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Prognostic Significance of Tumor Volume and Microvessel Density in Squamous Cell Carcinoma of Uterine Cervix

Shingo MORIYAMA,[1] Kouhei KOTERA,[1] Khaleque Newaz KHAN,[1] Futaba SATO,[2] Yoko SO,[1] Akira FUJISHITA,[2] Katsuya MATSUDA,[1] Hisayoshi NAKAJIMA,[3] Tadayuki ISHIMARU,[4] Hideaki MASUZAKI[1]

[1] Department of Obstetrics and Gynecology, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan

[2] Department of Obstetrics and Gynecology, Nagasaki Municipal Hospital, Nagasaki, Japan

[3] Division of Nursing, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan

[4] Sasebo Chuo Hospital, Nagasaki, Japan

The purpose of the present study was to evaluate the prognostic significance and relationship between tumor volume and microvessel density in squamous cell carcinoma of the uterine cervix. The estimated tumor volume (TV) in 57 patients (22 stage Ib, 18 stage IIa, and 17 stage IIb) had radical hysterectomy was calculated on the assumption that the tumor mass was spheroid. The micro-vessel density (MVD) was evaluated as the ratio of endothelial area immunoreactive to factor-VIII related antigen (von Willebrand factor) to whole image area measured by computer-aided image analysis system. Tumor volume ranged from 0.1 to 41.0 cm^3 (median 3.6 cm^3) and MVD from 0.33 to 2.95 % (median 0.85 %). A significantly larger median TV was noted in women with positive pelvic node metastasis (6.3 vs 2.6 cm^3, P=0.0228), parametrial invasion (8.9 vs 0.8 cm^3, P<0.0001), and postoperative irradiation (5.4 vs 0.6 cm^3, P=0.0007). In contrast, these clinical parameters had no effect on MVD. There was no correlation between TV and MVD. The overall survival rate at 5 years was 93.1% and 60.7% (P=0.0037) between women with a TV of ≦4 cm^3 and >4 cm^3, respectively; and 96.2% and 61.3% (P=0.0022) between tissue specimens with a MVD of ≦0.8% and >0.8%, respectively. A combined TV of >4 cm^3 and MVD >0.8% further deteriorated 5 year survival rate (42.1% vs 94.7%, P<0.0001). Multivariate analysis indicated TV and MVD as independent risk factor in this series (P=0.041, P=0.03, respectively). Our current findings suggested that TV and MVD are independent prognostic factors in women with cervical carcinoma who underwent radical hysterectomy. These prognostic factors may be clinically useful for the selection of high-risk patients who need extensive adjuvant therapy.

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Keywords: Squamous cell carcinoma; Tumor volume; Micro-vessel density; Prognosis; Uterine cervix

Introduction

Cervical cancer is the second most common cancer in women worldwide[1] and the seventh in Japan with an incidence of 8,779 new cases and 2,481 deaths recorded in 2002. Screening for cervical cancer by cytology has reduced the incidence of invasive cervical cancer, however, the number of young patients with invasive cervical cancer has recently been increased.[2]

Radical hysterectomy is a standardized surgical procedure for patients with stage Ib-IIb cancer. In cases of advanced disease or in the presence of risk factors such as lymph node metastasis, parametrial invasion, and involved resection margins, additional therapy with radiation or concurrent chemoradiation is useful. However, since this therapy is complicated by side effects such as bladder or bowel dysfunction and sexual dysfunction, single modality treatment is preferred to keep morbidity low. On the other hand, a more radical treatment is necessary to improve overall survival in surgically treated patients with high risk factors. Therefore, it is necessary to ascertain further prognostic factors to improve the therapeutic outcome.[3]

Tumor size has been proposed as an important prognostic factor in patients with cervical cancer by many authors.[4-13] However, information on the measurement of tumor size or volume in these reports was ill-defined. Only a few centers have subjected cervical

Address correspondence: Shingo Moriyama, M.D., Department of Obstetrics and Gynecology, Nagasaki University Graduate School of Biomedical Sciences, 1-7-1 Sakamoto, Nagasaki, 852-8501 JAPAN
TEL: +81-(0)95-819-7363, FAX: +81-(0)95-819-7365, E-mail: moriyama@smh.or.jp

