

## ARTICLE

## Microvessel Count, Proliferating Cell Nuclear Antigen and Ki-67 Indices in Gastric Adenocarcinoma\*

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The aim of the present study was to immunohistochemically investigate the prognostic value of neovascularization (expressed as microvessel count-MVC) and tumor cell proliferation (expressed as PCNA labeling index - PLI and Ki-67 labeling index - KLI) in gastric adenocarcinoma. Correlations with clinicopathologic features were also evaluated. Tumor specimens from 74 patients diagnosed as gastric adenocarcinoma were included in this study. Formalin fixed, paraffin embedded tissue sections stained immunohistochemically with F-VIII, PC10 and MIB-1 monoclonal antibodies. By ocular grid subdivided into 100 areas, number of microvessels and PC10, MIB-1 positive and negative cells were counted at x400 magnification. Chi-square test, Kaplan-Meier method and cox regression analysis

were used for statistical analysis. The results showed that, MVC and PLI had a significant correlation with invasion and lymph node metastasis. The prognosis was significantly worse in patients with high MVC (>14) and with high PLI (>49%). However any relationship was not observed between KLI (38%) and clinicopathologic parameters, so KLI failed to predict the prognosis. Cox model showed that, MVC and PLI were independent prognostic variables. Ki-67 labeling index in gastric carcinomas has no prognostic relevance. However, the evaluation of microvessel count and proliferating cell nuclear antigen index in gastric carcinomas could be reliable indicators of prognosis. (Pathology Oncology Research Vol 6, No 1, 59-64, 2000)

**Keywords:** microvessel count, angiogenesis, proliferation, gastric carcinoma

### Introduction

Quantitative histological studies revealed that intratumoral neovascularization, as assessed by microvessel density and, cell proliferative kinetics are important determinants of tumor behaviour and prognosis.<sup>1,9,11,30,41</sup>

In gastric adenocarcinomas, a correlation between the extent of microvessel density and clinical outcome has been demonstrated.<sup>24,35,42</sup> However, determination of proliferation index in gastric adenocarcinomas by using Ki-67 antigen or proliferating cell nuclear antigen (PCNA)

showed differences in their relationship with clinicopathologic factors and prognosis.<sup>4,5,19,23,25</sup>

The objective of this study was to investigate the prognostic significance of neovascularization (expressed as microvessel count-MVC) and tumor cell proliferation (expressed as PCNA labeling index, PLI, and Ki-67 labeling index, KLI) in gastric adenocarcinomas and to determine the correlation of these parameters with clinicopathologic features.

### Materials and Methods

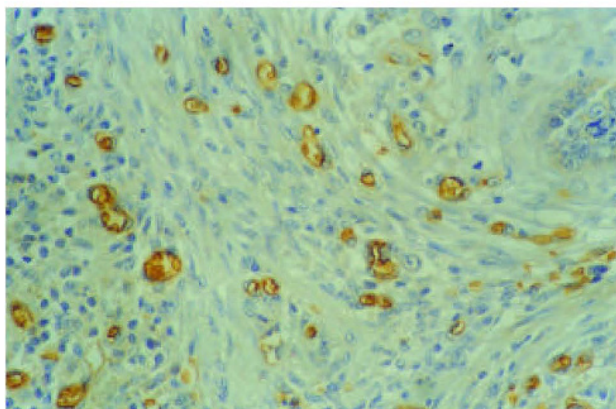
A total of 74 cases of gastric adenocarcinoma were included in this study. The patients had undergone subtotal or total gastrectomy combined with lymph node dissection or palliative resection at the Department of Surgery, Akdeniz University, Antalya from 1989 to 1997. The median age of the 42 men was 54 years (range 29-74 years) at the time of operation, and that of the 32 women, 59 years (range 32-65 years). Survival data were available on all patients and were obtained from case records.

