Angiogenesis in Stage IIIB Squamous Cell Carcinoma of Uterine Cervix: Reproducibility of Measurement and Preliminary Outcome as a Prognostic Factor

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This study was performed to determine the reliability and replicability of IMD analysis using the Factor VIII immunohistochemical method. The following purpose was determining the relationship between IMD and clinical outcome in individual cervical cancer patient treated with radical radiotherapy.

Twenty nine patients with stage IIIB cervical cancer were enrolled. Phase one was performed by using two pieces of tissue biopsy from different locations in the tumor from each patient. The IMD value was counted by the two pathologists after counterstaining by Factor VIII immunohistochemical method. No interobserver disagreement between the two pathologists was found (correlation coefficient = 0.92, 95% CI 0.82-0.96 for the first piece of tissue and 0.85, 95% CI 0.67-0.93 for the second piece). There was no variability in the IMD between the 2 pieces of tissue specimens from different locations of the tumor.

*Phase two followed to evaluate the relationship between IMD and clinical outcome in individual cervical cancer patients. Because of the small sample size, different patients' characteristics, different treatment protocol and short term follow up, there is no statistically significant conclusion.**Keywords: Cervical cancer, Angiogenesis*

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Tumor angiogenesis is a complex dynamic process leading to the formation of abnormal new blood vessels. Induction of angiogenesis is required for most tumors to grow beyond 1-2 mm in diameter, which is the limit of simple diffusion of nutrient and oxygen[1]. There is accumulating evidence that angiogenesis is controlled by a number of regulators, including proangiogenic and antiangiogenic factors.

Angiogenesis is considered essential for tumor growth and the development of metastases by increasing the opportunities for tumor cells to move into the bloodstream. The relationship between angiogenesis and increased risk of metastasis and/or decreased survival has been demonstrated in many types of cancer such as head and neck cancer, breast cancer, prostate cancer and also cervical cancer and other gynecologic malignancies[2-13].

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Intratumoral microvessel density (IMD) is assumed to reflect the intensity of tumor angiogenesis. With the use of immunohistochemistry, various antibody markers for endothelial cells have been used to identify intratumoral vessels. There are some variations in the immunohistochemical techniques used as well as differences in counting methods for assessing the IMD[14].

Cervical cancer is the most common cancer in Thai females[15]. Most patients present at an advanced stages. They have only 25-48% 5 year overall survival and 30-50% of patients have locoregional failure[16]. Tumor angiogenesis has been introduced into the assessment of cervical cancer to predict tumor control rate and progression of disease. High levels of angiogenesis will produce poor tumor control and a high rate of distant metastasis[2,4,5,7,16,17].

This study was performed in two phases. Phase one was undertaken to evaluate the reliability of IMD analysis using the Factor VIII immuno-

histochemical method. Phase two followed to determine the relationship between IMD and clinical outcome in individual cervical cancer patient treated with radical radiotherapy.

Material and Method

From June to November 2001, 29 patients with stage IIIB biopsy proven squamous cell carcinoma of uterine cervix were enrolled.

Phase one was performed to determine he reliability of IMD measurement using an immunohistochemical method. We first evaluated the replicability of tissue specimens. Two pieces of tissue biopsy were obtained from different locations in the tumor from each patient. The first piece was obtained from the outer most part of the tumor. The second was taken half way between the center of tumor and the outermost part. Secondly, we attempted to evaluate interobserver agreement between the two pathologists in IMD counting.

Immunohistochemical method

The 3 micron paraffin sections were deparaffinized and then treated with 3% hydrogen peroxide to block endogenous peroxidase activity. Then they were incubated overnight in a humidity chamber at room temperature with the primary antibody (Factor VIII monoclonal mouse related antigen,IgG1, kappa, DAKO Glostrup,Denmark; working dilution 1:2000), followed by the second antibody (biotinylated anti-mouse immunoglobulin, DAKO Glostrup, Denmark; working dilution 1:500). Any nonspecific reaction was blocked by incubating with 10% normal rabbit serum. Counterstaining was performed with hematoxylin.

