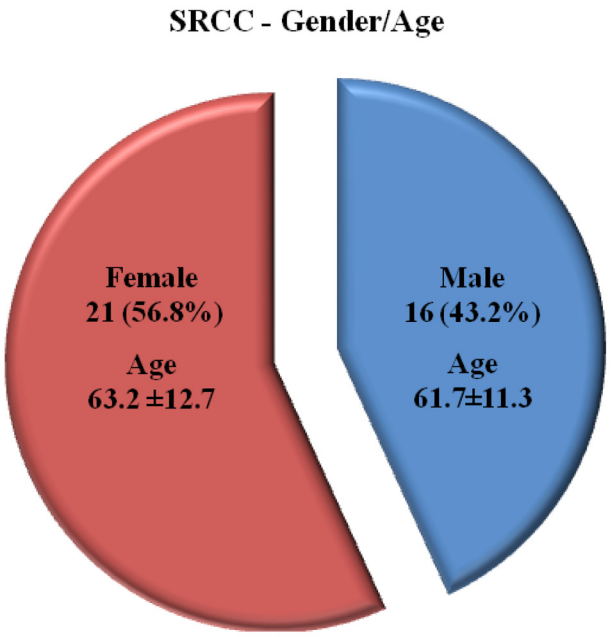

Figure 1. Distribution of patients with SRCC of the stomach by gender and age

Results

The tumors were composed of single or groups of malignant cells with intracytoplasmic mucin and with the nucleus displaced to the cell periphery. At low power, the cells diffusely infiltrated the lamina propria, spreading between the glands and foveolae. Focally, the malignant cells were localized in small groups in the lamina propria between normal and dysplastic or metaplastic glands. The tumor cells mostly formed a trabecular or solid growth pattern and in isolated cases there was conspicuous desmoplasia in the deeper layers of the gastric wall.

The distribution of patients with SRCC showed that there were more female patients, about 57%, in contrast to 43% of male patients, but this was not statistically significant at the accepted level of confidence (x^2 test, p=0.411; Figure 1).

The average age of women with gastric carcinoma was about 63 years (the youngest patient was 32, the oldest 84), while men were 62 years old on average (the youngest 41, the oldest 84). Again, this difference was not statisticaly significant (t-test, p=0.702; Figure 1).

Immunohistochemical expression of Ki67 and CD105 in signet ring cell gastric carcinoma

Absolute values of the densitiy of positive cells per mm^2 were obtained by immunohistochemical examination of expression of the nuclear proliferative antigen Ki67 and CD105. The basic caracteristics of those values (median, minimal and maximal values, deviations), are shown in the boxplot diagram (Figures 2 and 3).

The median number of Ki 67 positive cells per mm^2 in the cases of SRCC was significantly higher than the median Ki 67 positive cells per mm^2 in the adjacent non-tumor tissue (2081.8 vs 487.7, p<0.001, Mann-Whitney U test; Figure 2).

The median number of CD105 positive cells per mm^2 in SRCC was also significantly higher than the median number of CD105 positive cells per mm^2 in the adjacent non-tumor tissue (749.5 vs 71.4, p<0.001, Mann-Whitney U test; Figure 3).

Relationships between expression of Ki67 and other clinicopathological variables

When the patients with gastric SRCC were grouped by the level of expression of Ki67 relative to the median, there was no significant difference in the distribution of cases regarding age and gender in the group with a high proliferation index (proIDX) compared to the group with a low index level (Table 1).

A high proIDX was noted in each case of poorly differentiated SRCC. Simultaneously, a low proIDX was present in 55.6% of poorly differentiated tumors and 44% of moderately differentiatied tumors, which was not statistically significant (p=0.116; Table 1).

High proIDXs were noted in a significantly higher number of the pT3 (73.7%) and pT4 (26.3%) tumors; moreover, 100% of tumors with a high proIDX were pT3 or pT4 (Table 1). A low level of proIDX was noted in 38.9% of pT3 and 5.6% of pT4