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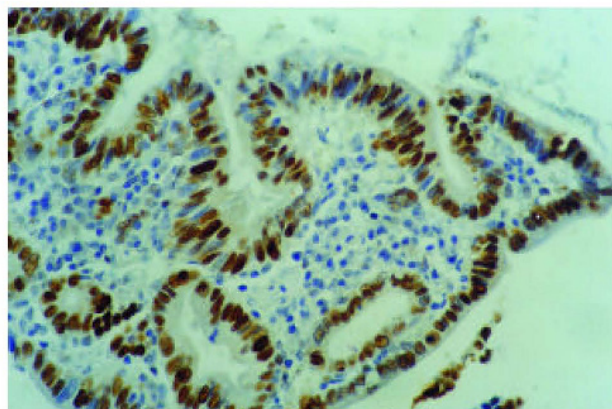
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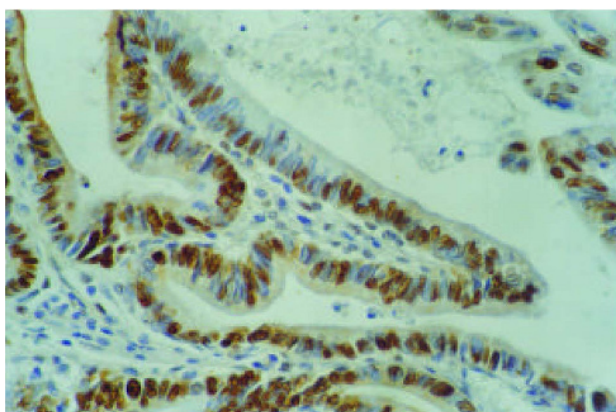
Abbreviations: MVC: Microvessel count; PLI: Proliferating cell nuclear antigen labeling index; KLI: Ki-67 labeling index.



**Figure 1.** Immunohistochemical staining of endothelial cells for FVIII-related antigen in gastric carcinoma tissue (x 400).



**Figure 3.** MIB-1 immunohistochemical staining of gastric carcinoma, nuclear positivity (x 400)



**Figure 2.** PC10 immunohistochemical staining of gastric carcinoma, nuclear positivity (x 400)

Twenty-nine of 74 patients (31,9%) have survived, with a mean survival duration of 49 months (range 32 to 62 months). Forty five cases died in 2 to 44 months (mean, 20 months). Peritoneal metastasis occurred in 23 patients. Fourteen were detected at the time of surgery and 9 developed after surgery. Eleven liver metastases were observed at the time of operation and 18 developed during the follow-up. Three lung, two brain and four bone marrow metastases were occurred.

Histologic classification and evaluation of tumor stage were performed according to Lauren's<sup>20</sup> and IUCC/AJCC<sup>13</sup> classification, respectively. Four  $\mu\text{m}$  thick hematoxylin and eosin stained tissue sections from the surgical specimens fixed in 10% formalin and embedded in paraffin were reviewed and representative tissue blocks were selected. Sections were deparaffinized and heated in a microwave oven for 10 minutes to retrieve antigens. Slides were immunostained with FVIII-Rag (F8/86, dilution 1:25, Dako, Glostrup, Denmark), Ki-67 (MIB-1, dilution 1:80, Immunotech, Marseille, France) and PCNA (PC10, dilution

1:50, Dako) monoclonal antibodies by the avidin-biotin immunoperoxidase technique. Finally, all slides were treated with DAB reagent to develop color and counterstained with hematoxylin. Slides were interpreted for MVC, KLI and PLI by a pathologist who had no knowledge of the clinicopathologic data.

For determination of MVC, the stained sections were screened at low power (x100) to identify the areas of the highest vascularization within the tumor. Microvessel counts were performed at x400 magnification by using an ocular grid subdivided into 100 areas in four fields of vision.<sup>8</sup> Assessment of PLI and KLI were performed at the highest proliferative areas. In each case positive and negative nuclei were counted at x400 magnification. KLI and PLI were calculated as the percentage of positive tumor cells relative to the total number of cells counted.

Chi-square was used to compare frequencies. The difference in numerical data between two groups was analyzed by using Mann-Whitney U test. Correlations among various parameters were tested by calculating Spearman's correlation coefficient. Survival was calculated from the day resection was performed to the day of death or the day of last follow-up. Univariate and multivariate survival analysis were performed by using Kaplan-Meier method and Cox regression analysis, respectively. A significance level of 0.05 was used throughout the analysis.

## Results

All endothelial cells were stained with anti-F-VIII-Rag antibody (*Figure 1*). Vascularization was most frequently observed at the invasive proportions of tumors. Ki-67 and PCNA immunostaining were diffuse or granular and confined to the nucleus (*Figure 2 and 3*). *Table 1* shows mean, standard deviation and range of values for MVC, PLI and KLI. Cases were also divided into two groups according to the median value of MVC, PLI and KLI for further analysis.

