

## TGF- $\alpha$ , c-erbB-2 Expression and Neoangiogenesis in Vulvar Squamous Cell Carcinoma

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**Abstract.** *Background:* TGF- $\alpha$  and c-erbB-2 are important mediators of tumor neoangiogenesis linked to the epidermal growth factor receptor. Thus, we analyzed TGF- $\alpha$  c-erbB-2 and tumor neoangiogenesis in vulvar carcinoma and determined their prognostic significance. *Patients and Methods:* Seventy-five carcinomas were evaluated for histological parameters. Tumors were stained immunohistologically for TGF- $\alpha$ , c-erbB-2 and factor VIII antigen. Results were analyzed statistically by  $\chi^2$ -test, Kaplan-Meier survival curves and log-rank-test. *Results:* Sixty-five % of the carcinomas were positive for TGF- $\alpha$  in >50% of the tumor cells. C-erbB-2 overexpression occurred in 47% of the tumors, but there was frequently a high cytoplasmic staining. Twenty-nine % showed high microvessel density. These tumors were more likely to have vascular space involvement ( $p=0.02$ ). In carcinomas with TGF- $\alpha$  expression in >50% of the tumor cells, microvessel density was increased ( $p=0.05$ ). Overall and disease-free survival tended to be reduced for tumors with high TGF- $\alpha$  expression and microvessel density, but differences were not significant. *Conclusion:* Tumor neoangiogenesis is an important event in vulvar carcinogenesis which is related to the expression of growth factors.

The induction of neoangiogenesis is an important event in the development of malignant tumors involved in tumor growth and the development of metastases (1). Neoangiogenesis is a multistep process. First, tumor proliferation with an insufficient vascular system results in tissue hypoxia and lack of nutritive substances leading to induction of angiogenesis (2). This is implemented by the secretion of angiogenetic and antiangiogenetic factors with predominance of angiogenetic factors in tumors (3).

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*Key Words:* Vulvar carcinoma, TGF- $\alpha$ , c-erbB-2, neoangiogenesis, immunohistology, prognosis.

Through these mediators, endothelial cells of existing blood vessels secrete enzymes which attack the basal membrane. New blood vessels lack the structure of mature vessels. Frequently there are no pericytes, vessels are dilatated and the basal membrane is incomplete and characterized by transcellular fenestrae (4).

Important angiogenetic factors are vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF), which act synergistically (5). Another important mediator, which stimulates endothelial cell proliferation by binding to epithelial growth factor receptor, is transforming growth factor  $\alpha$  (TGF- $\alpha$ ) (6). Further overexpression and amplification of human epidermal growth factor 2 (Her2/neu, c-erbB-2) elevates VEGF levels and stimulates neoangiogenesis (7).

Microvessel density and expression of angiogenetic factors are of prognostic significance in several carcinomas (8-10). The aim of the present study was to evaluate neoangiogenesis and regulation by mediators linked to the epidermal growth factor receptor in vulvar squamous cell carcinoma and their prognostic significance.

### Patients and Methods

*Patients and treatment.* We investigated 75 patients with primary squamous cell carcinoma of the vulva which were treated with local excision or vulvectomy. Forty-six patients (61%) received ipsi- or bilateral inguinal lymphadenectomy. Follow-up data were available for 73 patients with a median follow-up time of 69 months. Five vulvar biopsies without histopathological changes were used as controls.

*Histology.* The whole surgical specimen was examined histologically using hematoxylin and eosin-stained large scale sections. For carcinomas, grading according to the Gynecologic Oncology Group (GOG) (11), count of mitotic figures, inflammatory infiltrate and vascular space involvement were analyzed. Depth of infiltration was measured between the base of the most superficial dermal papillae and the deepest point of infiltration (12).

*Immunohistochemistry.* Tissues were fixed in formalin and embedded in paraffin. Slides were deparaffinized in xylene for 10 minutes and

hydrated in decreasing concentrations of ethanol and finally phosphate-buffered saline. Monoclonal antibodies, mouse-anti-TGF- $\alpha$ , clone 213-4.4 (Oncogene Science, Hamburg, FR Germany) diluted 1:70 and mouse-anti-c-erbB-2, clone 3B5 (Immunotech, Krefeld, FR Germany) prediluted, were used after microwave pretreatment in sodium citrate buffer pH 6.0 (Merck, Darmstadt, FR Germany) for 20 minutes and blocking of endogenous peroxidase with 3% hydrogen peroxide for 10 minutes. Factor VIII-antigen was stained by polyclonal rabbit antibody A 0082 (Dako, Hamburg, Germany) diluted 1:400. Staining was performed using the avidin-biotin-complex (ABC) method and peroxidase-diaminobenzidine. For counterstaining, Mayer's hemalaun was used. Sections were then dehydrated and mounted in permount. For each slide, a negative control with the same antibody idio-type as the primary antibody was performed. As positive control a breast cancer was used.