Vascular assessment

After scanning the immunostained section at low magnification the area of clear cut cancer tissue with the greatest number of distinctly highlighted microvessels (hot spot) was selected. The IMD was then determined by counting all vessels at a total magnification of x400 and examination area of 0.1964 mm2

The criteria for counting the stained endothelial cells was agree by the two pathologists[1,5,6,7,12-14]. Individual microvessels, seen as brown stained endothelial cell clusters not necessarily having lumens, were counted as shown in Fig. 1. Vessels with thick media were excluded from the count.

The homogeneity between 2 pieces of tissues from different locations in the tumor (replicability) was tested using non-parametrics. The intraclass correlation coefficient was used to analyze interobserver variability between the 2 pathologists.

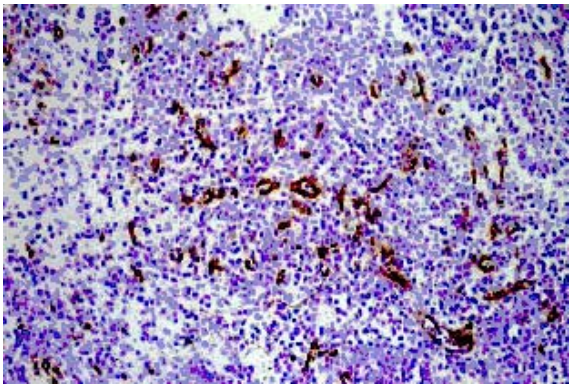

Fig. 1 Example of hotspot showing numerous discrete microvessels from formalin fixed, paraffin-embedded cervical cancer tissue stained with antifactor VIII monoclonal antibody. The vascular spaces are highlighted as irregular dark lines (x40)

Phase two of the study followed. All the patients received radical radiation therapy. External radiation and intracavitary radiation therapy were given to all patients. Some patients received concurrent chemoradiation therapy with cisplatinum based regimens. Some patients received alter-fractionation radiation therapy. Some patients were treated with radiation therapy alone. External radiation therapy was performed using standard AP/PA pelvis field using Co 60 machine. Intracavitary radiation therapy was performed using Cesium 137 medium dose rate in 1-2 fractions depending on tumor size.

Statistical analysis

The relationship between the IMD and patients' characteristics as well as clinical outcome were evaluated by Spearman's who non parametric correlation test. One year disease free survival and overall survival were analyzed by the Kaplan Meier method. Chi square test was used to compare the outcome between high and low IMD groups.

Results

Twenty nine patients were enrolled. The age ranged from 35 to 84 years old, with a median of 51.5 years old. The median size of tumor was 5.4cms (range 3.9-9.1cms). All patients were followed for 1 year.

In phase one of the study, adequate tissue specimens for evaluation of tumor homogeneity for IMD were obtained from 22 out of 29 patients. The IMD was counted from the two different sites of the

Table 1. IMD value of tissue specimens assessed by two pathologists

IMD value	Result			
	First specimens*		Second specimens+	
	1st pathologist	2nd pathologist	1st pathologist	2nd pathologist
mean	24.19	22.65	24.48	21.96
SD	18.10	18.36	22.26	18.44
range	4-86	5-71	3-97	2-90
	r = 0.92 95%CI 0.82-0.96		r = 0.85 95%CI 0.67-0.93	

* Tissues from periphery of tumors
\+ Tissues from halfway between the center and the periphery of the tumors
r = correlation coefficient

tumor for each patient. The remainders were excluded because they had only one adequate sample to count.

No interobserver disagreement between two pathologists was found (correlation coefficient = 0.92, 95% CI 0.82-0.96 for the first piece of tissue and 0.85, 95% CI 0.67-0.93 for the second piece). (Table 1) There was no variability in the IMD between the 2 pieces of tissue specimens from different locations of the tumor. (Table2).

The IMD values of twenty four patients were evaluated. Three patients were excluded because the specimens were inadequate. Two patients were excluded because they did not complete the course of treatment. The IMD value were determined by the first pathologist only. The median IMD was 17.50 (SD $\pm$ 17.61, range 4-86).

IMD was not correlated to patient's age or size of tumor (due to small sample size and large variation). After one year follow up, no correlation between IMD and local control or presence of distant metastases was found (Table 3). The median value of IMD (19.5) was used to divide the patients into 2 groups: high IMD (12 patients) and low IMD groups(12 patients).There was no significant difference in one year recurrence free and distant metastasis free survival between the high and low IMD groups as shown in Fig. 2 and 3, respectively.