**Table 1. MVC, PLI, KLI and stages (S)**

	MVC				PLI				KLI			
	Mean	Median	SD	Range	Mean	Median	SD	Range	Mean	Median	SD	Range
All cases	18.1	14	6.2	7-48	57.8	49	21.6	11-94	42.6	38	17.2	9-82
SI	10.8	12	2.9	7-25	39.5	36	8.4	11-42	40.7	36	11.6	9-61
SII	14.3*	15	4.3	8-27	58.7**	52	15	21-53	41.1	40	14.3	7-68
SIII	15.7	15	3.5	8-33	62.6***	61	21.9	34-81	43.6	38	16.2	27-82
SIV	29.2	23	7.4	9-48	69.9	67	21.8	37-94	44.8	42	18.7	33-82
Mann Whitney U-test	p<0.05				p<0.05				p>0.05			
n.s. not significant	*S II vs. S III n.s.				**S II vs. S III and S IV n.s.				***S II vs S IV n.s.			

The hypervascular group consisted of 45 tumors with MVC 14 or higher, and hypovascular group consisted of 29 tumors with MVC less than 14. Lymph node metastases, serosal invasion, peritoneal and liver metastases were more frequent in hypervascular group ( $p<0,05$ ) (Table 2). Stage III and IV tumors were more frequent in hypervascular group. According to Mann-Whitney-U test mean MVC in stage I disease was lower compared to stage

II, III and IV disease, and this difference was significant ( $p<0,05$ ). However, mean MVC of stage II disease was not different from MVC of stage III (Table 1).

High PLI was correlated only with lymph node metastasis and serosal invasion (Table 2). Mean PLI among patients with stage I disease was less than those with stage II, III and IV disease ( $p<0,05$ ) (Table 1). However PLI was not different in stage II, III and IV disease. Any

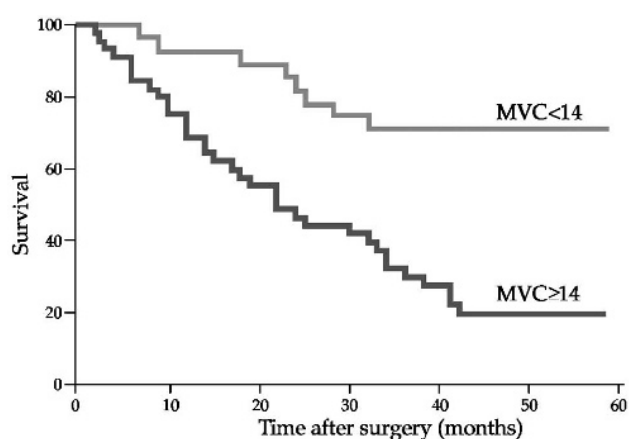
**Table 2. Correlation between MVC, PLI, KLI and clinicopathologic factors**

Clinicopathologic factors	Hypovascular (n: 29)	Hypervascular (n: 45)	p value	Low PLI (n: 27)	High PLI (n: 47)	p value	Low KLI (n: 30)	High KLI (n: 44)	p value
Age <sup>a</sup>	48 ± 11.5	46 ± 14.4	ns <sup>b</sup>	49 ± 16.3	53.8 ± 19.1	ns <sup>b</sup>	50.8 ± 16.4	47.6 ± 13.2	ns <sup>b</sup>
Gender (male/female)	14/15	28/17	ns <sup>c</sup>	14/13	28/19	ns <sup>c</sup>	17/13	25/19	ns <sup>c</sup>
Tumor diameter <sup>a</sup>	6.4 ± 2.3	7.8 ± 4.7	ns <sup>b</sup>	7.3 ± 2.8	8.9 ± 3.7	ns <sup>b</sup>	6.8 ± 3.1	6 ± 2.9	ns <sup>b</sup>
Histologic type									
Intestinal /diffuse	16/13	21/24	ns <sup>c</sup>	13/14	24/23	ns <sup>c</sup>	14/16	23/21	ns <sup>c</sup>
Serosal invasion (absent/present)	20/9	12/33	0.0001 <sup>c</sup>	17/10	15/32	0.009 <sup>c</sup>	11/19	21/23	ns <sup>c</sup>
Lymphatic invasion (absent/present)	13/16	33/12	ns <sup>c</sup>	19/8	27/20	ns <sup>c</sup>	18/12	28/16	ns <sup>c</sup>
Venous invasion (absent/present)	22/7	41/4	ns <sup>c</sup>	22/5	41/6	ns <sup>c</sup>	25/5	38/6	ns <sup>c</sup>
Lymph node metastasis (absent/present)	21/8	10/35	0.0001 <sup>c</sup>	18/9	13/34	0.001 <sup>c</sup>	10/20	21/23	ns <sup>c</sup>
Peritoneal metastasis (absent/present)	26/3	25/20	0.002 <sup>c</sup>	17/10	34/13	ns <sup>c</sup>	19/11	32/12	ns <sup>c</sup>
Liver metastasis (absent/present)	23/6	22/23	0.009 <sup>c</sup>	15/12	30/17	ns <sup>c</sup>	18/12	27/17	ns <sup>c</sup>