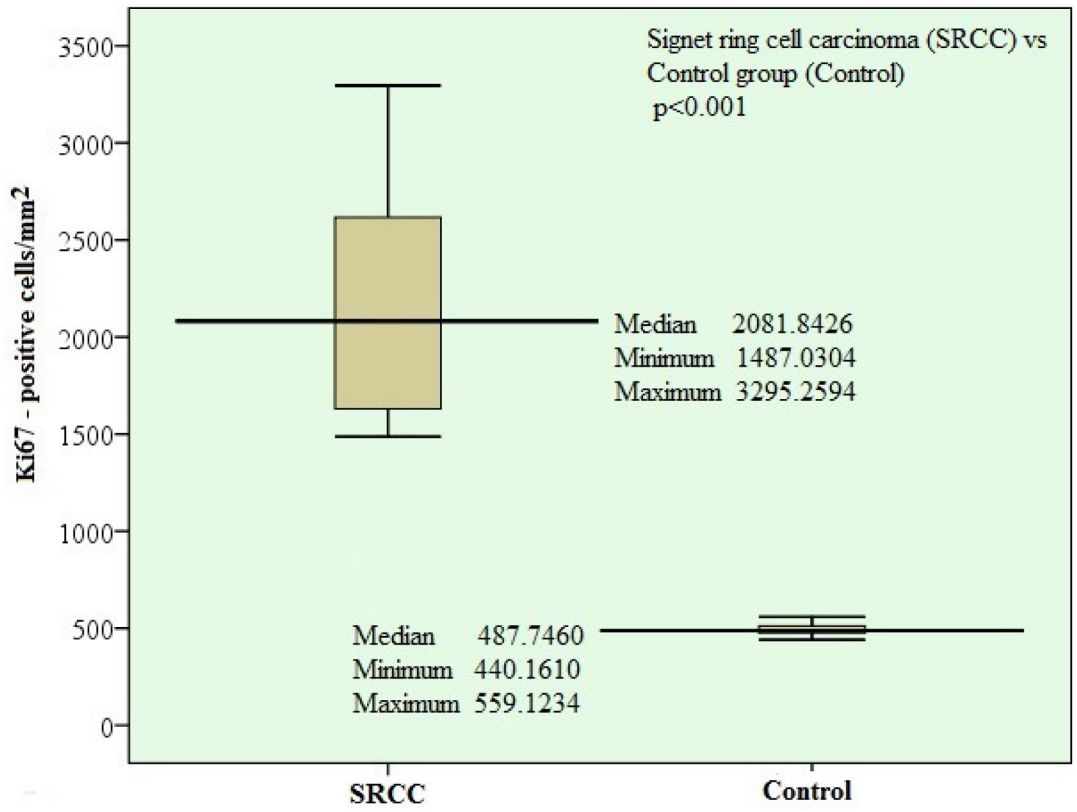

Figure 2. Ki67 positive cells per mm² of the investigated tissues. The median number of Ki67 positive cells/m² in the SRCC cases was significantly higher compared to the adjacent non-tumor tisue (Mann-Whitney U test, p<0.001).

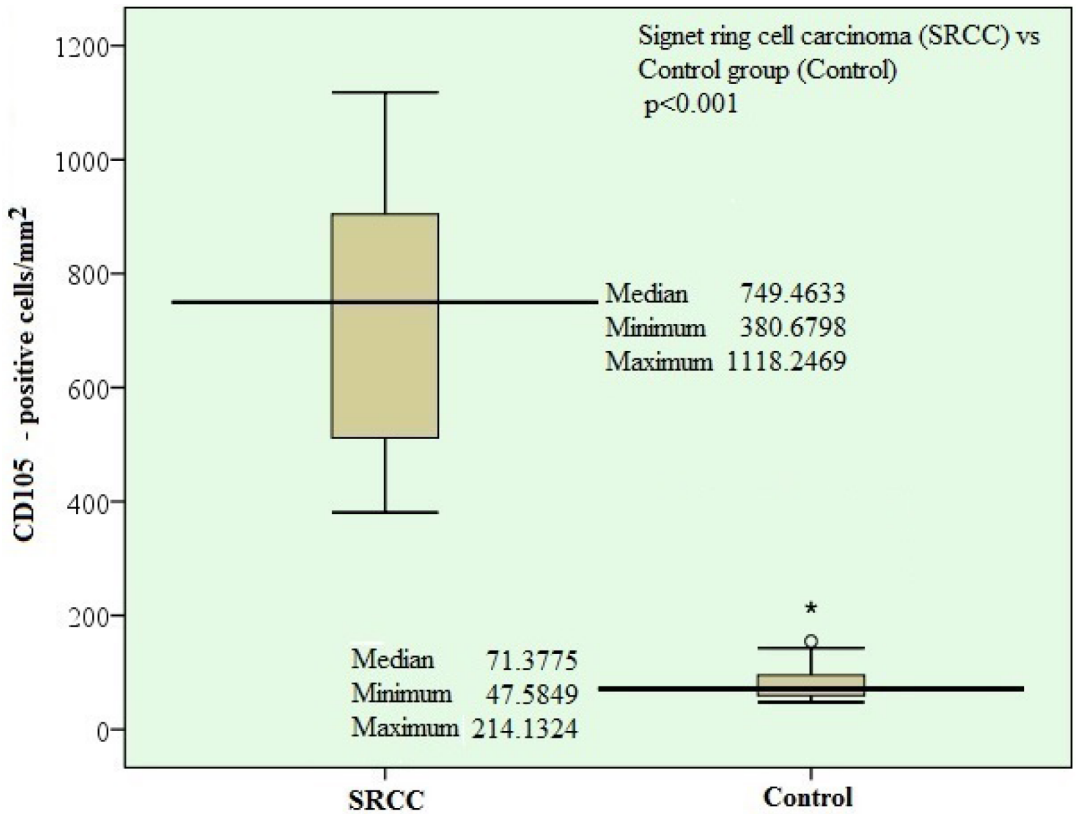

Figure 3. CD105 microvascular density in mm² of the investigated tissues. The median number of CD105 positive cells/mm² was significantly higher compared to adjacent non-tumor tissue (Mann-Whitney U test, p<0.001). *median number of CD105 positive cells/mm² in the control group.

tumors; 44.5% of the cases with a low proIDX were pT3 or pT4, which was not significantly different from the proportion of low index tumors that were pT1 or pT2 (55.5%, p=0.117). Based on this distribution of pathologic tumor stages, groups with low and high proIDXs were significantly different (p<0.001; Table 1).

