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Evaluation of angiogenesis in urothelial carcinoma of bladder and its relation with prognostic factors

Mesane ürotelyal karsinomlarında anjiogenezin değerlendirilmesi ve prognostik faktörlerle ilişkisi

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Summary

Introduction: The aim of this study was to evaluate the relationship between the established prognostic indicators and angiogenesis in urothelial carcinoma of the bladder.

Materials and Methods: Archival samples from a total of 51 radical cystectomy specimens (seven pTa, eight pT1, eleven pT2, seventeen pT3 and eight pT4) were analysed. For each case clinicopathologic parameters such as, age, sex, grade, pathologic stage, lymph node metastasis, vascular/lymphatic invasion, perineural invasion and the presence of carcinoma-in-situ were detected. To evaluate angiogenesis, vessels were immunohistochemically highlighted using CD34 antibody. Vascular surface density (VSD) and number of vessels per square millimetre of stroma (NVES) values were analysed.

Results: As a result no relation between angiogenesis and the prognostic factors studied other than the presence of carcinoma in situ could be established.

Key Words : *Bladder; Bladder Neoplasm; Carcinoma, Transitional Cell; Neovascularization*

Özet

Giriş: *Bu çalışmada mesane ürotelyal karsinomlarında anjiogenez ile bilinen prognostik faktörler arasındaki ilişkiyi araştırmayı amaçladık.*

Gereç ve Yöntem: *51 radikal sistektomi materyalinde (yedi pTa, sekiz pT1, onbir pT2, 17 pT3 ve sekiz pT4) klinikopatolojik parametrelerden yaş, cinsiyet, patolojik evre, lenf nodu metastazı, lenfatik/vasküler invazyon, perinöral invazyon ve karsinoma in situ varlığına bakıldı. Anjiogenezi değerlendirmek amacıyla immunohistokimyasal olarak CD34 boyası ile damarlar belirlendi. Vasküler alan yoğunluğu (VSD) ve stromada milimetre kare başına düşen damar sayısı (NVES) analiz edildi.*

Bulgular: *Sonuç olarak anjiogenez ile karsinoma in situ haricindeki hiçbir prognostik belirleyici arasında ilişki bulunmadı.*

Anahtar Kelimeler: *Mesane; Mesane Neoplazm; Karsinom, Transizyonel Hücreli Karsinom, Neovaskülarizasyon*

Introduction

Urothelial carcinoma (UC) of the bladder is the second most common malignant neoplasm of the genitourinary tract and the most common bladder cancer occurring in about %90 of the cases (1). Approximately 20-30% of patients with bladder cancer present with invasive tumour. Patients with invasive cancers are at significant risk of tumour progression and metastases (2). Today specific treatment decisions are made according to some established prognostic parameters such as tumour stage, grade and lymph node status (3). However these prognostic indicators are not always enough to determine best management as tumours of similar stage and grade are sometimes associated with widely varying outcomes (2, 4). More prognostic indicators are needed to aid clinicians to help predict tumour behaviour and treatment

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strategies with accuracy (3). Recently, microvessel density, the commonly used histologic measure of angiogenesis is found to be significantly correlated with poor outcome in several human cancers including urological cancers (5).

Here in we examined 51 radical cystectomy specimens with urothelial carcinoma to investigate the relationship between tumor associated angiogenesis and established prognostic factors such as pathologic stage, grade, lymph node status, vascular/lymphatic invasion, perineural invasion and presence of carcinoma in situ.

Material And Method

Tissues from 62 radical cystectomy specimens diagnosed as UC between January 1998 to September 2001 were examined. Early cystectomy is the generally preffered treatment in our urology department. In pTa tumors, cystectomy was perfomed i) if there was a high tumor volume which was unresectable by transurethral resection (TUR) ; ii) if frequent recurrences devoloped after TUR of the bladder tumor . In pT1 tumors cystectomy was the therapy of choice if there was a recurrence after the first course of intravesical BCG treatment.