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cancers obtained at surgery to precise morphometric study and thus can relate patient outcome to tumor size reliably.[7,9,10] Meanwhile the growth of solid tumors beyond 2mm diameter depends on tumor angiogenesis, which may also permit tumor progression and metastasis.[14,15] So far many studies have shown a statistically significant relationship between increased intratumoral microvessel density (MVD) and risk of metastasis and/or decreased survival of patients with solid tumors including cervical cancer.[16,17] While some investigators have shown that MVD counts might be an independent prognostic factor, whereas others have been unable to reproduce those findings. These differences in findings on MVD counts may be caused by variability in staining and counting of microvessels.[18] Furthermore studies are still limited on the relationship between tumor size and MVD and their individual or combined effect on the survival of women with cervical cancer.

Based on these reasons, we planned to evaluate the prognostic significance of tumor volume and MVD in cervical squamous cell carcinoma in comparison to other prognostic factors, and objectively examined the relationship between these two factors.

Materials and Methods

Patients and Tissues

Fifty-seven patients with squamous cell carcinoma of the uterine cervix were treated initially by radical hysterectomy from 1981 to 1990 at Nagasaki University Hospital. Clinical information was reviewed from the patients' medical records. Availability of adequate tissue material and clinical follow-up data was the only criterion for selection of patients. A fraction of these patients underwent adjuvant radiotherapy when risk factors for recurrence such as lymph node metastasis, parametrial invasion, deep cervical stromal invasion, and vessel permeation were present. An external beam radiation with a total dose of 50 Gy was applied for radiotherapy. All tumor specimens were routinely formalin-fixed and paraffin-embedded. Serial sections from each paraffin block having deepest invasive lesion per tumor specimen were subjected to immunohistochemical study.

Tumor Volume

Assuming the collected tumor as spheriod shape, the estimated volume of the primary tumor was calculated retrospectively by the approximation formula as shown in Figure 1. Briefly, the depth of the deepest invasive lesion (a) in the vertical direction was considered as sum of both deepest lesion (x) and most superficial lesion (y). The width (b) of the widest invasive lesion along the direction of cervical canal was evaluated from all blocks (median 12, range 10-20) of each tumor and using both macroscopic and microscopic samples. Finally, the tumor volume (TV) was calculated according to the formula as shown below:

$$TV\ (cm^3)=(4\pi/3)(a/2)^2\ (b/2)$$

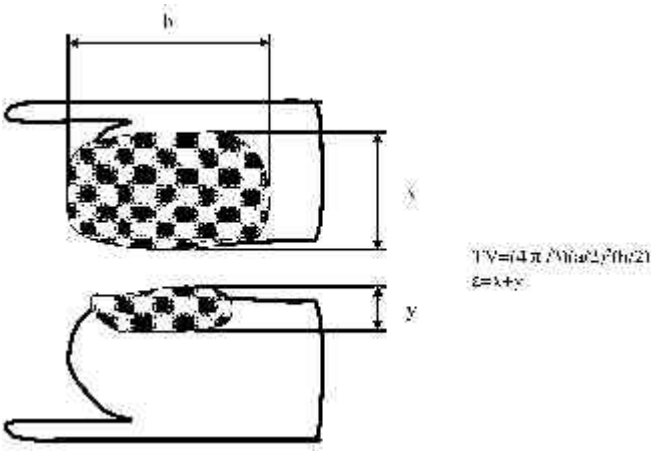

Figure 1. The estimated volume of primary tumor was measured by formula as mentioned here. The detail of tumor volume calculation is described in the text. TV indicates tumor volume (cm^3); 'a' denotes depth of the tumor (x+y); 'b' denotes width of the tumor.

