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Comparative study of tumor angiogenesis and immunohistochemistry for p53, c-ErbB2, c-myc and EGFr as prognostic factors in gastric cancer

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Summary. Gastric cancer (GC) continues to be a highly aggressive malignancy with poor prognosis and low survival rates. The survival of patients with GC depends mainly on the stage of the disease, with early GC having a 5 year survival of 90-100% and advanced tumors a 5 year survival of 15-25%. The role of other prognostic factors in these tumors is still under investigation. 28 gastric dysplasia, 45 Early GC and 98 Advanced Gastric Cancers were evaluated for expression of the oncogenes p53, c-ErbB2, c-myc and the EGFr in paraffin-embedded material utilizing Avidin-Biotin immunohistochemistry techniques. In 34 cases of GC microvessel density (MVD) was determined in CD34 stained sections. Statistical correlations with stage, histologic type, differentiation degree, location, size, ploidy patterns and overall survival were done. The Mantel-Cox test was performed to evaluate which factors had an independent prognostic value.

Both, tumor angiogenesis and p53 protein expression were statistically associated (95% confidence intervals) with overall survival in patients with GC. p53 protein expression was also correlated with cardial location, nodal involvement and tumor stage. c-ErbB2 may recognize a group of highly aggressive well differentiated adenocarcinomas with worse prognosis. c-myc was also significantly enhanced in well differentiated tumors. EGFr showed no significant associations.

Mantel-Cox was performed to compare the prognostic value of tumor stage, p53 protein expression and tumor angiogenesis. Tumor angiogenesis was the most important prognostic indicator to predict overall survival in our series. p53 expression was not independent and did not provide additional prognostic information to tumor stage. Our study suggests that angiogenesis as demonstrated by microvessel counts in CD34 stained sections is a significantly important prognostic factor for predicting survival in gastric cancer.

Key words: Angiogenesis, p53, Gastric cancer, Prognosis

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Introduction

Gastric cancer (GC) continues to be a highly aggressive malignancy with poor prognosis. Survival rates depend mainly on the stage of the disease when gastrectomy is performed. Patients with early gastric cancer have a 5 years survival of 90-100% while advanced gastric cancer have a 5 years survival of 15-25% (Ming and Goldman, 1992). However, further characterization of the prognosis of patients within these two groups is necessary in order to avoid excessive treatment, morbidity and costs. Immunohistochemical analyses of the expression of oncogenes, suppressor genes and growth factors have been proposed as prognostic indicators that routinely could be incorporated to diagnosis and may provide additional prognostic information to the histological evaluation of tumors. The expression of the p53 tumor suppressor gene, c-ErbB2 and c-myc oncogenes, as well as the Epidermal Growth Factor receptor (EGFr) have been independently proposed to play a role in the prognosis of gastric cancer (Ninomiya et al., 1991; Martin et al., 1992; Sasano et al., 1992; Kiu et al., 1993; Motojima et al., 1994; Gabbert et al., 1995; Tokunaga et al., 1995). Recently, tumor angiogenesis has been found to be a predictor of recurrence and metastasis in GC (Maeda et al., 1995; Araya et al., 1997).

Tumor cells require angiogenesis for nutritive supply. The newly formed vessels play an important role in tumor progression and metastatic dissemination (Weidner et al., 1993). As shown by Liotta et al. (1974) a large number of tumor vessels increases the opportunity of the tumor cells to enter the circulation. Indeed, the newly formed capillaries usually have a fragmented basement membrane, making easier the possibility of invasion (Nagy et al., 1989). The putative role of angiogenesis in

tumor growth and progression is also remarked by the fact that inhibitors of angiogenesis which are not cytostatic to tumor cells in vitro can suppress tumor growth in vivo (Nguyen et al., 1993; Fotsis et al., 1994). Thus, loss of suppressor genes in cancer can favour the uncontrolled expression of angiogenic factors as shown in experimental assays (Rastinejad et al., 1989; Chen et al., 1990; Hulevy et al.,1990).