**Immunohistological evaluation.** Evaluation of the immunohistochemical results was performed retrospectively and blindly. C-erbB-2 was categorized according to standard procedure as 0, +, ++, ++++. For TGF- $\alpha$  staining, the percentage of positive cells was determined by counting 200 cells. The percentage was classified in subgroups of 0-50% and >50%. For evaluation of microvessel density (MVD), three hot spots were identified under low magnification (40x) and the vessels counted in the stroma adjacent to the invasive cancer under high magnification (200x) (13, 14). The results were classified as low, medium and high MVD (15).

**Statistics:** The significance of the association between staining results and semiquantifications with histological and clinical tumor parameters was analysed by X<sup>2</sup>-test at a significance level of  $\alpha=0.05$ . For disease-free survival and overall survival, Kaplan-Meier analysis using log-rank-test was performed. For analysis of overall survival, patients who died because of intercurrent disease were not included in the study.

## Results

All 5 biopsies without histopathological changes showed TGF- $\alpha$  expression with low intensity in the cytoplasm of basal and parabasal epithelial layers. Twelve % of the carcinomas showed TGF- $\alpha$  expression in 0-10% of the cytoplasm of the tumor cells, 23% in 11-50% and 65% in >50%. Most tumors exhibited a diffuse staining, but in 13% TGF- $\alpha$  expression was restricted to basal areas of infiltrating tumor aggregates (Figure 1 A, B). Further the cells of the inflammatory infiltrate were positive for TGF- $\alpha$  (Figure 1C). Correspondingly, there was a significant correlation with the severity of the inflammatory reaction ( $p=0.02$ ), but not with other histological parameters (Table I).

Membrane c-erbB-2 overexpression was present in 47% (++) and 21% (+++) with a strong cytoplasmic background staining in several cases. C-erbB-2 overexpression was correlated with high count of mitotic figures in the tumor ( $p=0.02$ ) and high-grade tumors ( $p=0.04$ ) (Table I).

Twenty-nine % of the tumors had a high MVD (Figure 2), 39% had low MVD and 32% medium MVD. High MVD was significantly correlated with vascular space involvement

( $p=0.02$ ). Tumors with high MVD had a significantly stronger inflammatory reaction ( $p=0.004$ ) and lower count of mitotic figures ( $p=0.002$ ) (Table I). In carcinomas with TGF- $\alpha$  expression in >50% of the tumor cells, MVD was significantly higher ( $p=0.05$ ). There was no correlation of c-erbB-2 overexpression with MVD or TGF- $\alpha$  (Table II). Disease-free and overall survival seemed to be reduced for tumors with high TGF- $\alpha$  expression and MVD according to Kaplan-Meier curves, but the differences were not significant (Figures 3, 4).

## Discussion

The 5 cases of vulvar epithelium without histopathological changes analyzed showed TGF- $\alpha$  expression with low intensity in the basal and parabasal epithelial layers accounting for less than 50% of the epithelial cells. Weak TGF- $\alpha$  expression has been observed previously in 100% of normal epithelium (16). Contradictory results have been obtained for squamous epithelium of the cervix uteri, with total lack of TGF- $\alpha$  expression in normal cervical epithelium (17).

TGF- $\alpha$  overexpression is defined in previous studies by expression in more than 20% of the tumor tissue (17, 18). According to this definition, 85% of the carcinomas would show TGF- $\alpha$  overexpression. Since normal vulvar epithelium expressed TGF- $\alpha$  in <50% of the epithelial cells and immunohistologically there was a big difference between tumors with expression in 0-50% and >50% of the tumor cells, we defined overexpression as positive staining in >50%. Thus 65% of the carcinomas showed an elevated expression of TGF- $\alpha$ . An immunohistological study on vulvar epithelial disorders recognized TGF- $\alpha$  in 100% of normal epithelium, vulvar intraepithelial neoplasia (VIN) and invasive cancer, but there was an increase of staining intensity from normal epithelium to cancer (16). Further, TGF- $\alpha$  mRNA assay was positive in all analyzed vulvar carcinomas (19). The high percentage of TGF- $\alpha$  overexpression supports an important effect of TGF- $\alpha$  in vulvar carcinogenesis. Further, the positive correlation of TGF- $\alpha$  expression with MVD indicates that TGF- $\alpha$  is an important mediator of neovascularisation in vulvar cancer, which has been shown previously for esophageal squamous cell carcinoma (20).