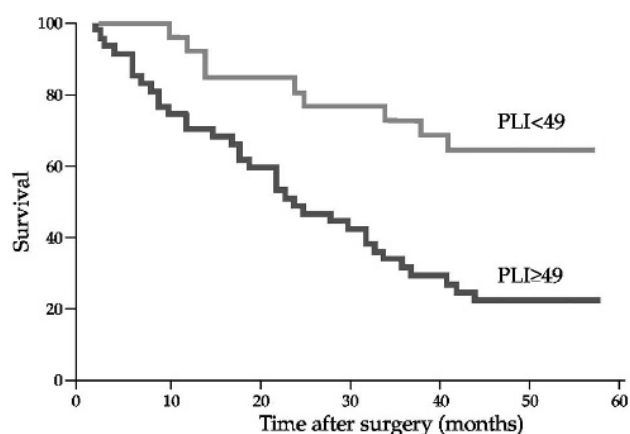
<sup>a</sup> mean ± standart deviation, ns : not significant.

<sup>b</sup> p values were evaluated by using the Mann-Whitney U-test

<sup>c</sup> p values were evaluated by using the Chi-square test



**Figure 4.** Survival curves according to MVC status. Patients were stratified by median microvessel count of series.



**Figure 5.** Survival curves according to PLI status. Patients were stratified by median microvessel count of series.

correlation between KLI and clinicopathologic factors was not detected.

Spearman correlation test revealed a strong correlation between MVC and PLI ( $r=0,617$ ). However, we failed to find a significant correlation between KLI and PLI ( $r=0,012$ ).

Univariate analysis based on the log rank test revealed that the prognosis of patients in hypervascular group was significantly worse than hypovascular group. The 5-year survival rates of the hypovascular and hypervascular groups were 71% and 20%, respectively (Figure 4). Similarly 5-year survival rate of patients in low PLI group (64%) was significantly better than high PLI group (22%) (Figure 5). In addition to MVC and PLI, the prognosis of the patients was adversely affected by serosal invasion, lymph node metastasis, peritoneal and liver metastases. (Table 3). Multivariate analysis with covariates that showed statistical significance in the univariate analysis, MVC and PLI were found to be independent prognostic factors in addition to liver metastases (Table 3).

## Discussion

Neovascularization is an important step in metastasis of solid tumors.<sup>1,9,41</sup> Many studies have demonstrated that the degree of tumor angiogenesis is correlated with increased risk of metastasis and clinical outcome in a number, including gastric carcinomas.<sup>24,35,36,42</sup> The results of our investigation confirm the association of high microvessel counts in gastric adenocarcinoma with the presence of metastasis and survival. In our study microvessels were highlighted by using F-VIII-Rag and the mean MVC was  $18 \pm 6,2$ . This value was lower when compared to the results of the studies performed on gastric carcinomas which evaluated tumor vascularization using antibodies against CD31 and CD34.<sup>14,35,36</sup> Although with these antibodies it is possible to detect a greater number of microvessels than F-VIII-Rag, this does not exclude the significance of our findings that showed a close correlation between MVC and the presence of metastasis and survival in gastric carcinoma.

In this study higher microvessel count was significantly associated with parameters of advanced disease. Hypervascular tumors were more frequent in cases with nodal and serosal involvement than in those without. In addition, microvessel count increased with histologic stage, suggesting that enhanced vascular supply might reflect an increased malignant potential. In our series, the prognosis of patients with hypovascular tumor was more favorable than the hypervascular ones ( $p<0,005$ ). Furthermore, multivariate analysis showed that microvessel count was a significant and independent prognostic factor to predict the probability of survival. Tumors that developed liver and peritoneal metastases were more frequent in hypervascular group. Although tumor angiogenesis is not the only factor responsible for metastasis, our data support previous reports showing that microvessel count is a valuable predictor of distant metastasis in patients with gastric carcinomas.<sup>24,35,36</sup>

It is well established that, beside angiogenesis, cell proliferative kinetics are also important to predict the biolog-

**Table 3. Multivariate analysis indicating independent factors correlated with survival in patients with gastric carcinoma**