Angiogenesis can be assessed on histologic sections by counting the density of microvessels (MVD) in the tumor stroma. Clinical studies have found a significant association between MVD and metastasis in histological sections stained with different vascular immunohistochemical markers (Factor VIII, *Ulex europaeus*, CD31 and CD34) in cutaneous melanoma (Vacca et al., 1993; White et al., 1994), breast cancer (Weidner et al., 1992; Horak et al., 1993; Toi et al., 1993; Gasparini et al., 1994a,b; Vartanian and Weidner, 1994), non-small-cell lung cancer (Macchiarini et al., 1992), prostate (Brawer et al., 1994), head and neck carcinomas (Gasparini et al., 1993; Igarashi et al., 1998), ovary (Hollisworth et al., 1994), esophagus and stomach (Maeda et al., 1995; Araya et al., 1997). In some studies MVD had an independent prognostic value for predicting overall survival or disease free survival. Determination of EGFr expression provides additional prognostic information to measuring tumor angiogenesis in breast cancer (Weidner and Gasparini, 1994).

We made a comparative study of tumor angiogenesis and the expression of p53, c-myc, c-ErbB2 and EGFr as prognostic indicators for overall survival in gastric cancer. All those immunohistochemically assessed parameters were compared with classical histological prognostic factors. Tumor angiogenesis was determined by counting microvessel density (MVD) within the invasive primary carcinoma in CD34 stained sections.

Materials and methods

143 cases of gastric cancer were obtained from the files of the Hospital Universitario "San Carlos", Madrid, Spain. Patients ranged in age from 36 to 96, median 65. 45 cases corresponded to early gastric cancer (EGC) and 98 to advanced gastric cancer (AGC). According to the International Union Against Cancer (UICC) classification, the 98 patients with advanced gastric cancer were distributed as follows: 4% IA, 8% IB, 30% II, 18% IIIA, 21% IIIB, 18% IV. All patients with EGC and AGC underwent total (53%) or subtotal (47%) gastrectomy and lymphadenectomy as follows: 11.3% R0, 49% R1 and 40% R2. Additionally, 16 cases of low grade gastric dysplasia and 12 of high grade gastric dysplasia were analyzed.

Histologically, the tumors were classified following Lauren's classification as intestinal (75.5%) and diffuse (24.5%); and by the TNM staging system as stage I+II (41.9%) versus stage III+IV (58.1%). Additionally, tumors were classified as those without evidence of metastases (36.7%), tumors with lymph node metastases (46.9%) and tumors with distant metastases (16.3%).

Ploidy patterns were analyzed by Flow Cytometry (Epics profile II, Coulter) and classified as aneuploid (35.5%) or diploid (64.5%), as previously described (Sanz-Ortega et al., 1995).

Patients overall survival was calculated from the time of surgery to death or last follow up (from 12-60 months).

Antibodies

Novocastra NCL-p53-DO7, C-ErbB2 (CB11-Medac), Novocastra NCL-c-myc, EGFr (BIogenex) and CD34 (Clone QBEND/10; AMAC, Westbrook ME) monoclonal antibodies were used.

Immunohistochemistry (Fig. 1)

Tissue samples were routinely fixed in 10% formalin, embedded in paraffin and cut at 5 μm. The Vectastain ABC kit (Vector Laboratories, Burlingame, CA) was used.

Microvessel quantitation

Quantification of vessels was done following the technique described by Weidner et al. (1991) and modified by Bosary et al. (1992). In each case the three most vascular areas (neovascular "hot spots") were assessed by light microscopy (Olympus BH2; Olympus America, Lake Success, NY), avoiding areas close to ulcerations or high inflammatory infiltration. A x200 (0.74 mm^2) and a x400 (0.37 mm^2) field in each of these regions were counted. The average of the three areas counting with x200 (AVG200) and x400 (AVG400) magnifications were recorded. As discussed by Bossari et al. (1992) large vessels with lumina greater than approximately eight red blood cells were excluded from the count. Any staining of single or clustered endothelial cells, clearly separated from adjacent microvessels or tumor cells was considered a countable microvessel (Fig. 2).

34 cases of gastric carcinoma were selected for MVD determination. Reasons to exclude cases from the study were the association of tumor cells with ulceration and granulation tissue, less than five H-E slides available for review per case or tumor tissue sample not enough to make a reliable assessment of MVD. It was not possible to assess MVD in dysplastic lesions.