A high proIDX was present in 100% of the cases with metastases in at least 3, 7 to 15 and in over 16 lymph nodes, while a low proIDX was present in a significant number of cases (77.8%) without metastasis in the regional lymph nodes. Based on this distribution of metastasis in the regional lymph nodes, groups with low and high proIDXs were significantly different (<0.001; Table 1).

100% of tumors with a high proIDX showed lymph vessel invasion, whilst there was no significant difference between the presence or absence of lymph vessel invasion in tumors with a low proIDX (55.6 vs 44.4%, p=0.116).

High proIDX was present in a larger number of tumors with blood vessel invasion, but not significantly in comparison with the number of tumors without vascular invasion (57.9% to 42.1%, p=0.216). A low proIDX was noted in a significant number of tumors with no blood vessel invasion (77.8%; Table 1).

A high proIDX was present in 100% of the cases of stage III and IV SRCC while a low proIDX was present in 100% of stage I-II tumors; this difference was statistically significant (Table 1).

The distribution of macroscopic type and tumor localization relative to the level of proIDX expression did not show a significant difference (Table 1), but it was noted that a higher proIDX level was present more often in the ulceroinfiltrative macroscopic tumor type. It was also noted that a high proIDX was present in a larger number of tumors that were located in the lower half of the stomach (57.9%).

Relationships between expression of CD105 and other clinicopathological variables

No significant difference was noticed between the group with high mvdIDX and the group with low mvdIDX by distribution of cases according to age and gender and relative to MVD index (mvdIDX) (Table 2).

High expression of mvdIDX was present in all cases (100%) of poorly differentiated signet ring carcinoma. Low mvdIDX was present in 55.6% of poorly differentiated tumors and in 44.4% of moderately differentiated tumors, which was not statistically significant (p=0.116). However, mvdIDX was significantly associated with lesion grade overall (p= 0.020; Table 2).

High mvdIDX was noted in a significantly higher number of pathological stage pT3 (68.4%) and pT4 (31.6%) tumors (100% of cases in total). A low mvdIDX was present in 44.4% of pT3 stage tumors, which did not differ significantly relative to 55.6% of the remaining pT1 and pT2 stages in this group (p= 0.116; Table 2).

High mvdIDX was present in 89.5% of the cases of signet ring carcinoma with metastasis in at least 3, 7 to 15 and over 16 lymph nodes. Low mvdIDX was present in a significant number of cases (66.7%) in signet ring carcinomas with no regional lymph node metastasis. Based on this distribution of regional lymph node metastasis, groups with low and high mvdIDX differed significantly (<0.001; Table 2). 94.7% of tumors with a high mvdIDX showed lymphovascular invasion while lymphovascular invasion was present or absent equally (50% each) in cases with a low mvdIDX (Table 2).

Blood vessel invasion was present in a greater number of tumours with a high mvdIDX but not significantly relative to tumors without vascular invasion (63.2 vs 36.8%, p=0.251), while no blood vessel invasion was found in a significant number of tumors with a low mvdIDX (83.3%)(Table 2).

Expression of high mvdIDX in stage III-IV tumors was significantly higher (89.5%) than in stages I-II, while the expression of low mvdIDX in stages I-II was significantly higher compared with stages III-IV (Table 2). The distributions of macroscopic type and localization of tumor relative to the level of expression of mvdIDX did not show a significant difference (Table 2), but it is noticeable that a higher mvdIDX was more often present in tumors located in the lower half of the stomach (18/27;66.7%).

As in the previous analysis of the proliferative index, it can be concluded that the presence of distant metastasis was a remarkably rare occurrence (3 cases), and for this reason it was not subjected to a special analysis. It can be concluded that, in each of these cases, there were high values of both indexes.

Correlation analyses of Ki67 and CD105 expression

The similarity of the results and the established significance of other variables in the previous analysis between proIDX and mvdIDX initiated the need to examine the mutual relation between these two indexes. As can be seen in Table 3, the use of Spearman`s rank correlation showed that, in gastric SRCC there was a highly positive correlation between proIDX and mvdIDX that was highly significant (correlation coefficient=0.784, p<0.001).

Table 1. Relationships between levels of expression Ki67 (Low/High) and other clinicopathological variables