Of these 24 patients, the one year recurrence free survival and distant metastasis free survival, one year overall survival and disease free survival were 70.37%, 88.89%,100 % and 59.26%, respectively.

Discussion

Tumor angiogenesis is abnormal neovascularization caused by an imbalance between proangiogenic and antiangiogenic factors. These are derived from genetic effects such as mutation of p 53 tumor suppressor gene, ras oncogene, src oncogene. Hypoxia within a large tumor also influences this effect. The IMD is the parameter used to indicate the level of angiogenesis in the tumor.

There have been many studies demonstrating a significant difference in the IMD between pre-invasive and invasive cervical cancer[2,6,16,18]. However, there is no significant difference in IMD between each stage of cancer[8,17,19]. The relationship between IMD and some pathological characteristics such as

Table 2. Comparison of IMD value (tissue variability) between 2 pieces of tissues from the same tumor performed by two pathologists

	Pair difference*			P -value
	Mean	SD	95% CI of difference	
First pathologist	-3.59	16.15	-10.75-3.57	0.309
Second pathologist	-3.55	10.52	-8.21-1.12	0.129

* Pair difference of IMD between two pieces of tissues

Table 3. Correlation among median value of IMD, initial patients' characteristics and one year clinical outcome

	Correlation coefficient to IMD	P value
Age	0.144	0.502
Tumor size	0.021	0.922
Local control	0.119	0.578
Distant control	0.264	0.212

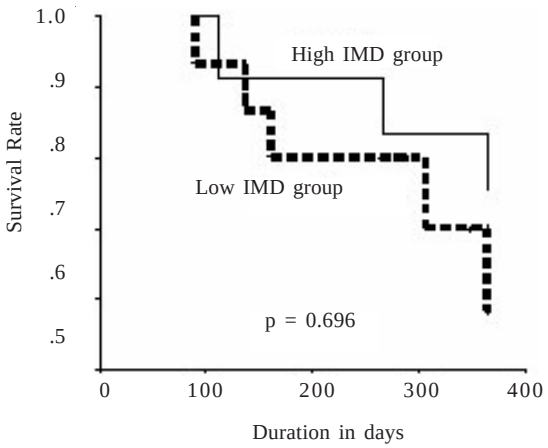

Fig. 2 One year local recurrence free survival of low (IMD < 19.5) and high IMD (IMD>= 19.5) groups

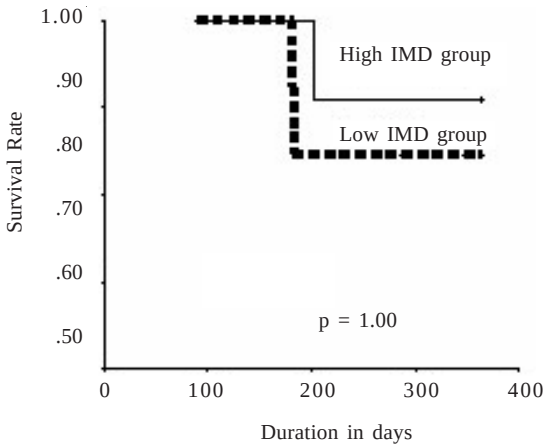

Fig. 3 One year distant metastasis free survival of low (IMD < 19.5) and high IMD (IMD>= 19.5) groups

depth of stromal invasion, lymphovascular invasion and IMD is controversial[2,6,8,18,20]. So in this study we studied stage IIIB cervical cancer because of its poor clinical outcome.

There is still variable concerning the best immunohistochemical method to stain endothelial cells[14]. Anti CD 31 or anti CD 34 immunostaining have been used in the detection of microvessels in tumors, however they may counterstain inflammatory cells or stromal cells as well as endothelial cells[3,23,24]. In this study we choose monoclonal antibody to factor VIII antigen,which is one of the acceptable agents used in many studies and is available in our institute[5,8,9,18,20].