Statistics

Statistical analysis was first performed to determine whether each of the two angiogenesis counts (AVG200, AVG400), p53, c-ErbB2, c-myc or EGFr expression had any association with histologic type, tumor location, stage of the disease, evidence of metastasis or ploidy status. The Wilcoxon rank sum test was used to evaluate the difference between two factor levels while the Kruskal-Wallis test was used to compare factors among

three groups.

The probability of survival was calculated for all the parameters using the Kaplan-Meier method (1958) and the significance of the difference between pairs of Kaplan-Meier curves was calculated using the Mantel-Haenszel procedure (1996). The Cox proportional hazards model was used to identify which factors are jointly significant in their association with survival (Cox, 1972).

Results

The incidence of p53, c-ErbB2, c-myc and EGFr expression in low grade dysplasia, high grade dysplasia, early gastric cancer and advanced gastric cancer is shown in Table 1. p53 expression (Fig. 1) increases

Table 1. p53, c-ErbB2, c-myc and EGFr expression.

	% POSITIVE CASE			
	p53	c-ErbB2	c-myc	EGFr
Low grade dysplasia	0%	31.3%	40%	50%
High grade dysplasia	25%	41.7%	16.7%	75%
Early cancer	24.45%	20%	34.1%	53.3%
Advanced cancer	41.8%	29.6%	28.3%	50%

Table 2. Clinicopathological features in early gastric cancer. Correlations with p53, c-ErbB2, c-myc and EGFr expression.

	n	p53		c-ErbB2		c-myc		EGFr	
		+	p	+	p	+	p	+	p
Depth invasion			0.06		1		1		1
Mucosa	23	47%		18%		48%		30%	
Submucosa	22	72%		24%		55%		35%	
Differentiation			0.66		0.40		0.09		0.52
Differentiated	22	37%		27%		68%		27%	
Undifferentiated	23	27%		13%		39%		42%	
Lymph nodes			0.05*		0.02*		0.85		0.75
Not affected	37	19%		13%		51%		31%	
Affected	8	62%		50%		71%		50%	
Endoscopic type			0.26		0.40		0.64		0.60
Type I+II	22	32%		27%		68%		28%	
Type III	23	17%		13%		48%		40%	

n: number of cases; +: percentage of positive cases; p: p2 value; *: statistically significant.