Variables	*Ki67 -Low n=18 (48.6%) n (%)*	*Ki67- High n=19 (51.4%) n (%)*	*p value*
Gender			0.603**
Male	7 (38.9)	9(47.4)	
Female	11 (61.1)	10 (52.6)	
Age (years), mean±SD			
Male	64.0±12.7	59.9±10.5	0.489*
Female	63.0±10.8	63.5±15.1	0.931*
Macroscopic tumor types			0.634***
Ulcerated	7 (38.9)	3 (15.8)	
Ulcero-infiltrative	4 (22.2)	9 (47.4)	
Vegetating-infiltrative	4 (22.2)	6 (31.6)	
Infiltrated	3 (16.7)	1 (5.3)	
Histological grade			0.001***
Well differentiated	-	-	
Moderately differentiated	8 (44.4)	0 (0.0)	
Poorly differentiated	10 (55.6)	19 (100.0)	
Localization			0.886**
Upper half of the stomach	8 (44.4)	8 (42.1)	
Lower half of the stomach	10 (55.6)	11 (57.9)	
Pathological tumor stage			<0.001***
T1	4 (22.2)	0 (0.0)	
T1b	3 (16.7)	0 (0.0)	
T2	3 (16.7)	0 (0.0)	
T3	7 (38.9)	14 (73.7)	
T4	1 (5.6)	5 (26.3)	
Metastasis (how many regional lymph nodes show signs of cancer)			<0.001***
Not spread to the regional lymph nodes	14 (77.8)	0 (0.0)	
1 to 2 regional lymph nodes	4 (22.2)	0 (0.0)	
3 to 6 regional lymph nodes	0 (0.0)	4 (21.1)	
7 to 15 regional lymph nodes	0 (0.0)	8 (42.1)	
16 or more regional lymph nodes	0 (0.0)	7 (36.8)	
Lymphatic invasion			<0.001**
Not present	10 (55.6)	0 (0.0)	
Present	8 (44.4)	19 (100.0)	
Venous invasion			0.045**
Not present	14 (77.8)	8 (42.1)	
Present	4 (22.2)	11 (57.9)	
Distant metastasis			0.230**
Not present	18 (100.0)	16 (84.2)	
Present	0 (0.0)	3 (15.8)	
Tumor stage grouping			<0.001***
IA	6 (33.3)	0 (0.0)	
IB	5 (27.8)	0 (0.0)	
IIA	5 (27.8)	0 (0.0)	
IIB	2 (11.1)	0 (0.0)	
IIIA	0 (0.0)	5 (26.3)	
IIIB	0 (0.0)	11 (57.9)	
IV	0 (0.0)	3 (15.8)	

*Student's t-test, **x^2 test (Fisher's Exact test), ***Mann-Whitney U test. Low levels of Ki67 expression (values ≤ the median value). High levels of Ki67 expression (values > the median value).

Table 2. Relationships between levels of expression CD105 (Low/High) and other clinicopathological variables

Variables	*CD105- Low n=18 (48.6%) n (%)*	*CD105- High n=19 (51.4%) n (%)*	*p value*
Gender			0.603**
Male	7 (38.9)	9 (47.4)	
Female	11 (61.1)	10 (52.6)	
Age (years), mean±SD			
Male	61.0±9.1	62.2±13.2	0.838*
Female	60.9±9.5	65.8±15.6	0.391*
Macroscopic tumor types			0.118***
Ulcerated	7 (38.9)	3 (15.8)	
Ulcero-infiltrative	6 (33.3)	7 (36.8)	
Vegetating-infiltrative	4 (22.2)	6 (31.6)	
Infiltrated	1 (5.6)	3 (15.8)	
Histological grade			0.020***
Well differentiated	-	-	
Moderately differentiated	8 (44.4)	0 (0.0)	
Poorly differentiated	10 (55.6)	19 (100.0)	
Localization			0.419**
Upper half of the stomach	9 (50.0)	1 (5.3)	
Lower half of the stomach	9 (50.0)	18 (94.7)	
Pathological tumor stage			<0.001***
T1	4 (22.2)	0 (0.0)	
T1b	3 (16.7)	0 (0.0)	
T2	3 (16.7)	0 (0.0)	
T3	8 (44.4)	13 (68.4)	
T4	0 (0.0)	6 (31.6)	
Metastasis (how many regional lymph nodes show signs of cancer)			<0.001***
Not spread to the regional lymph nodes	12 (66.7)	2 (10.5)	
1 to 2 regional lymph nodes	4 (22.2)	0 (0.0)	
3 to 6 regional lymph nodes	2 (11.1)	2 (10.5)	
7 to 15 regional lymph nodes	0 (0.0)	8 (42.1)	
16 or more regional lymph nodes	0 (0.0)	7 (36.8)	
Lymphatic invasion			0.003**
Not present	9 (50.0)	1 (5.3)	
Present	9 (50.0)	18 (94.7)	
Venous invasion			0.007**
Not present	15 (83.3)	7 (36.8)	
Present	3 (16.7)	12 (63.2)	
Distant metastasis			0.230**
Not present	18 (100.0)	16 (84.2)	
Present	0 (0.0)	3 (15.8)	
Tumor stage grouping			<0.001***
IA	6 (33.3)	0 (0.0)	
IB	5 (27.8)	0 (0.0)	
IIA	4 (22.2)	1 (5.3)	
IIB	1 (5.6)	1 (5.3)	
IIIA	2 (11.1)	3 (15.8)	
IIIB	0 (0.0)	11 (57.9)	
IV	0 (0.0)	3 (15.8)	

*Student's t-test, **x^2 test, Fisher's Exact test, ***Mann-Whitney U test. Low levels of CD105 expression (values ≤ the median value). High levels of CD105 expression (values> the median value).