In phase one, we found that the IMD detection by using this immunohistochemical method was reliable. There was no interobserver disagreement in IMD values between the two pathologists. Regarding to Revesz et al[21] and de Jong et al[22], they found that there were neither significant differences in IMD among cervical cancer patients nor significant differences within each patient. Our study showed similar results. We found that the IMD value of one piece of tumor could represent the IMD of the whole tumor because there was no significant difference in the IMD value between 2 pieces of tissues from the same tumor.

Clinically, angiogenesis has been shown to be a significant and independent prognostic factor for survival and local control following radiotherapy in cervical cancer patients[1,4-8,15]. For example, Tjalma et al reported that 5 years overall survival was low in the high IMD group when compared with the low IMD group (42% vs 63%, $p < 0.005$).

In phase two of our study, the relationship between IMD and clinical outcome was determined. We found that there were no significant correlation between IMD and age of patient, size of tumor, local control rate and distant control rate. After the median value of IMD was used to divide the patients into 2 groups, there was also no significant difference between the high and low IMD group in terms of one year recurrence free and distant metastasis free survival.

However, a sample size was small and there were no definite inclusion criteria for the patients characteristic or treatment protocol. Also, the follow up time was only one year. This would reduce the validity of the data.

Comment

This first study can be used as a reference immunohistochemical method to determine IMD.

The second part of the study looking at IMD related to clinical outcome, could be repeated, ensuring that patient characteristics were limited and that all patients received the same treatment protocol. The sample size should be large enough to provide sufficient statistical proves to evaluate any differences in outcome related to IMD values. Lastly, the follow up time should be long enough for the differences to be evident.

Conclusion

This study was the first study in our institute to measure IMD in cancer. We concluded that the pathological assessment is reliable. One piece of tissue can represent IMD for whole tumor in

cervical cancer. This study can be a reference study to generate a new more effective study to determine the relationship between IMD and clinical outcome in the future.

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การศึกษาภาวะ Angiogenesis ในผู้ป่วยมะเร็งปากมดลูกชนิด Squamous cell carcinoma ระยะที่ 3B: การทดสอบความน่าเชื่อถือในการทำซ้ำและความสัมพันธ์กับการพยากรณ์โรค

จันจิรา เพชรสุขศิริ, เตือนใจ ช่วงสุวนิช, พิทยภูมิ ภัทรนุธาพร, สมรมาศ กันเงิน

คณะผู้ศึกษาได้ทำการตรวจชิ้นเนื้อมะเร็งปากมดลูกในผู้ป่วยมะเร็งปากมดลูกชนิด squamous cell carcinoma ระยะที่ 3B ตาม FIGO staging จำนวน 29 ราย โดยได้ทำการตัดชิ้นเนื้อจำนวน 2 ชิ้น จาก 2 ตำแหน่งในผู้ป่วยแต่ละราย แล้วนำมาทำการย้อมติดสีด้วยวิธี immunohistochemistry ด้วย Factor VIII monoclonal antibody เพื่อตรวจหาภาวะ angiogenesis ในเนื้อมะเร็งปากมดลูกและนับวัดค่าออกมาในรูป IMD (Intratumoral microvessel density) ทั้งนี้ได้ทำการศึกษาโดยพยาธิแพทย์ 2 คน และจากการศึกษาพบว่าไม่มี interobserver disagreement ระหว่างพยาธิแพทย์ทั้ง 2 คน (correlation coefficient = 0.92, 95% CI 0.82-0.96 สำหรับชิ้นเนื้อที่ 1 และ 0.85, 95% CI 0.67-0.93 สำหรับชิ้นเนื้อที่ 2) นอกจากนี้ยังไม่พบความแตกต่างของค่า IMD ระหว่างชิ้นเนื้อ 2 ชิ้นจากคนละตำแหน่งในผู้ป่วยรายเดียวกัน

ผู้ป่วยทุกรายได้รับการรักษาด้วยการฉายรังสีและมีการติดตามผลการรักษาต่อเนื่องเป็นระยะเวลา 1 ปี แต่อย่างไรก็ตามยังไม่สามารถสรุปความสัมพันธ์ของ angiogenesis กับลักษณะการดำเนินโรคทางคลินิกได้ในขณะนี้ เนื่องจากผู้ป่วยมีจำนวนจำกัด และมีลักษณะพื้นฐานและวิธีการรักษาที่แตกต่างกัน