Immunohistochemistry

Expression of factor VIII-related antigen (von Willebrand factor, VWF) was examined with the labeled strepto-avidine biotin procedure (LSAB kit, DAKO JAPAN Co., Ltd., Kyoto, Japan) in formalin fixed paraffin-embedded tissue specimens. Four-μm thick sections were cut and mounted on silane-coated glass slides. After deparaffinization, the sections were immersed in xylene and rehydrated through a series of graded ethanol and were washed three times with phosphate-buffered-saline (PBS, pH 7.2). The samples for VWF expression were initially treated with 0.1% trypsin (DIFCO Laboratories, Detroit, Michigan, USA) in tris-HCL buffer (pH 7.6) at 37℃ for 15 min. Slides were immersed in a 10mM citrate buffer (pH 6.0) and then micro-waved (MC-T5, NEC Corp., Tokyo, Japan) for a total of 10 min (90℃). Anti-human VWF antibody (clone F8/86, code M0616; DAKO, A/S, Denmark) is a mouse monoclonal antibody and was used as a primary antibody (1:50 dilution) and incubated for 3 hours at room temperature. The slides were subsequently incubated with biotinylated second antibody for 10 min, followed by incubation with avidin-peroxidase for 10 min and visualized with $NiSO_4/CoCl_2$/3,3'-diaminobenzidine tetrahydrochloride (DAB, Wako Pure Chemicals Industries, Ltd., Osaka, Japan) and hydrogen peroxide for color appearance[19]. Finally, the tissue sections were dehydrated with serial alcohols, cleared in xylene, and mounted with cover slip.

Microvessel Density

Microvessel density (MVD) was evaluated quantitatively by computer-aided image analysis system which was comprised of a optical microscope (Nikon, Tokyo, Japan) with 3CCD color vision camera module (Sony, Tokyo, Japan), high resolution image monitor (Sony), Image Grabber 1.2 color video digitizer software (Neotech, Ltd., London, UK) and Mac Scope 2.5.3 image analysis software (Mitani Co., Ltd., Fukui, Japan). The staining of vessels in the