TGF- $\alpha$  was positive in the cytoplasm of tumor cells and inflammatory cells, indicating that both cell populations are important for the production of this mediator. Analogous results have been obtained for adenocarcinoma of the gastro-esophageal junction (21). Corresponding TGF- $\alpha$  expression was correlated with the severity of the inflammatory infiltrate. The Kaplan-Meier curve analysis with non-significant reduction in disease-free and overall survival, which may be explained by the low patient number, suggests a prognostic significance for TGF- $\alpha$ . An association with reduced overall survival has previously been shown for squamous cell carcinomas of head and neck, esophageal and

Table I. Correlation of TGF- $\alpha$ , c-erb B2-staining and MVD with histological parameters.

	TGF- $\alpha$			c-erbB-2			MVD			
	<50%	>50%	X <sup>2</sup> -test	0/+ / ++	+++	X <sup>2</sup> -test	low	medium	high	X <sup>2</sup> -test
Tumor type										
keratinizing	22	37		47	12		21	20	18	
basaloid/condylomatous	4	12	ns	12	4	ns	8	4	4	ns
Inflammatory reaction										
no	1	2		3	0		2	1	0	
weak	8	4		10	2		7	3	2	
intermediate	6	19		16	9		12	9	4	
strong	8	21		24	5		7	10	12	
extremely strong	3	3	<i>p</i> =0.02	6	0	ns	1	1	4	<i>p</i> =0.004
Grading										
G1	5	20		23	2		10	5	10	
G2	12	22		25	9		13	12	9	
G3	9	7	ns	9	7	<i>p</i> =0.04	6	7	3	ns
Count of mitotic figures										
0-3/10hpf	6	16		19	3		7	3	12	
4-5/10hpf	11	18		24	5		13	9	7	
>5/10hpf	9	15	ns	14	10	<i>p</i> =0.02	9	12	3	<i>p</i> =0.002
Vascular space involvement										
no	10	29		31	8		20	11	8	
yes	16	20	ns	26	10	ns	9	13	14	<i>p</i> =0.02
pN										
pN0	5	20		20	5		8	10	7	
pN1,2	7	14	ns	19	2	ns	5	9	7	ns

colorectal carcinoma (20, 22, 23). However, there are also contradictory results in other carcinomas, for example laryngeal squamous cell carcinoma (24).

Overexpression of c-erbB-2 occurred in 47% (+ + / + + +). These results are consistent with findings in other carcinomas (25, 26) and in vulvar carcinoma with overexpression in 46% (27). C-erbB-2 was characterized by a high cytoplasmatic staining, which has been described for squamous cell carcinoma of the head and neck (28). Overexpression was correlated with high-grade tumors and high count of mitotic figures, which has been shown for breast carcinomas (29). However, the correlation of c-erbB-2 overexpression with MVD in breast cancer (15) could not be reproduced for vulvar cancer. Supporting this observation, an inverse correlation has been analyzed for squamous cell carcinoma of head and neck (28). Further, there was no prognostic value of c-erbB-2 overexpression, which is contradictory to previous findings (27), although studies on different carcinomas could not show a prognostic value, for example in squamous cell carcinoma of the head and neck (28). Nevertheless, there is an important predictive value regarding the therapeutic effect of trastuzumab. Thus, in case of generalized disease, the use of this substance should be reflected in the 47% of c-erbB-2-overexpressing vulvar carcinomas.

High MVD was significantly correlated with vascular space involvement. This could be explained by the immature

vascular structures in tumor-induced neoangiogenesis, which increase the risk of vascular infiltration (1). Further, there was a positive correlation of high MVD with low count of mitotic figures, perhaps as a result of low stromal components in these tumors, and with the severity of the lymphoplasmacytic infiltrate, which has been observed previously in laryngeal squamous cell carcinoma (30) but could not be reproduced in vulvar cancer.