Correlation analysis of the examined variables

With the aim of establishing the degree and trend of the mutual association of other variables that were the objectives of observation in this study, the results of correlation analysis are shown in Table 3. Both indices (proIDX and mvdIDX) showed a significant, moderate positive correlation with histological tumor grade. The proliferative index showed a very highly significant positive correlation with lymph node metastasis (cc=0.924). Furthermore, a highly significant positive correlation existed between proIDX and the stage of disease (cc=0.911). A slightly weaker, but still significant positive correlation (cc=0.593) existed between proIDX and the pathological tumor stage (pT).

A good significant positive correlation also existed between proIDX and angioinvasion (cc=0.363). ProIDX correlated with a highly significant positive coefficient with lymph vessel invasion (cc=0.625). The neoangiogenesis index was linked to lymph node metastasis with a highly significant positive coefficient of correlation (cc=0.788). Between mvdIDX and the tumor stage there was also a high positive correlation (cc=0.832). The pathological T stage of disease and mvdIDX showed a high level of dependence that was defined by the correlation coefficient of 0.669. With a highly significant positive correlation coefficient the mvdIDX was associated with angioinvasion (cc=0.483) and lymph vessel invasion (cc=0.503).

As the previous analysis implied, there was no significant difference between low and high values of proIDX and mvdIDX according to the distribution of macroscopic type and tumor localization. In the correlation analysis this fact was confirmed by the absence of significant correlation of those parameters according to both indices. The absence of correlation of demographic variables – gender and age of patients - with expression of proIDX and mvdIDX was determined by absence of significant correlation (Table 3).

Receiver operating characteristics (ROC) analysis

ROC curve analysis was used to determine cut off values of nuclear proliferative index (Ki67) and marker of angiogenesis (CD105), above which progression and metastatic potential of SRCC can be determined with high confidence (Figure 4).

Table 3. Correlation analysis - the sign and strength of the association between Ki67 (Low/High), CD105 (Low/High) and other clinicopathological variables

Variables	*Spearman's ρ*			
	Ki67		*CD105*	
	Correlation coefficient	*p value*	*Correlation coefficient*	*p value*
Ki67	1.000		0.784**	<0.001
CD105	0.784	<0.001	1.000	
Gender	-0.086	0.615	-0.086	0.615
Age	-0.068	0.690	0.134	0.429
Macroscopic tumor type	0.050	0.768	0.275	0.100
Histological grade	0.540	0.001	0.540	0.001
Localization	0.024	0.890	0.133	0.433
Pathological stage	0.593	0.148	0.669	<0.001
Lymph node metastasis	0.924	<0.001	0.788	<0.001
Lymphatic invasion	0.625	<0.001	0.503	<0.001
Venous invasion	0.363	0.027	0.483	0.003
Distant metastasis	0.289	0.083	0.289	0.083
Tumor stage grouping	0.911	<0.001	0.832	<0.001

Table 4. Cut-off and AUC values

Test result variables	*Cut off*	*AUC*	*Std. Error*	*Asymptotic Sig.*	*Asymptotic 95% Confidence Interval*	
					Lower bound	*Upper bound*
Ki67	2159.17*	0.970	0.021	<0.001	0.929	1.012
CD105	761.36*	0.936	0.030	<0.001	0.877	0.996