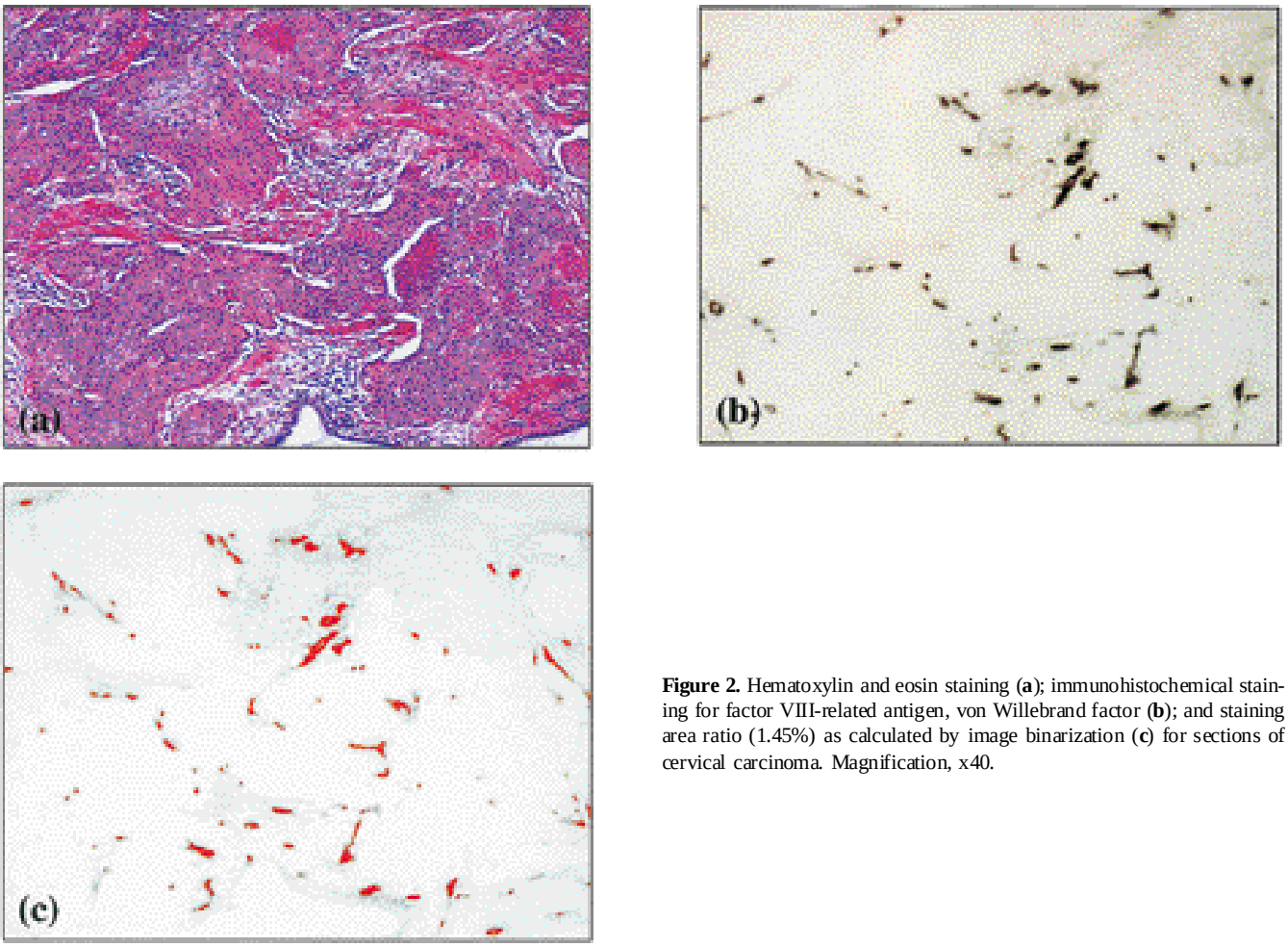

Figure 2. Hematoxylin and eosin staining (**a**); immunohistochemical staining for factor VIII-related antigen, von Willebrand factor (**b**); and staining area ratio (1.45%) as calculated by image binarization (**c**) for sections of cervical carcinoma. Magnification, x40.

stroma outside the tumors was used as an internal positive control. Negative controls were sections incubated with PBS only without the primary antibody. Because there was no counterstaining for VWF, a serially sectioned slide stained with hematoxylin and eosin (H & E) (Figure 2-a) was used as a map to identify vessels in VWF immunostained slide and to accurately target areas of carcinoma for analysis. Vessels were analyzed only in the area of invasive carcinoma. Slides were scanned at x40 magnification to determine 3 to 6 highly vascularized areas or "hot spots", which indicates area with higher neovascular potential.[13,20,21] The one imaging total area was 1.07 mm^2. The original color image was input into the computer and converted to gray image (Figure 2-b), so vessels were observed as dark gray lines. A binary image (Figure 2-c) was generated by computing a single adaptive threshold that was adopted from the staining intensity of area with internal positive control. An optimum threshold level to demarcate distinction between microvessels and background was adjusted by a single operator. MVD was measured automatically as the ratio (%) of VWF immunostained endothelial area to the whole image area.

Statistical analysis

Association among patient characteristics, tumor volume and microvessel density was tested by Mann-Whitney's U test or regression analysis. Survival was estimated using Kaplan-Meyer method, and comparison between study groups was performed with log-rank test. Multivariate analysis was performed using Cox's proportional hazard model. For all statistical analyses, the Stat View system of personal computer was used and $P<0.05$ was considered as statistically significant.

Results

Patients were classified according to the clinical staging of the International Federation of Gynecology and Obstetrics (FIGO), 22 were stage Ib, 18 were stage IIa and 17 were stage IIb. The mean age of the patients at surgery was 55.9 years (range 33-72 years). The tumor was classified for histological types (35 large cell non-keratinizing, 10 small cell non-keratinizing, 12 keratinizing). Pelvic lymph nodes were positive in 19 patients. Parametrial invasion and vessel permeation were seen in 34 patients and 20 patients, respectively. Mean follow-up period was 66.0 $\pm$ 32.7 months, with a range of 10.5-174.3 months. A summary of tumors collected after radical hysterectomy is listed in Table 1.

Table 1. Characteristics of tumors with cervical carcinoma after radical hysterectomy.