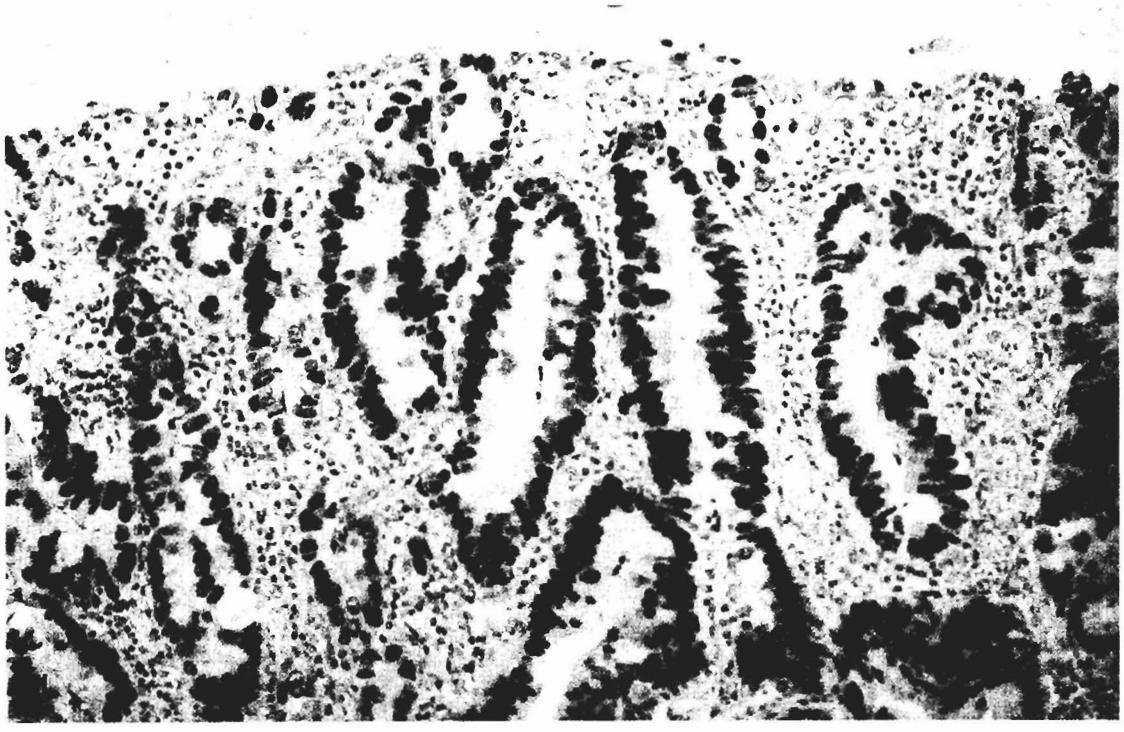

Fig. 1. p53 expression. Positive immunostaining for p53 located in the nuclei. x 200

Table 3. Clinicopathological features in advanced gastric cancer. Correlations with p53, c-ErbB2, c-myc and EGFr expression.

	n	p53		c-ErbB2		c-myc		EGFr	
		+	p	+	p	+	p	+	p
Location			0.01*		0.80		0.80		1
Body-Antrum	73	34%		26%		48%		29%	
Cardia	25	64%		40%		56%		25%	
Lauren			0.01*		0.00*		0.01*		1
Intestinal	74	46%		38%		57%		28%	
Diffuse	24	29%		4%		29%		27%	
Differentiation			0.55		0.00*		0.01*		1
Well-Moderate	53	46%		41%		59%		29%	
Poor	45	29%		15%		32%		26%	
TNM stage			0.01*		0.23		0.21		0.52
Stage I+II	41	27%		22%		41%		34%	
Stage III+IV	57	53%		35%		56%		24%	
Metastases			0.04*		0.09		0.24		0.45
None	36	30%		17%		39%		38%	
Lymph node	46	43%		37%		56%		21%	
Hepatic	16	62%		37%		40%		25%	

n: number of cases; +: percentage of positive cases; p: p2 value; *: statistically significant.

progressively from low grade dysplasia to advanced cancer while c-ErbB2, c-myc and EGFr expression show a high incidence in dysplastic lesions and do not follow a sequential pattern. Table 2 shows the clinicopathological features of the early gastric cancer group and their statistical correlations with oncogenes expression. A significant association ($p<0.05$) was found between lymph node metastases and both, p53 and c-ErbB2 expression. Table 3 shows the same analysis for the advanced cancer group. p53 expression correlates with cardial location, stage III+IV of the disease, and lymph node and distant metastases. c-ErbB2 and C-myc expression are associated with well-moderate differentiated and intestinal type carcinomas. EGFr shows no significant associations.

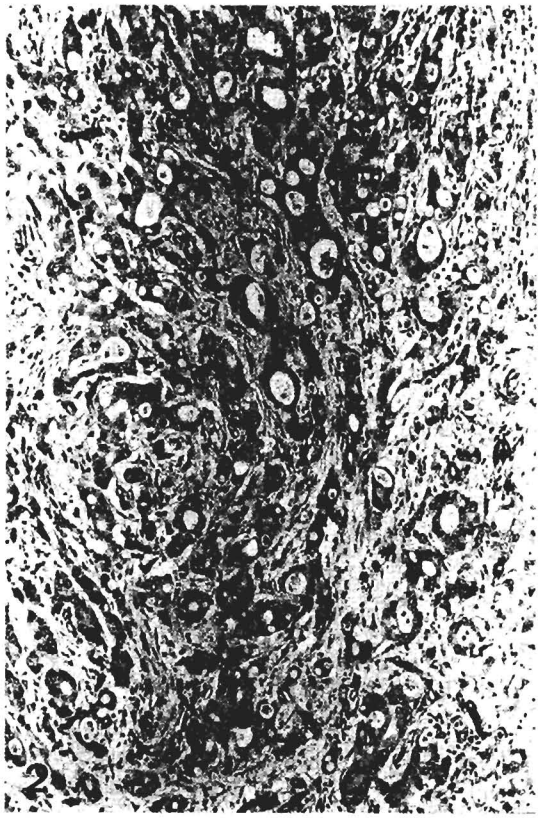

Fig. 2. Tumor angiogenesis in a high vascular density area. CD34 stained section. x 200