Kaplan-Meier curve analysis seems to show reduced disease-free and overall survival for tumors with high MVD, but the differences were not significant, which may be explained by the small number of patients. The prognostic relevance of MVD has been demonstrated for carcinoma of the vulva (31) and other organs, for example keratinizing squamous cell carcinoma of the uterine cervix and breast cancer (15, 32), although there are contradictory results (33). Also, an increase of MVD has been observed from normal epithelium over low- and high-grade intraepithelial neoplasia to invasive cancer for cervix, vulva and anus (14, 34, 35). MVD may become a predictive factor identifying VIN lesions which are more likely to become invasive (36).

In conclusion, angiogenesis is an important event in vulvar carcinogenesis, being regulated by angiogenetic factors like TGF- $\alpha$ . Further studies are required to clarify the independent prognostic significance of neoangiogenesis in vulvar cancer.

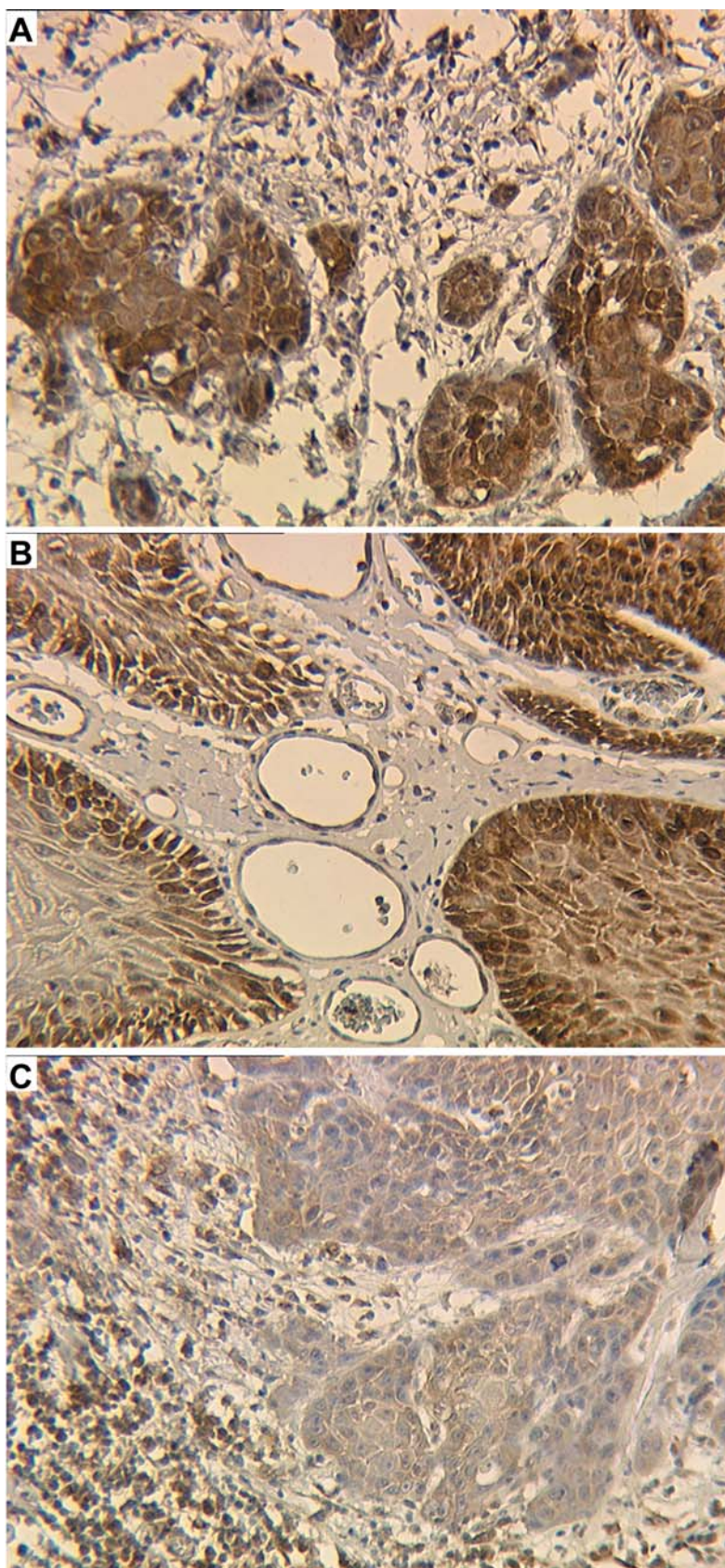


Figure 1. *TGF- $\alpha$*  staining in squamous cell carcinomas.  
*A* - diffuse in >50% of the tumor cells  
*B* - restricted to basal areas of infiltrating tumor aggregates  
*C* - in the lymphoplasmacytic infiltrate