Characteristics	cases
FIGO stage	
Ⅰb	22
Ⅱa	18
Ⅱb	17
Histological type	
large cell non-keratinizing	35
small cell non-keratinizing	10
keratinizing	12
Pelvic lymph node metastasis	
Negative	38
Positive	19
Parametrial invasion	
No	23
Yes	34
Vessel permeation	
No	37
Yes	20
Postoperative irradiation	
No	10
Yes	47

Tumor Volume and Microvessel Density

Tumor volume (TV) assessed in 57 patients with primary cervical carcinoma ranged from 0.1 to 41.0 cm^3 (median 3.6 cm^3), and MVD ranged from 0.33 to 2.95 % (median 0.85 %). As shown in Table 2, significantly larger median TV was noted in cases with positive pelvic node metastasis (2.6 versus 6.3 cm^3, P=0.0228), parametrial invasion (0.8 versus 8.9 cm^3, P<0.0001) and postoperative irradiation (0.6 versus 5.4 cm^3, P=0.0007). In contrast, neither of these clinical parameters had any impact on MVD. In this series of 57 patients there was no significant correlation between TV and MVD (Figure 3).

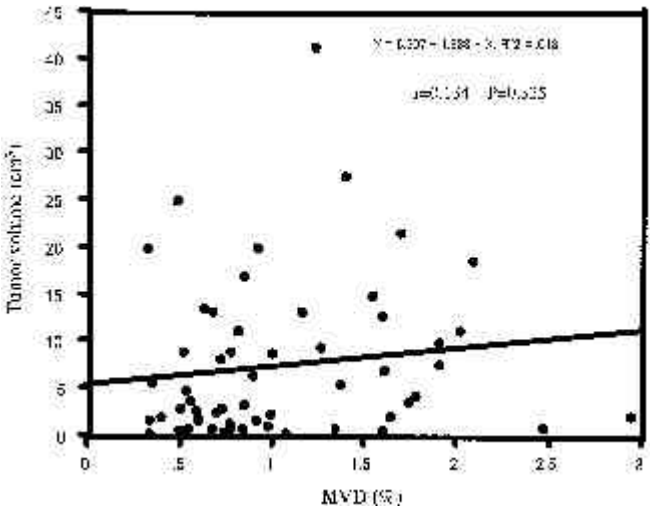

Figure 3. Shows the relationship between microvessel density (MVD %) and tumor volume (cm^3) of 57 patients with primary cervical carcinoma. No relationship was observed between them (r=0.134; P=0.325).

Table 2. Median tumor volume and microvessel density of 57 patients with cervical carcinoma who underwent radical hysterectomy.

Characteristics	TV(cm^3) (range)	P-value	MVD(%) (range)	P-value
FIGO stage		NS		NS
Ⅰb	2.6(0.1−21.4)		0.81(0.34−1.78)	
Ⅱa	5.4(0.5−41.0)		0.77(0.35−2.95)	
Ⅱb	6.3(0.6−27.3)		0.89(0.33−2.03)	
Histological type		NS		NS
large cell non-keratinizing	5.3(0.1−41.0)		0.97(0.33−2.95)	
small cell non-keratinizing	3.7(0.2−19.8)		0.87(0.34−1.78)	
keratinizing	2.7(0.3−24.8)		0.76(0.40−1.91)	
Pelvic lymph node metastasis		0.0228		NS
Negative	2.6(0.1−24.8)		0.83(0.33−2.47)	
Positive	6.3(1.0−41.0)		0.92(0.33−2.95)	
Parametrial invasion		<0.0001		NS
No	0.8(0.1−11.1)		0.78(0.34−2.95)	
Yes	8.9(0.5−41.0)		0.87(0.33−2.09)	
Vessel permeation		NS		NS
No	2.9(0.1−41.0)		0.84(0.33−2.47)	
Yes	5.8(0.2−27.3)		0.87(0.33−2.95)	
Postoperative irradiation		0.0007		NS
No	0.6(0.1−13.1)		0.84(0.50−2.47)	
Yes	5.4(0.2−41.0)		0.85(0.33−2.95)	

TV, tumor volume; MVD, microvessel density; NS, not significant.

