Microvessel density as a prognostic factor in preinvasive and invasive cervical lesions

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Summary

Objective: To assess angiogenesis in preinvasive and invasive cervical lesions and its prognostic value in squamous cell carcinoma (SCC).

Methods: Twenty-seven cervical intraepithelial lesions (CIN I, II and III), 27 Stage Ib-IIa SCC and 12 normal cervical epithelium were included in the study. Clinico-pathological prognostic factors were re-evaluated from the patients' files and previous tissue sections. Microvessel density (MVD), a marker for angiogenesis, was assessed from new tissue blocks by an immunohistochemical staining method. Statistical tests included Kruskall-Wallis analysis, the Mann-Whitney U-test, Fisher's exact t-test to analyse the categorical data and Cox regression and Kaplan-Meier survival analyses to define the effect of prognosticators on survival.

Results: CIN II and III lesions had significantly higher MVD counts than normal epithelium and CIN I lesions, both of which had similar MVD count. Compared to preinvasive lesions invasive SCC had significantly higher MVD counts. Among SCC cases, only pelvic lymph node involvement appeared to be independent risk factor on unvariate analysis. However, MVD, as a cut-off value of 21 determined by ROC analysis, was found to be an independent prognosticator in early stage SCC cases by multivariate analysis.

Conclusion: Despite the small number of enrolled cases, the results of this study suggest that angiogenesis involved in the development and progression of cervical neoplasms and MVD might be used as a prognostic factor.

Key words: Cervical intraepithelial neoplasia; Cervical cancer; Angiogenesis; Microvessel density.

Introduction

Angiogenesis, the process in which new capillaries are formed from pre-existing vessels, takes part in a substantial number of physiologic events in the female genital tract such as folliculogenesis, menstrual cycles and embryogenesis [1, 2]. It also has been shown to be involved in both the initialisation and development of all tumoral processes in the body [3, 4].

Cells in a growing tumor gradually remain in a hypoxic and ischemic environment as in one part, new capillary formation continues. This state enables a milieu that triggers the chemotaxis of several cells such as macrophages, T-lymphocytes, mast cells and the release of growth factors, cytokines, matrix metalloproteinases, and vascular permeability factors, most of which are potentially angiogenic [5, 6]. Recently, cervical tissue has been the topic of the research of angiogenetic processes. All of these events are also expected to occur in the development and progression of cervical neoplasms that result in increased angiogenesis, which may be correlated to the malign potential of the tumor [7-16]. In neoplastic differentiation, the microenvironment in the cervix undergoes a series of cellular events that render the tumor cells to proliferate and disseminate [10, 11].

This study is an attempt to elucidate the role of microvessel density, a marker for angiogenesis, on the pathogenesis of cervical neoplasias and to assess its prognostic value in cases with invasive carcinoma.

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Materials and Methods

This retrospective study, covering the period from 1990 to 1998, included 27 preinvasive cervical lesions including CIN I, II and III, and 27 clinical Stage Ib and IIa invasive squamous cervical carcinoma (SCC). Twelve samples of normal cervical epithelium adjacent to preinvasive lesions served as controls. All cases with invasive cancers were staged clinically and then underwent a standard surgical procedure that consisted of a Ruthledge type III radical hysterectomy, bilateral salpingo-oophorectomy, pelvic and paraaortic lymph lymphadenectomy. Clinical and pathological prognostic factors of all cases including surgical findings, tumor grade, tumor diameter, lympho-vascular space involvement (LVSI), depth of stromal invasion, pelvic or paraaortic lymph node metastasis were re-evaluated from the patients' files and previous tissue sections by two co-author pathologists who were both blinded to previous clinical and pathological results. New tissue blocks were obtained from all cases to assess the microvessel density, a marker for angiogenesis, by an immunohistochemical staining method consisting of the treatment of tissue with FVIII monoclonal antibodies (Biogenex) via labelled streptavidive-biotin peroxidase (Zymed Universal Kit) [7].