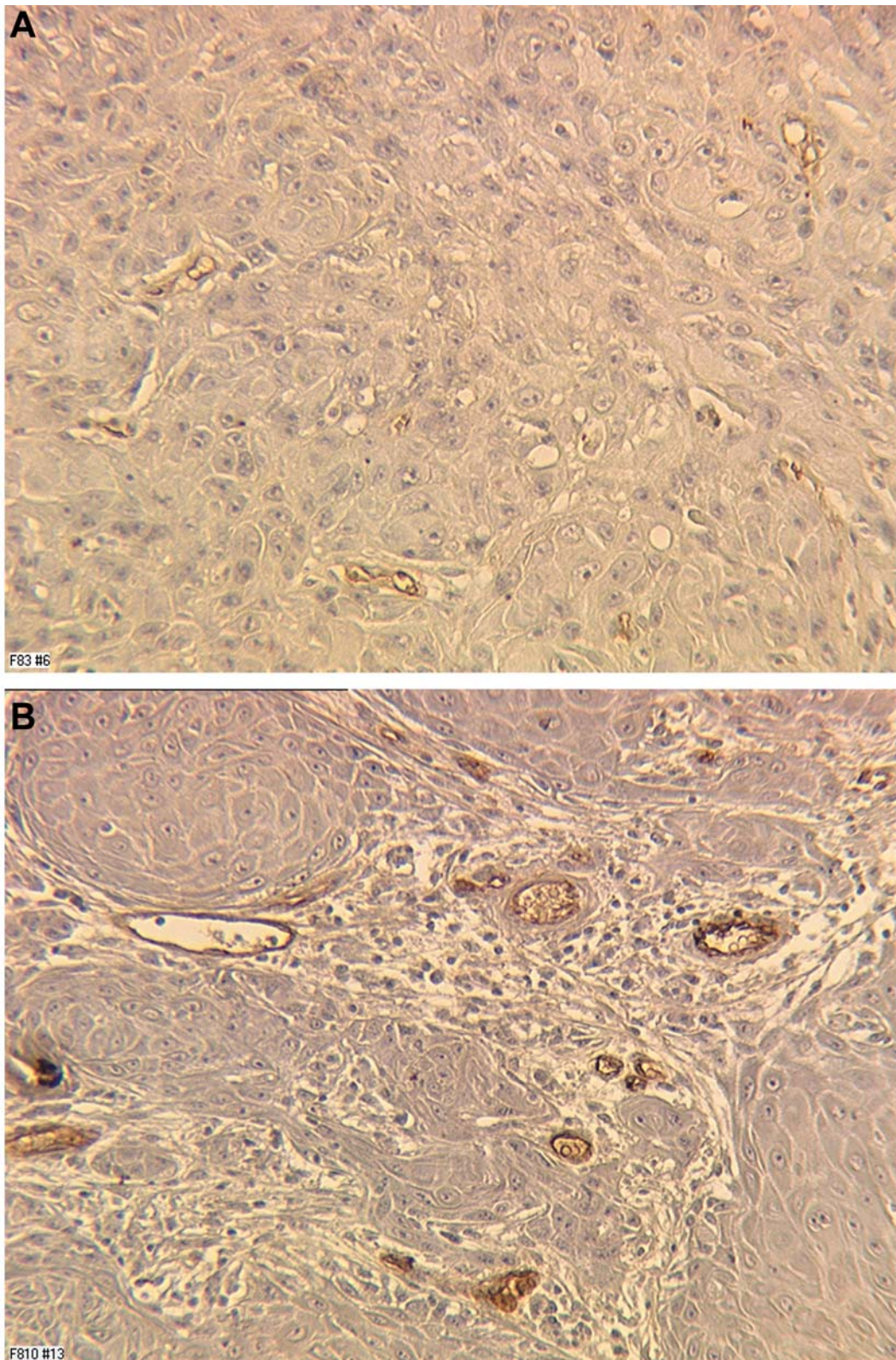


Figure 2. Immunohistological analysis of the microvessel density (MVD).  
A - low MVD (6 vessels / high-power field)  
B - high MVD (18 vessels / high-power field)

Table II. Correlation of the immunohistological staining results for TGF- $\alpha$ , c-erbB-2 and MVD.