Log-Rank Analysis

Patients with small (≦4cm^3) TV had a significant good prognosis when compared with patients with large TV (>4cm^3) (Figure 4). Five-year overall estimated survival rates were 93.1 % versus 60.7 %, respectively (P=0.0037). Again, patients with less MVD (≦0.8%) displayed a significant good prognosis when compared to patients with higher MVD (>0.8%) (Figure 5). Five-year overall estimated survival rates were 96.2 % versus 61.3 %, respectively (P=0.0022). FIGO stage, pelvic lymph node status, parametrial invasion, and vessel permeation had no significant impact on prognosis of patients as evaluated by univariate analysis in this series.

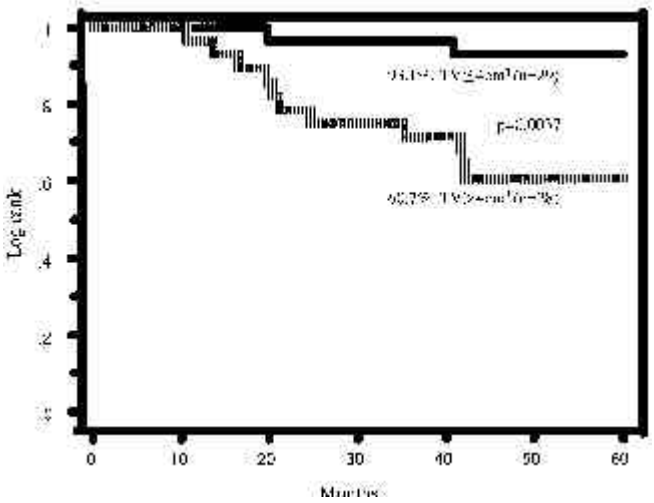

Figure 4. Overall estimated 5 years survival of 57 patients with primary cervical carcinoma according to tumor volume (TV). Patients with a TV of ≦4cm^3 had a good survival rate (93.1%) when compared with women having a TV >4cm^3 (60.7%) (P=0.0037).

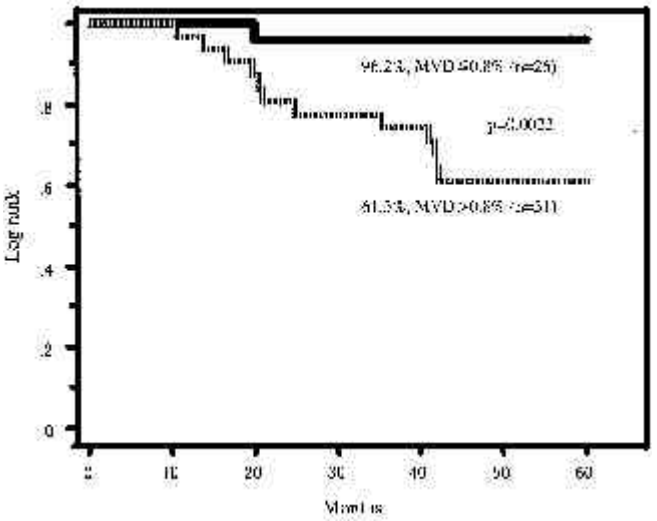

Figure 5. Overall estimated 5 years survival of 57 patients with primary cervical carcinoma according to microvessel density (MVD). Specimens of patients with a MVD of <0.8% showed a significantly better survival (96.2%) when compared with cancer specimens of patients with a MVD of >0.8% (61.3%) (P=0.0022).

Multivariate Analysis

A Cox's proportional hazards model for overall survival was carried out incorporating FIGO stage, pelvic node status, TV, and MVD (Table 3). Only variables with P values of <0.30 in the univariate analysis (log-rank analysis) were included in the multivariate analysis. Tumor volume and MVD were the only independent prognostic factors by multivariate analysis (Table 3). Patients with combined large TV (>4cm^3) and high MVD (>0.8%) had the poorest prognosis (Figure 6) when compared with other factors. Five-year overall estimated survival rates were 42.1 % versus 94.7 % for combined small TV (<4cm^3) and less MVD (<0.8%) (P<0.0001).

Table 3. Univariate and multivariate analysis of tumors with Cox's proportional hazards model to predict prognostic factors in patients with cervical carcinoma.