The average of MVD in GC was 100.36 for x200 fields, ranging from 55 to 200; and 28.3 for x400 fields, ranging from 28.6 to 110. The statistical study showed that there was no correlation between angiogenesis (AVG200 or AVG400) and intestinal or diffuse histologic type, Stage I+II versus stage III+IV, tumors with distant, lymph node or without metastases, tumors located in body-antrum or cardias, and diploid or aneuploid tumors (Table 4)

Univariate statistical analysis of overall survival showed that stage of the disease (p=0.0007), evidence of metastases (p=0.02) and p53 expression (p=0.03) were significant prognostic indicators for overall survival. A second univariate statistical analysis was performed in the group of patients in which MVD was assessed. Again, patients with tumor stage III+IV had a significantly worse overall survival than patients with tumor stage I+II (p=0.03). Thus, tumors with AVG200 greater than 99.1 and AVG400 greater than 52 had significantly worse prognosis compared to tumors with AVG200 counts lower than 99 and AVG400 counts lower than 52, p=0.008 and 0.009, respectively (Table 5). Tumors with lymph node or distant metastases, cardial location, diffuse type, poorly differentiated or aneuploid

Table 4. Angiogenesis and prognostic factors.

GROUP	n	MEAN	SE	MIN	MAX	p VALUE
AVG200 All	28	100.4	6.0	55	200	
AVG400 All	28	46.2	3.2	28.3	110	
AVG200- Histologic type						
Intestinal	20	106.0	7.5	60	200	
Diffuse	8	86.2	8.2	55	132.2	0.10
AVG400-Histologic type						
Intestinal	20	49.2	4.2	28.3	110	
Diffuse	8	38.6	2.5	30.6	49.3	0.10
AVG200- Metastases						
No metastases	9	87.9	7.9	55	121.6	
Lymph Node	13	113.8	10.4	67	200	0.17(KW)
Distant	6	90.0	7.5	60	106.6	
AVG400- Metastases						
No metastases	9	40.5	2.3	31.6	54.6	
Lymph Node	13	52.9	6.1	29.6	110	0.18(KW)
Distant	6	40.3	3.0	28.3	49.6	
AVG200-Stage						
Stage I+II	10	86.6	7.2	55	121.6	
Stage III+IV	18	108	8.1	60	200	0.11
AVG400-Stage						
Stage I+II	10	39.7	2.2	31.6	54.6	
Stage III+IV	18	49.8	4.6	28.3	110	0.11
AVG200- Ploidy						
Diploid	15	96.3	7.5	60	105.3	
Aneuploid	13	105.1	9.9	55	200	0.55
AVG400- Ploidy						
Diploid	15	44.4	5.3	28.3	110	
Aneuploid	13	48.3	3.3	35.6	81.6	0.12
AVG200- Location						
Body-antrum	18	107.7	7.3	66.6	200	
Cardias	8	93.3	11	60	84	0.24
AVG400- Location						
Body-antrum	18	46.2	2.9	30.6	81.6	
Cardias	8	48.6	9.3	28.3	110	0.54

MVD: microvessel density; n: number of cases; SE: standard error; MIN: minimum counts; MAX: maximum counts; p: p value by the Wilcoxon rank sum test used to evaluate the significance of the difference in angiogenesis between two factor levels or by Kruskal-Wallis test to compare factors among 3 groups (KW).

DNA patterns were not associated with lower survival rates when compared with tumors without evidence of metastases, body-antrum location, intestinal type, well-moderate differentiated or diploid DNA, respectively ($p>0.05$).

The multivariate analysis demonstrated that AVG200 was the most important independent prognostic factor for predicting overall survival when compared with both stage and metastasis or with any of them separately. p53 expression was associated with tumor stage and it was not an independent prognostic indicator (Table 5).

Discussion

In gastric cancer, tumor stage continues to be the histological feature most reliable as a prognostic indicator. Immunohistochemical analysis of the expression of different oncogenes, growth factors, proliferative markers, cell adhesion related substances, and other parameters such as ploidy patterns determined by flow cytometry have been proposed as new prognostic factors but contradictory results have been reported.