*positive cells per mm², AUC: area under the curve

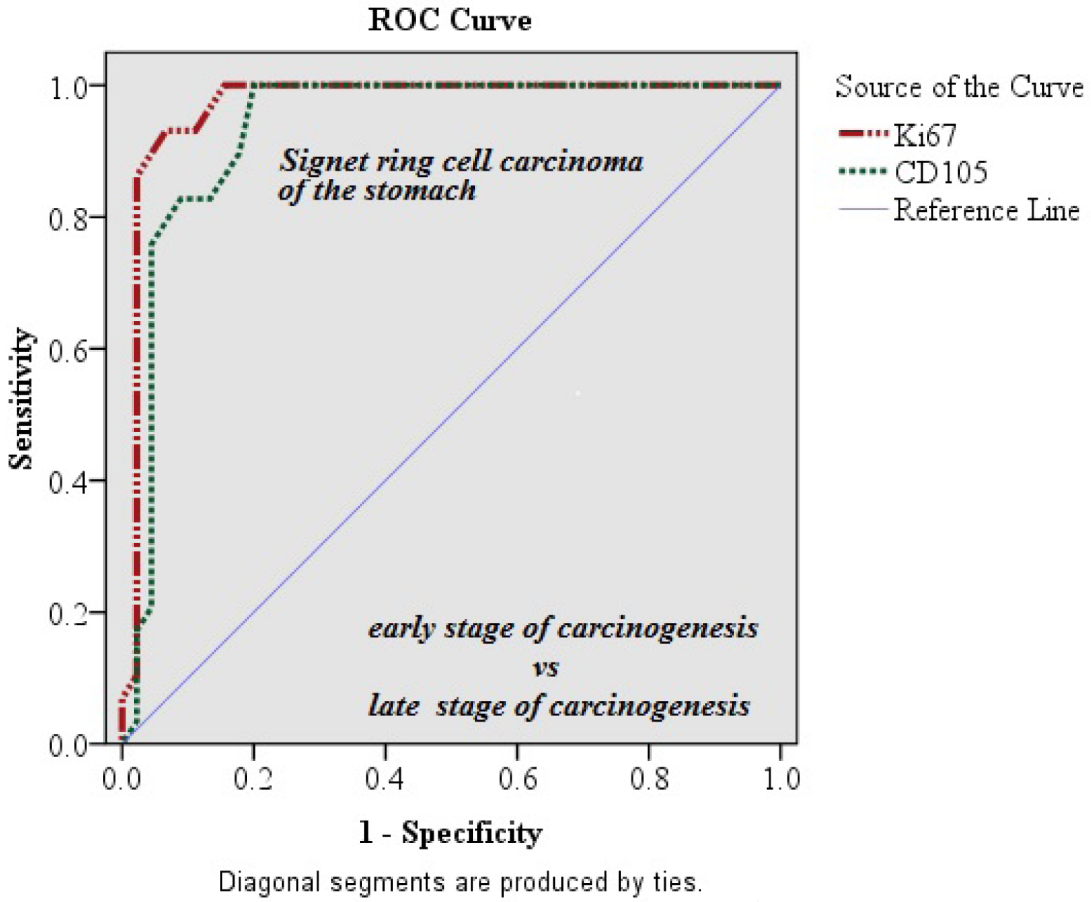

Figure 4. Receiver operating characteristic curves which identified cut off values for Ki67 and CD105 in early vs. late stage of carcinogenesis and progression and metastatic potential of SRCC, determined with high confidence.

A highly significant value of the area under the curve (AUC) showed that the obtained cut off values were, with high sensitivity and specificity, reliable diagnostic markers of gastric SRCC (Table 4).

Discussion

The most important biological mechanism of carcinogenesis is cell proliferation. The prognostic importance of the proliferative activity has been discovered in many tumors [12-14], but the reports about the prognostic importance of proliferation in gastric carcinoma are rather inconsistent [16-18]. In this study, we used the Ki67 antibody, which reacts exclusively with nuclear structures present in proliferating cells, for the examination of proliferative activity in SRCC. The Ki67 antigen can be visualized in the nuclei of cells during middle-late G1, S, G2 and M phases of the cell cycle. It cannot be detected in G0 phase when the cell is in quiescence [19].

Two decades ago, MacCallum and Hall noted that Ki67 plays a crucial role in the stabilization of nuclear chromatin, because it is bound to the thick fibrillar material of the nucleolus. The same authors showed the structural role of Ki67 in the nucleolus and emphasized its ability to bind to RNA and DNA and that it represents an essential factor in the process of ribosome synthesis during cell division [20,21].

The expression of Ki67 protein is strictly controlled; it goes through phosphorylation and dephosphorylation during mitosis. Its quantity is regulated by proteolytic pathways, probably by including a complex of proteases, which results in its short half-life of 60-90 min [22]. It is clear that this protein is of great importance for cell proliferation, since its suppression by antisense oligonucleotides stops proliferation [23,24].

Examining the expression of nuclear proliferative antigen Ki67, the absolute values of density of positive cells per unit area were established and, from the absolute values, the index of proliferation was calculated by deviation from the median. As expected, a statistically significant difference in the expression of this antigen (proliferation index) between tumor and surrounding tissue was noted.

In our study we verified the expression of Ki67 in all cases of SRCC, whereby a high index of Ki67 expression was found in 51.4% of the cases, which

is in accordance with the work of Lazar et al. [18].

A high index of expression of Ki67 was verified in all poorly differentiated tumors, while a low proIDX was present in 55.6% of poorly differentiated and 44.4% of moderately differentiated SRCCs, which indicates that a high proliferation index is a significant occurrence in poorly differentiated gastric SRCCs. A high proIDX was also significantly associated with pT3 (73.7%) and pT4 (26.3%) stages, being present in 100% of the cases in these stages.

A significant presence of high proIDX was noted in SRCC with metastasis in at least 3 lymph nodes, as well as in tumors with lymph vessel invasion and in stage II and IV SRCCs.

In tumors with vascular invasion a high proIDX was noted in the majority of cases but not to a significantly greater extent than in tumors without vascular invasion.

It is noted that a high proIDX was found more often in the ulcero-infitrative macroscopic type of tumor and in tumors located in the lower part of the stomach, but in both cases without statistical significance.

Immunohistochemical examination of Ki67 expression has proven to be useful in many pathologic conditions, first of all in tumors. It can be used for assessment of proliferative activity of tumors, but also for differentiating benign from malignant tumors. Namely, the mitotic activity is usually absent in benign tumors or it is very low, while it is high in malignant tumors and correlates with the level of tumor progression [13-15,25].

In 1971, Folkman stated that the growth and metastasis of malignant tumors depend on the formation of new blood vessels. Namely, once the tumor has grown, any further increase in tumor size must be preceded by an increase in the number of new blood vessels that converge toward the tumor and supply oxygen and nutrients to the tumor and drain away catabolites. At the same time, the endothelial cells produce growth factors with paracrine effects on tumor cells [26]. Induction of angiogenesis happens when an imbalance between pro and antiangiogenic factors occurs. A disorder in this balance can be provoked by hypoxia or genetic alteration that activates oncogenesis and/or inactivates tumor-suppressor genes [27].