Factors	Univariate	Multivariate[a]		
	P-value	P-value	Relative risk	95%CI
FIGO (stage Ⅰ)	0.0539	0.1015	0.278	0.060～1.287
Pelvic lymph node status (negative)	0.2998	0.8338	1.131	0.357～3.589
Parametrial invasion (negative)	0.4456	—	—	—
Vessel permeation (negative)	0.3121	—	—	—
TV (≦4 cm^3)	0.0037	0.0410	0.103	0.013～0.803
MVD (≦0.8%)	0.0022	0.0300	0.194	0.040～0.936

TV, tumor volume; MVD, microvessel density; CI, confidence interval.
[a] Only variables with P<0.30 in the univariate analysis are included in the multivariate analysis.

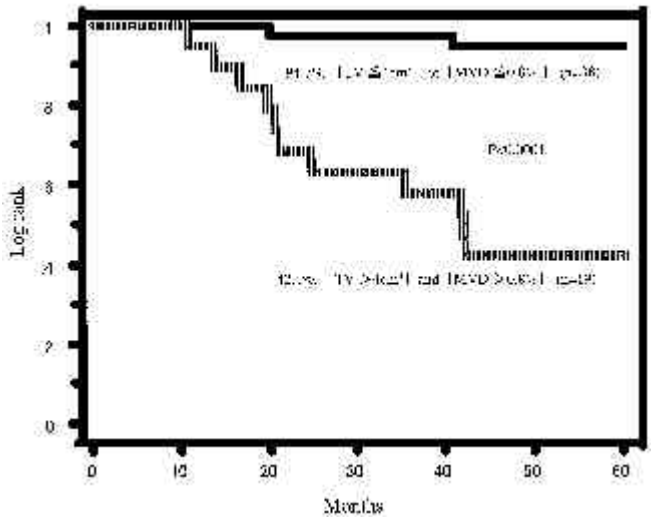

Figure 6. Overall estimated 5 years survival of 57 patients with primary cervical carcinoma according to combined tumor volume (TV) and microvessel density (MVD). Tumor of patients with a TV of >4cm^3 and MVD of >0.8% displayed worse prognosis (42.1%) when compared with those of <4cm^3 TV and <0.8% MVD (94.7%) (P=<0.0001).

Discussion

We demonstrated in our current study that estimated large tumor volume and increased microvessel density were independent prognostic factors to predict the survival of patients with carcinoma of the uterine cervix when compared with a number of other clinical parameters. This was confirmed by both univariate and multivariate analysis together with estimation from life-time survival curve.

Median tumor volume (4cm^3) was a significant prognostic indicator to isolate high- risk patients in this series. Tumor diameter has been proposed as a prognostic factor for survival of patients with cervical carcinoma by many authors[3-12]. It is postulated that patients with early stage Ib carcinoma of the cervix may have a survival of 90% or greater, while patients with stage Ib "bulky" tumor may have no more than a 50 or 60% survival. These patients have a poorer survival than some with stage II disease. In order to adjust this gap in the prognosis of patients with same stage Ib disease, FIGO Congress in Montreal, the Gynecologic Oncology Committee proposed some changes in the staging of cervical cancer. In 1995, a new International Federation of Gynecology and Obstetrics (FIGO) staging system for carcinoma of the cervix was published with a proposal to divide patients with stage Ib into two separate subgroups based on tumor extension and diameter: Ib1, tumor limited to the cervix and ≦4.0 cm diameter; and Ib2, tumor limited to the cervix and >4 cm diameter. However, the FIGO committee realized that this subdivision of stage Ib is a step trying to further delineate a wide spectrum of disease so that hopefully the best treatment modality may be ascertained.[22]

Based on these facts, it remains a concern of debate whether clinical tumor size can be considered as an actually important predictor of survival or not. This issue is still unclear. Finan et al.[23] showed that the FIGO staging system for Ib1 and Ib2 disease did not have an independent impact on survival in surgically treated patients. They said that disease stage acted only through nodal status in its impact on survival. Rutledge et al. subsequently demonstrated that the prognosis in stage Ib carcinoma was mostly influenced by the presence of lymphovascular space involvement and depth of stromal invasion and not by tumor size.[24] The reason for this is that clinical estimation of tumor size is a poor surrogate for the actual tumor volume, because measurement of tumor size is not standardized and obviously varies between various investigators.[9,10]