The role of angiogenesis as a prognostic factor has been most widely analyzed in breast cancer. Weidner et al. showed a highly significant association of MVD with overall and relapse free survival in all women ($p<0.001$) and in the subset of node positive women ($p=0.007$). Several independent studies have confirmed these findings (Horak et al., 1993; Toi et al., 1993; Gasparini et al., 1994a,b; Vartanian and Weidner, 1994) and tumor angiogenesis has been proposed as an independent and reliable prognostic indicator in breast cancer. In gastric cancer, a previous study (Maeda et al., 1995) suggested an important prognostic role of tumor angiogenesis as a predictor of recurrence.

Counting microvessels has proven to be reproducible though some discrepancies in the prognostic value of this technique in certain tumors have been reported (Bosari et al., 1992). These discrepancies have been related to different sensitivity and specificity of the analyzed markers for endothelial cells. Prior studies of angiogenesis were performed with anti-FVIII, a marker for endothelial cells and megakaryocytes. Horak et al. (1993) reported CD31 to be a marker with higher sensitivity for endothelial cells than anti-FVIII marker, although occasionally mildly cross reactivity with plasma cells, fibroblast and tumor cells can be seen. CD34, a marker of hematopoietic progenitor cells and endothelial cells, has been also found to be more sensitive than anti-FVIII. However, mild crossreactivity has been also reported with fibroblasts and tumor cells. In our experience with this marker however, only 2 out of 28 cases showed mild fibroblast and tumoral cell positivity.

The present study shows that p53 expression correlates with overall survival. However, the prognostic significance of p53 expression depends on its association with tumor stage as demonstrated the Cox's proportional hazards model. This prognostic dependance has been already reported by others authors (Starzynska et al., 1992; Motojima et al., 1994). c-ErbB2 may recognize a group of highly aggressive well differentiated adenocarcinomas as found by other groups (Motojima et al., 1994; Tokunaga et al., 1995). Both, p53 and c-ErbB2 were associated with lymph node metastasis in early gastric cancer suggesting that a closer follow-up should be performed in that group of patients. c-myc and EGFr showed no remarkable association in this study with gastric cancer prognosis.

Angiogenesis as demonstrated by MVD in CD34 stained sections is the most reliable immunohistological factor to correlate with prognosis in our series and does not depend on intestinal or diffuse histologic type, esophago-gastric junction or body-antrum location, the degree of differentiation or the tumor stage.