Several antibodies are used for the assessment of angiogenesis, such as CD31, CD34, von Willebrand factor, CD105 and others [28,29]. In this study, we used CD105 (Endoglin), first of all because it has been shown that CD105 antibodies react with newly formed endothelial cells that take part in angiogenesis and that it reacts with only 20% of preformed non-neoplastic blood vessels. It is also noted that CD105 does not react with inflammatory and stromal cells which lower the number of false positive results [28,30].

In our study, endoglin expression was present in tumor and peritumoral vessels of SRCC. High expression was present in 51.4% of the tumors. A high mvdIDX was noted in all poorly differentiated tumors, which is a significant fact for SRCCs.

High mvdIDX was present in a significantly greater number of pT3 stage (68.4%) and pT4 (31.6%) tumors, in 89.5% of SRCCs with metastasis in at least 3 or more lymph nodes, in 94.7% of tumors with lymph vessel invasion and 89.5% of stage III and IV tumors. It was also noted that a high mvdIDX was more often present in SRCCs with vascular invasion and in tumors located in the lower part of the stomach, but with no statistical significance relative to a low mvdIDX.

Comparative analysis of parameters from our study points to important mutual relationships between the analyzed variables. Correlation analysis has shown the true measure of their relationships, as demonstrated by the significance of the correlation coefficients. Both indices (of proliferation and microvascular density) showed a significant moderate positive correlation with the histological grade of SRCCs.

Similarly, the proIDX showed a highly significant positive correlation with lymph node metastasis, lymph vessel invasion and tumor stage. There was a slightly weaker positive correlation between the proIDX and pathologic tumor stage, as well as between the proIDX and vascular invasion. In this study, a relationship between proIDX and macroscopic type and localization of SRCC was not established.

mvdIDX was linked to metastasis in lymph nodes, with lymph vessel invasion, with vascular invasion and with tumor stage, with a highly significant positive correlation coefficient.

Regarding the value of the correlation coefficient, the proIDX showed a slightly greater strength of positive association relative to the metastatic potential of the tumor, presented by the number of involved lymph nodes, appearance of lymph vessel invasion and stage of tumor, compared with the angiogenesis index. However, in the case of angiogenesis index, the correlation with the mentioned variables was also high and significant.

In the correlation analysis there was no significant correlation of mvdIDX with macroscopic tumor type and localization of SRCC. Also, no link between the demographic variables – gender and age – and the expression of proIDX and mvdIDX was noted. There was, however, a high positive correlation between proIDX and mvdIDX, which was highly significant.

The use of ROC curve analysis in the determination of cut off values for proliferative antigen (Ki67) and marker of angiogenesis (CD105) was aimed to verify these markers as a standard test with sufficient accuracy for the assessment of progression of gastric SRCC. The cut off or cut off point is the value of a test above which a case can be considered positive; in the case of gastric SRCC, this aimed to determine whether a case is likely to be at advanced stage, including metastasis.

The diagnostic performance of ROC analysis revealed its ability to identify patients who are really in advanced stage of disease (sensitivity), as well as its ability to exclude the presence of progression of carcinoma with metastasis where, in fact, it does not exist (specificity). The AUC of the ROC curve can be used as a measure of diagnostic accuracy (efficacy), taking into account all the characteristics of ROC analysis. Comparing the AUC for markers of proliferation and angiogenesis analyzed in this study, it can be concluded that the proIDX, as a marker of identification of progression in gastric SRCC, showed better performance than angiogenesis (AUC=0.970 vs. 0.936). It should be emphasized that both indices are highly significant and reliable as markers of disease progression ($p<0.001$ and AUC close to 1). However, the proIDX showed relatively better performance (lower number of false positive and false negative results).

Conclusion

Although both the proIDX, verified by Ki67 immunostaining, and the mvdIDX are reliable as markers of progression of gastric SRCC, the former showed better performance, with higher sensitivity and specificity.

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Conflict of interests

The authors declare no conflict of interests.