Variable	Multivariate analysis		
	Regression coefficient	p value	Risk Ratio
Liver metastasis <sup>a</sup>	1.2723	0.0071	3.545
MVC <sup>b</sup>	0.9090	0.0130	2.387
PLI <sup>c</sup>	0.7923	0.0425	2.173

<sup>a</sup> absent vs. present

<sup>b</sup> <14 vs.  $\geq 14$

<sup>c</sup> <49 vs.  $\geq 49$

ical behaviour of tumors. Many studies have been addressed that higher tumor proliferative rates assessed by various techniques, correlate with increased risk of metastasis and clinical outcome in a number of malignancies.<sup>18,27,33,34</sup> However previous reports on the evaluation of cell proliferation by using Ki-67 and proliferating cell nuclear antigen (PCNA) in gastric carcinomas, revealed discordant results.<sup>4,5,16,17,19,23,25,28,43</sup>

Although a significant relation between KLI and wall invasion or nodal metastasis has been observed in a few studies,<sup>16,28</sup> most investigators have not demonstrated a relationship between KLI and clinicopathologic factors in patients with gastric carcinomas.<sup>4,5,19,43</sup> In our study, KLI was not associated with clinicopathologic factors, such as lymph node metastasis, serosal or venous vessel invasion. We found no difference between KLI of diffuse and intestinal type carcinomas. A higher KLI has been described in intestinal type carcinomas.<sup>16,40</sup> However in more recent studies no significant difference was observed between the KLI of these two histological subtypes.<sup>29,31</sup> Survival of patients with high and low KLI in our series was not different. Therefore, the results of our investigation show that KLI fails to demonstrate any correlation with clinicopathologic parameters and has no prognostic relevance in patients with gastric carcinomas.

On the other hand, our PLI findings are in accordance with those reported by many investigators who observed a significant relation between PLI and clinicopathologic factors as well as prognosis, in gastric tumors.<sup>15,17,23</sup> In our study high PLI correlated with the presence of lymph node metastases and serosal invasion as well as advanced stages. Moreover, patients with higher PLI, had significantly poorer prognosis, and PLI was found to be effective as a prognostic factor.

In this study the mean PLI was higher than KLI and the correlation between these two proliferation markers was not significant ( $r=0.021$ ). Many other studies have also failed to demonstrate a correlation between PCNA and Ki-67 indices in different tumors as well as in gastric carcinomas.<sup>12,18,21,26,32</sup> These findings might be attributed to a number of factors, as previously described. It is suggested that Ki-67 might act as a timer molecule in the regulation of cell proliferation but at present its exact role is unknown.<sup>2</sup> This antigen is expressed during the cell cycle, but not present in quiescent cells ( $G_0$ ) and its amount varies during cell proliferation.<sup>10,32</sup> Moreover, nutrient supply could influence Ki-67 antigen expression, leading to considerable variations in the expression in different parts of a tumor.<sup>6</sup> On the other hand, PCNA is identified as an auxiliary protein of DNA polymerase delta that is directly involved in DNA repair and replication.<sup>7,38</sup> The long half life of this antigen results in detection of cells that have recently left the cycle and might contribute to the detection of greater number of cells than Ki-67.<sup>3</sup> Rosa et al<sup>32</sup> found no relationship between the

indices of these two proliferation markers in gastric carcinoma pointed out that the most likely explanation for this finding is the marked heterogeneity of these tumors.

No correlation between angiogenesis and KLI was detected in this study, corresponding with the data of a previous study on gastric carcinoma using Ki-67.<sup>22</sup> The correlation of angiogenesis with proliferation has been investigated in different tumors. In the gastrointestinal system, a strong association between MVC and proliferation has been reported in both oral<sup>37</sup> and colorectal<sup>39</sup> carcinomas. Regarding gastric carcinomas, the correlation between MVC and proliferation was investigated by using Ki-67 antigen in one study and no relationship was detected.<sup>22</sup> It was suggested that MVC and proliferation are independent. However, in our study a correlation between angiogenesis and PLI, an interesting finding, that contradicts the independence of angiogenesis and proliferation reported in previous studies, was detected.

Although our data remains to be elucidated in further large scale studies, our study shows that when proliferation and angiogenesis are to be compared in gastric adenocarcinomas many markers and methods should be used.

We conclude that microvessel count and proliferating cell nuclear antigen labeling index are independent prognostic factors in gastric adenocarcinomas and that Ki-67 labeling index has no additional prognostic information.

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