New paraffinised tissue blocks of 4 μ in thickness were prepared from all cases and then deparaffinised and rehydrated for the immunohistochemical staining method. Following the incubation period of one hour at 70°C in the oven, at 10-minute intervals, they were processed three times with 96% alcohol and xylol solution and finally, with distilled water. They were then treated with phosphate-buffered saline at pH 7.0 (Zymed, USA) for five minutes and dessicated. Following this procedure, they were kept at room temperature for ten minutes in hydrogen peroxide solution diluted with water. After treating with buffer solution, two drops of FVIII primary monoclonal antibodies

were added to the tissue blocks that were kept for two hours at room temperature. Following their treatment with buffer solution, secondary monoclonal antibodies were added to the tissue blocks. After the treatment with buffer solution for five minutes, they were processed with 3 amino 9-ethylcarbazole (AEC, Lab Vision Corporation), kept at room temperature for five minutes and then washed with distilled water. Hematoxylin solution was finally added to the blocks for an opposite dye effect. Tissue blocks were mounted with slides (Lab Vision Ultramount). According to Weidner *et al.*, any stained areas apart from tumor cells and connective tissue elements, whether a luminal structure is present or not, were considered as positively stained [7]. These positively stained areas were observed under a high magnified field (x20 objective lens and x10 ocular lens) for MVD count. Regions where MVD had the highest value among three hot areas were considered for evaluation.

Statistical analysis included the Mann-Whitney U-test and Kruskal-Wallis analysis for evaluation of MVD count between preinvasive and invasive lesions, and normal epithelium. A receiver operating curve (ROC) was constructed for MVD count value and a cut-off value of 21 was found to have the best sensitivity (35.7%) and specificity (92.3%) for differentiation of preinvasive and invasive cancers. MVD count changes among various prognosticators were evaluated by Fisher's exact chi square test by using 21 as the cut-off value. Cox's multiple regression analysis was performed to assess the contribution of each variable to the survival by controlling all the other variables when necessary. Kaplan-Meier survival curves were constructed to analyse the relationship between various prognosticators including the MVD count and survival periods. A Statistical Package Program (SPSS 10.0, Chicago, IL, USA) was performed when analysing the data. Values were expressed as mean ± standard error of mean (SEM); $p < 0.05$ was considered statistically significant.

Results

Mean ages of cases with preinvasive and invasive cervial lesions were 50.5 ± 1.9 years (34-74 years) and 47.1 ± 1.9 years (27-72 years), respectively. As shown in Figure 1, the mean MVD counts of the normal epithelium, the CIN I lesions and the CIN II or CIN III lesions were found to be 1.2 ± 0.1, 2.1 ± 0.3, 14.1 ± 0.9, respectively. Invasive lesions had a significantly higher MVD count compared to the preinvasive counterparts ($p < 0.001$). MVD count did not differ statistically between the normal epithelium and CIN I lesions ($p = 0.08$). However, CIN II or CIN III lesions demonstrated a significantly higher MVD count compared to the normal epithelium and CIN I lesions ($p < 0.001$). The mean MVD counts of Stage Ib and IIa cancers were 33.1 ± 4.1 and 32.1 ± 3.8, respectively.

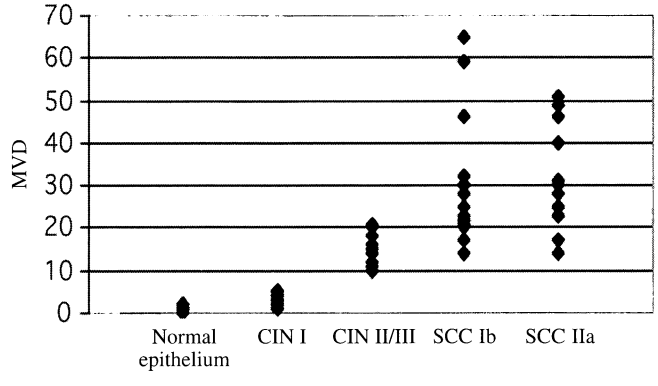

Figure 1. — Microvessel density (MVD) count among normal cervical epithelium (control), preinvasive lesions and squamous cell carcinoma (SCC).