References

1. Ferlay J, Soerjomataram I, Dikshit R et al. Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. Int J Cancer 2015;136:E359-86.
2. Markovic-Denic L, Cirkovic A, Zivkovic S, Stanic D, Skodric-Trifunovic V. Cancer mortality in central Serbia. JBUON 2014;19:273-7.
3. Bamboat ZM, Tang LH, Vinuela E et al. Stage-stratified prognosis of signet ring cell histology in patients undergoing curative resection for gastric adenocarcinoma. Ann Surg Oncol 2014;2:1678-85.
4. Taghavi S, Jayarajan SN, Davey A, Willis AI. Prognostic significance of signet ring gastric cancer. J Clin Oncol 2012;30:3493-8.
5. Fenoglio-Preiser C, Carniero F, Correa P et al. Gastric carcinoma In: Hamilton SR,Aaltonen LA (Eds): World Health Organisation Classification of Tumours: Tumours of the Digestive System, IARC Press, Lyon, 2000, pp 37-53.
6. Lauwers GY, Carneiro F, Graham DYet al. Gastric carcinoma. In: Bosman FT, Carniero F,Hruban RH,Theise ND (Eds): WHO Classification of Tumours of the Digestive System. (4th Edn). IARC press, Lyon 2010,pp 48-58.
7. Kwon KJ, Shim KN, Song EM et al. Clinicopathological characteristics and prognosis of signet ring cell carcinoma of the stomach. Gastric Cancer 2014;17:43-53.
8. Bielenberg DR, Zetter BR. The Contribution of Angiogenesis to the Process of Metastasis. Cancer J 2015;21:267-73.
9. Yoo SY, Kwon SM. Angiogenesis and its therapeutic opportunities. Mediators Inflamm 2013; 127170. Published online 2013 July 28. doi: 10.1155/2013/127170.
10. Aprile G, Ongaro E, Del Re M et al. Angiogenic inhibitors in gastric cancers and gastroesophageal junction carcinomas: A critical insight. Crit Rev Oncol Hematol 2015;95:165-78.
11. Weibel ER, Kistler GS, Scherle WF. Practical stereological methods for morphometric cytology. J Cell Biol 1966;30:23-38.
12. Mouton PR. Unbiased Stereology: A Concise Guide. Baltimore, John Hopkins University Press, 2011, pp 1-171.
13. Gerring Z, Pearson JF, Morrin HR, Robinson BA, Harris GC, Walker LC. Phosphohistone H3 out performs Ki67 as a marker of outcome for breast cancer patients. Histopathology 2015;67:538-47.
14. Kim H, Park CY, Lee JH, Kim JC, Cho CK, Kim HJ. Ki-67 and p53 expression as a predictive marker for early postoperative recurrence in pancreatic head cancer. Ann Surg Treat Res 2015;88:200-7.
15. Lawrence NF, Hammond MR, Frederick DT et al. Ki-67, p53, and p16 expression, and G691S RET polymorphism in desmoplastic melanoma (DM): A clinicopathologic analysis of predictors of outcome. J Am Acad Dermatol 2016;75:595-60.

16. Tsamandas AC, Kardamakis D, Tsiamalos P et al. The potential role of Bcl-2 expression, apoptosis and cell proliferation (Ki-67 expression) in cases of gastric carcinoma and correlation with classic prognostic factors and patient outcome. Anticancer Res 2009;29:703-9.
17. Xiao LJ, Zhao S, Zhao EH et al. Clinicopathological and prognostic significance of Ki-67, caspase-3 and p53 expression in gastric carcinomas. Oncol Lett 2013;6:1277-84.
18. Lazar D, Taban S, Sporea I et al. Ki-67 expression in gastric cancer. Results from a prospective study with long-term follow-up. Rom J Morphol Embryol 2010;51:655-61.
19. Zhou Y, Li Y, Zheng J, Liu K, Zhang H. Detecting gastric cancer by Bcl-2 and Ki67. Int J Clin Exp Pathol 2015;8:7287-90.
20. MacCallum DE, Hall PA. The location of pKi67 in the outer dense fibrillary compartment of the nucleolus points to a role in ribosome biogenesis during the cell division cycle. J Pathol 2000;190:537-44.
21. MacCallum DE, Hall PA. The biochemical characterization of the DNA binding activity of pKi67. J Pathol 2000;191:286-98.
22. Li LT, Jiang G, Chen Q, Zheng JN. Ki67 is a promising molecular target in the diagnosis of cancer (review). Mol Med Rep 2015;11:1566-72.
23. Rubenstein M, Hollowell CM, Guinan P. Suppression of BCL2 by Antisense Oligonucleotides and Compensation by Non-Targeted Genes May Enhance Tumor Proliferation. In Vivo 2015;29:687-93.
24. Wang XM, Xu J, Cheng ZQ et al. Study on effects of microRNA-21 antisense oligonucleotide in vivo and in vitro on bionomics of human cervical squamous carcinoma cell lines SiHa. Zhonghua Bing Li Xue Za Zhi 2012;41:254-9.
25. Foltyn W, Zajęcki W, Marek B et al. The value of the Ki-67 proliferation marker as a prognostic factor in gastroenteropancreatic neuroendocrine tumours. Endokrynol Pol 2012;63:362-6.
26. Folkman J. Tumor angiogenesis: therapeutic implications. N Engl J Med 1971;285:1182-6.
27. Rak J, Yu JL, Klement G et al. Oncogenes and angiogenesis: signaling three-dimensional tumor growth. J Investig Dermatol Symp Proc 2000;5:24-33.
28. Miyata Y, Sakai H. Reconsideration of the clinical and histopathological significance of angiogenesis in prostate cancer: Usefulness and limitations of microvessel density measurement. Int J Urol 2015;22:806-15.
29. Lekovic D, Gotic M, Skoda R et al. Bone marrow microvessel density and plasma angiogenic factors in myeloproliferative neoplasms: clinicopathological and molecular correlations. Ann Hematol 2017;96:393-404.
30. Miyata Y, Mitsunari K, Asai A, Takehara K, Mochizuki Y, Sakai H. Pathological significance and prognostic role of microvessel density, evaluated using CD31, CD34, and CD105 in prostate cancer patients after radical prostatectomy with neoadjuvant therapy. Prostate 2015;75:84-91.