		TGF- $\alpha$		
		<50%	>50%	
c-erbB2	0/+	16	24	ns
	++	3	16	
	+++	7	9	
		<50%	>50%	
MVD	low	14	15	$p=0.05$
	medium	6	18	
	high	6	16	

		MVD			
		low	medium	high	
c-erbB2	0/+	15	13	12	ns
	++	7	7	5	
	+++	7	4	5	

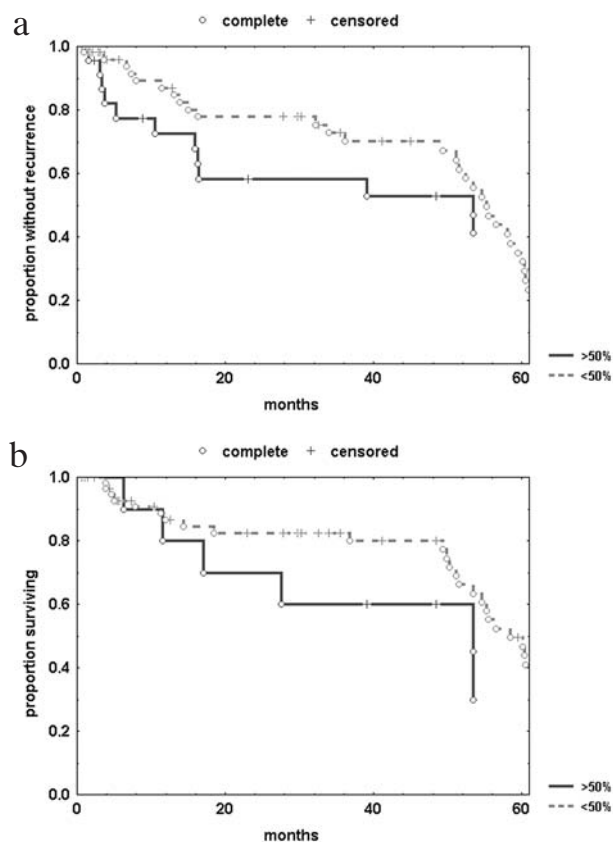


Figure 3. Kaplan-Meier curves for disease-free (a) and overall survival (b) analyzed by TGF- $\alpha$  expression in 0-50% and >50% of the tumor cells.

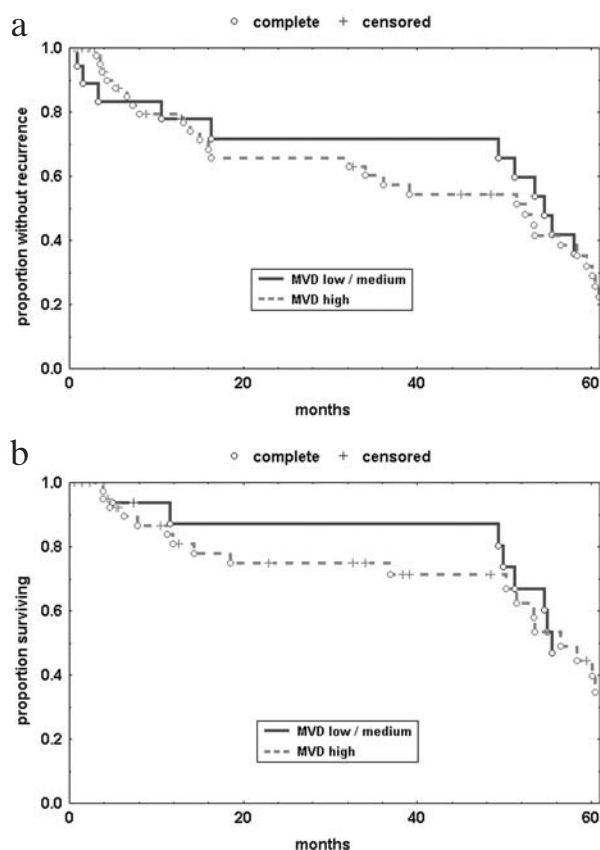


Figure 4. Kaplan-Meier curves for disease-free (a) and overall survival (b) analyzed by high MVD versus low and medium MVD.

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Received August 2, 2004  
Accepted February 8, 2005