Burghardt et al. demonstrated in a large series that tumor volumetry of surgical specimens obtained at radical hysterectomy permitted a more accurate assessment of therapeutic results in patients with cervical cancer than does the FIGO classification.[9] In parallel with this study, Trattner et al. has proposed that tumor volume calculated by assuming an ellipsoid using the area of the largest pathological tumor specimens might provide important prognostic information if lymph node status was not known or histopathological stage could not be assessed.[4] Our data supported those two studies and it appears reasonable to speculate that tumor volume, because of its 3-dimensional measurement, would relate even better to prognosis than a linear tumor size in clinical practice.

Microvessel density (MVD), as measured by endothelial stained area and expressed as percentage of the whole tissue area, was found to be another independent prognostic factor for carcinoma of the uterine cervix. In fact, we found that patients with high MVD of more than 0.8 % had significantly worse overall 5 years survival rate (62%) than those with low MVD (≦0.8 %) in this study. This overall survival rate in patients with cervical cancer further deteriorated (61% to 42%) when high MVD was combined with a large tumor volume.

Angiogenesis has been shown to be essential for the growth and progression of malignant tumors.[25] MVD and its influence on survival has been studied in squamous cell carcinoma of the cervix, but the results of those studies were inconsistent and the prognostic value of MVD was uncertain,[26] probably because of difficulties in quantifying vascularization. Several methods have been applied to quantify tumor vascularity irrespective of the manner of highlighting the blood vessels in tissue sections. These methods are vessel counts per unit area, relative amount of vascular volume, intercapillary distance, and distance from tumor cells to the closest microvessel.[15] An evaluation of vascularization has been demonstrated using the method by manually counting the number of immunohistochemically stained capillaries. Because blood vessel is not uniform within tumor, the most densely vascularized area (so-called "hot spot") often is selected.[14] Moreover it is necessary of distinguishing individual vessels for counting. Thus it was pointed out that manual counting was time consuming and the potential subjectivity made results uncertain. On the other hand, Schoell et al. demonstrated that image analysis system for measurement of MVD was useful in ovarian carcinoma.[27] This study described that method of com-

puter-aided image analysis might overcome some of the subjectivity inherent in the manual counting of vessels and may improve the ability to evaluate accurately the angiogenic potential in ovarian carcinoma. Our current study also evaluated MVD using image analysis system by measuring immunoreactive endothelial cells to von Willebrand factor. We assume that this method would be more objective to measure vascularity in a defined tumor area than that of manual counting of immunoreactive capillaries.

We did not find any significant correlation between tumor volume and microvessel density in this study, even both TV and MVD were independent prognostic factors for overall survival by multivariate analysis. This lack of association between TV and MVD is unclear but can be explained as follows. Most tumors become symptomatic and clinically detected only after neovascularization. It should be emphasized, however, that switching to the angiogenic phenotype dose not always result in a rapidly proliferating tumor.[23] In cervical cancer, the onset of angiogenesis is an early event in pre-malignant changes of the cervix due in part to enhanced expression of vascular endothelial growth factor by the abnormal epithelium.[28] Recently, soft tissue contrast of magnetic resonance imaging (MRI) enables clear delineation of tumors within the uterine cervix using an endovaginal or endorectal technique. Tumor volume measurements obtained from such images correlate well with volume measurements at histopathology and are valuable predictors of outcome.[29] Moreover evaluation of microvessel density by dynamic enhanced MRI has been evaluated in a recent report.[30]

In conclusion, we reported here for the first time that tumor volume and microvessel densities are independent prognostic factors to predict the survival of patients with cervical cancers who underwent radical hysterectomy. These two prognostic factors may be clinically useful for the selection of high-risk patients who need extensive adjuvant therapy. Further clinical study is necessary regarding association between tumor volume or vascularity at radiological image and that by histopathology in order to strengthen the significance of our current findings at tissue level.

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