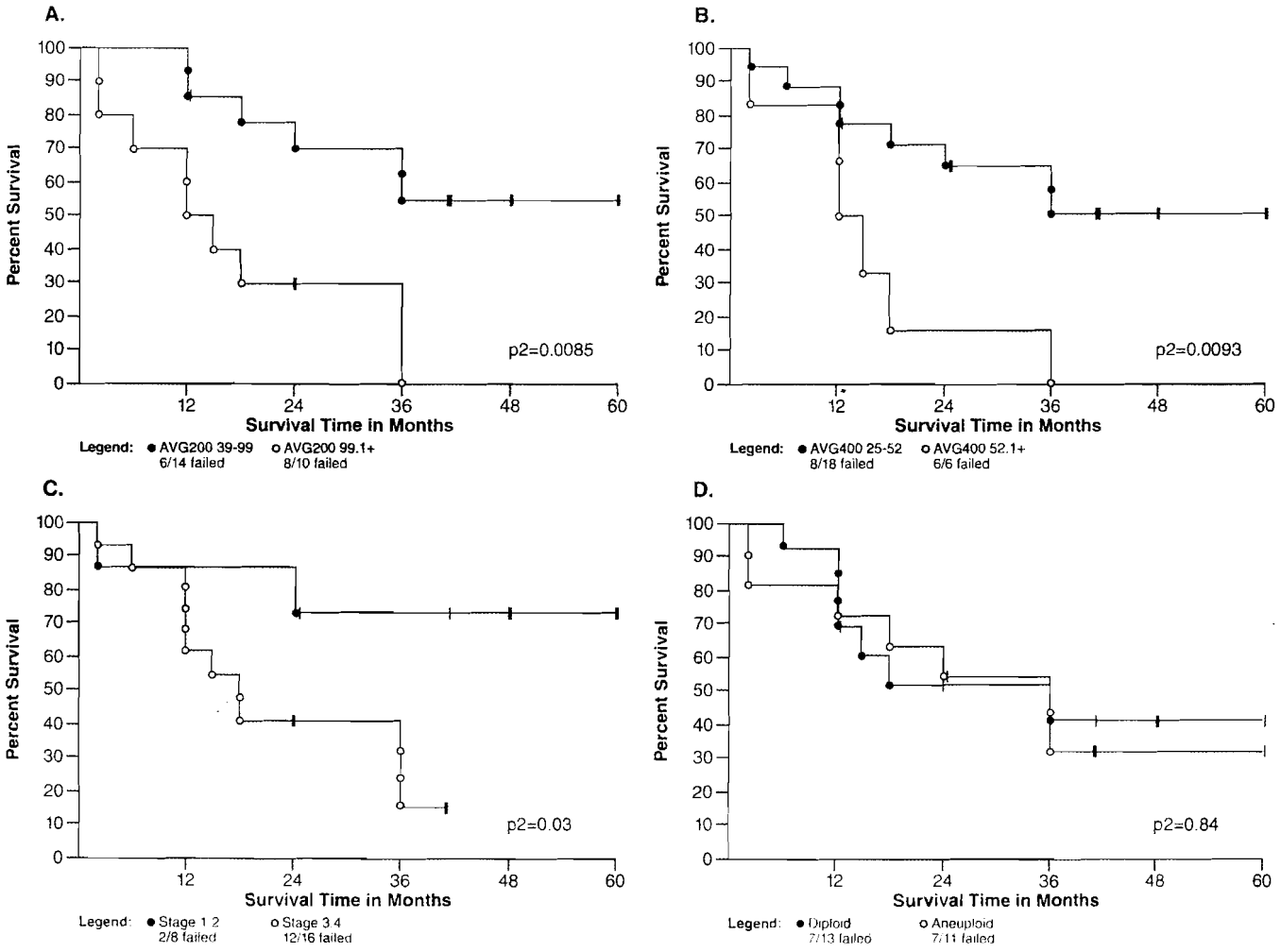

Fig. 3. Comparison of the Kaplan-Meier survival curves. **a.** AVG200 <99 and AVG200 >99. **b.** AVG400 <52 and AVG 400 >52. **c.** Stage I+II and Stage III+IV. **d.** diploid and aneuploid ploidy patterns.

Table 5. Overall survival and prognostic factors.

A.- UNIVARIATE ANALYSIS (Kaplan-Meier with Mantel-Haenszel p-values):

FACTOR VALUE	GROUP 1	GROUP 2	p
AVG200:	39-99	99+	0.0085
	39-115	115+	0.0019*
AVG400:	25-52	52+	0.0093
Stage:	I+II	III+IV	0.030*
Metastases:	Without	Lymph node or Distant	0.089
Differentiation	Well/moderate	Poorly	0.8
Histologic type	Intestinal	Diffuse	0.39
Location	Body-Antrum	Cardias	0.75
Ploidy	Diploid	Aneuploid	0.84

B.- MULTIVARIATE ANALYSIS (Cox proportional hazards model):

VARIABLE	RELATIVE RISK	STD ERROR	CHI-SQUARE	p-VALUE
AVG200 115+	3.19	0.58	3.94	0.047
Stage III+IV	2.9	0.81	1.82	0.18
AVG200 115+	3.6	0.58	4.86	0.028
Any Metastases	2.2	0.81	0.96	0.33
Stage III+IV		0.54	8.54	0.003
Any Metastases		0.22	0.47	0.50
p53 expression		0.36	2.02	0.15
c-ErbB2 expression		0.19	1.99	0.15

Furthermore, the Cox proportional hazards model showed that compared to other classical prognostic indicators angiogenesis was the most important independent factor for predicting overall survival. We suggest that microvessel quantitation as described is a useful technique that can provide important additional information in the prognosis of gastric cancer. We also want to encourage other pathologists to address the question of whether counting microvessels with the described technique is also a powerful prognosticator in their hands and can be included routinely in gastric carcinoma cases.

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