Among the SCC group, the distribution of clinicopathological prognostic factors are shown in Table 1. It was found that 59.3% of cases had several prognostic factors that necessitated adjuvant radiotherapy. Tumor stage distributions were evenly distributed. MVD count was > 21 in 77.8% of cases with invasive cancer. MVD distribution among these various indicated prognosticators are summarised in Table 1. As clearly demonstrated, MVD count did not show any statistically significant correlation with tumor stage, tumor grade, depth of stromal invasion, LVSI and pelvic or paraaortic lymph node involvement. However, tumors with diameters of > 2 cm

Table 1. — *Microvessel density count distribution among several prognostic factors.*

Prognostic Variables	Microvessel Count (MVD) ≤ 21	> 21	p
Stage			
Ib	4 (25%)	12 (75%)	
IIa	2 (18.2%)	9 (81.8%)	1.000
Grade			
I	3 (33.3%)	6 (66.7%)	
II-III	3 (16.7%)	15 (83.3%)	0.367
Depth of stromal invasion			
≤ 1 cm	4 (44.4%)	5 (55.6%)	
> 1cm	2 (11.1%)	16 (88.9%)	0.136
LVSI			
(+)	0 (0%)	2 (100%)	
(–)	6 (24%)	19 (76%)	1.000
Tumor diameter			
≤ 2 cm	3 (75%)	1 (25%)	
> 2 cm	3 (13.0%)	20 (87.0%)	0.025*
Pelvic or paraortic LN			
(+)	0 (0%)	7 (100%)	
(–)	6 (30%)	14 (70%)	0.155

LVSI: lympho-vascular space involvement; LN: Lymph node; * $p < 0.05$; Fisher's exact chi-square test.

Table 2. — *Effect of various independent prognostic variables on survival through univariate and multivariate analysis.*

	Univariate Analysis B	B/SE	p	Multivariate Analysis B	B/SE	p
Age	0.013	0.196	0.658	0.048	1.571	0.210
Tumor stage	0.348	0.390	0.532	1.313	1.628	0.202
Tumor grade	0.1431	0.132	0.716	0.506	0.211	0.646
Pelvic/paraaorticLN	–1.999	10.753	0.001*	–0.943	0.609	0.435
Tumor diameter	3.408	1.325	0.250	17.709	0.006	0.939
Stromal invasion	4.170	3.219	0.073	16.874	0.008	0.931
LVSI	–0.276	0.070	0.792	–0.635	0.140	0.708
Radiotherapy	0.030	0.003	0.958	1.083	1.630	0.202
MVD	0.0048[1]	0.71	0.790	–0.096	3.866	0.049*

LN: Lymph node; LVSI: lympho-vascular space involvement; MVD: Microvessel density.

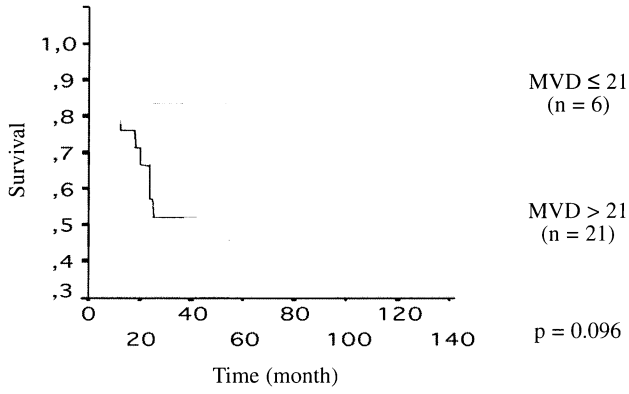

Figure 2. — Survival related to microvessel density (MVD) count with a cut-off value of 21.

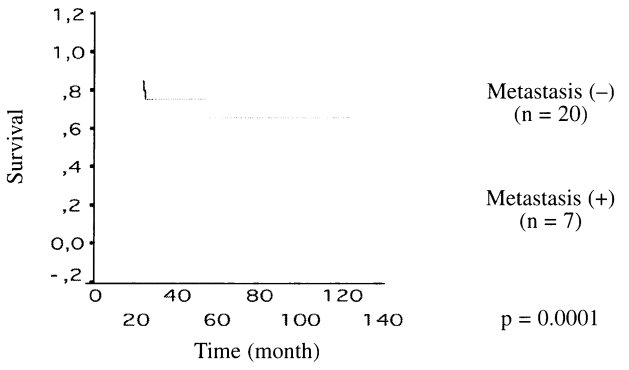

Figure 3. — Survival related to pelvic or paraaortic lymph node metastasis.

had significantly higher MVD count than those with diameters of ≤ 2 cm (p = 0.02).

Mean survival of the cases with invasive cancer was 43.7 ± 6.3 months (4-127 months). Fourteen (51.9%) of the 27 cases with invasive cancer died of disease during the follow-up with a mean survival of 20.3 ± 4.3 months. Among the cases with invasive cancer, only pelvic or paraaortic lymph node metastasis appeared to correlate with the survival on univariate analysis (p = 0.02). As is indicated in Table 2, when all other confounding factors were controlled, only MVD count, at a cut-off value of 21 determined by ROC analysis, was found to be an independent factor that affected survival (p = 0.001). Kaplan-Meier survival analysis demonstrated that MVD count did not reach a statistically significant value (p = 0.09) when comparing the survival curves at a cut-off value of 21 (Figure 2), however, pelvic or paraaortic lymph node metastasis appeared to significantly affect survival (Figure 3) (p < 0.001).

Discussion

Although there still appears to be a paradigm of the development of cervical cancer from normal epithelium, molecular, immunologic and viral factors have been advocated for the etiopathogenesis. Based on the angiogenic processes analysed hitherto in various cancers in the female genital tract, a recent body of evidence has shown that angiogenesis has a validated prognostic value for tumor recurrence, lymph node involvement, disease-free survival and need for adjuvant therapy [4, 8, 13-16]. Invasive cervical cancer with angiogenic processes at early stages may possess an intrinsic tendency to rapid growth and early nodal metastasis [8, 9].

During cervical neoplastic growth an angiogenetic process is assumed to occur prior to the invasion of the basal lamina, when the tumoral cells invade all the epithelial thickness. In this study, CIN II and CIN III lesions had higher MVD counts compared to CIN I lesions and normal epithelium. However, no difference was observed between CIN I lesions and normal epithelium. Contrary to this finding, Smith-McKune *et al.* found that MVD count did not show any difference between high and low-grade CIN lesions [10]. In another study, the degree of cervical preneoplastic changes was found to correlate with microvessel density and vascular endothelial growth factor expression, despite no differences depicted between low-grade lesions and normal epithelium, as also stated in our study [11]. These findings suggest that, as dysplastic cells integrate throughout the epithelium, angiogenic milieu becomes more predominant, rendering the cells to further invade the surrounding tissues.

There are controversies regarding the prognostic value of angiogenesis on disease-free survival of invasive cervical cancers [12, 13]. Nevertheless, recent contributions in this aspect address the fact that angiogenesis is one of the most important independent prognostic factors for the development of more invasive cervical cancer and poor prognosis [14, 15]. In our study, MVD count only correlated with tumor diameter as 2 x 2 contingency tables were analysed. Other prognosticators such as lymph node involvement and lympho-vascular space involvement (LVSI) were also detected to correlate with high MVD count which has been regarded recently as a prognostic factor for tumor recurrence and poor survival [16]. Our findings did not demonstrate an association of MVD count with other prognosticators except tumor diameter. We think that the number of cases enrolled to this study is fairly small to draw firm conclusions and the study should be repeated on a large data set.

Neoplastic growth of a tumor is thought to be determined by micro-environmental factors such as sustained hypoxia within the tumor, genomic alterations, angiogenic stimuli and other factors that lead to a vicious circle of malignant progression of the tumor to local or distant regions [17, 18]. Novel molecular or cellular interactions during angiogenic processes bring about new strategies to monitor local cervical milieu such as level of hypoxia in

early stages, to aid the selection of cases that would benefit from hypoxia modification efforts [19]. Angiogenic factor determination could also discriminate the subset of patients for whom antiangiogenic therapeutic approaches or adjuvant radiotherapy are indicated [20]. However, the validity of these findings in the literature should be demonstrated by more studies.

The results of this study suggest that angiogenesis is involved in both the development and progression of cervical neoplasms and despite the small number of enrolled cases, MVD may be used as prognostic factor, which can discriminate the need for further adjuvant therapies. However, further large series are needed to elucidate the contribution of MVD in the prognosis of cervical neoplasms.

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