11 specimens were excluded as tissue blocks were unavailable. For each case of the 51 UC clinicopathologic parameters such as, age, sex, lymph node metastasis, perineural invasion, vascular/lymphatic invasion, grade and pathological stage were detected. The presence of carcinoma in situ was investigated in the slides. The tumours were staged according the TNM staging system (6) and graded according to grading schema proposed by World Heath Organization (7).

All haematoxylin-eosin stained slides of tumour blocks were reviewed to find the most active areas of angiogenesis and chosen for the study. To highlighten the blood vessels 5 micron-thick sections from paraffin embedded, formalin-fixed tissue of these tumour blocks were immunostained with antibody CD34 using the avidin-biotin complex streptavidin biotin immunoperoxidase method (DAKO) (Figure 1).

Stereologic Measurements:

10 different areas containing the greatest neovascularization was chosen on CD34 stained tumoral slides to determine the vascular surface density (VSD) and microvessel number (NVES). Measurements were held by a steorologic method described by Barth et al in a recent study (8).

The microscopic image was projected to a monitor (Sony Trinitron, Japan) at a total width of 41 cm attached to a microscope (Olympus, Japan). A transparent grid with

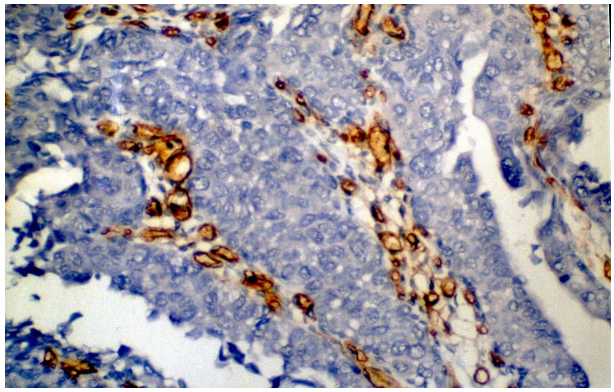

Figure 1. Vessels highlightened by staining with antibody CD34 (X220)

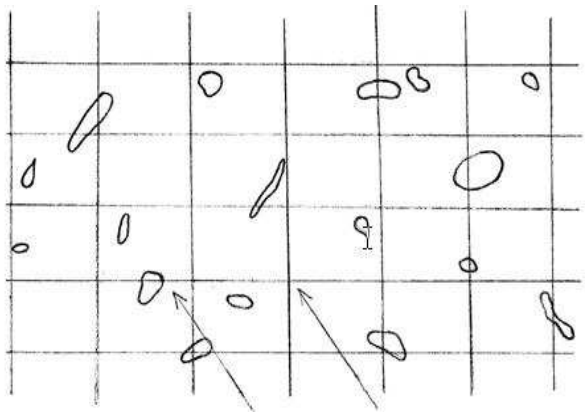

Figure 2. Schmatic drawing indicating In; intersection between test-lines and vessel walls and Istr; points superimposed on the stromal department.

121 points composed of 11 horizontal and 11 vertical test lines with known total test line length (Lr) 6,05 µm was superimposed on the test fields to be measured. The microscopic image obtained at X 10 objective was projected to the monitor and the final magnification of X 932 was obtained on the monitor screen. Labelled vessel walls (N), number of intersections between the test lines and vessel walls (In), and the number of lattice points hitting the stroma (I str) was counted (figure 2) (8). The volume portion of stroma of the tumour was computed according to the following formulas:

$$Vstr = Istr / 121.$$

Vascular surface density (VSD), equivalent to the vascular surface area per unit tissue (mm^2 / mm^3) and the number of vessels per mm stroma (NVES) was computed according to

$$VSD = €In \times 2 / Lr \times Vstr$$

$$NVES= N / Vstr$$

All statistical analysis were performed using the SPSS for Windows Version 11 statistical package on a personal computer. A P value of <0,05 was used to

indicate statistical significance. Associations of vascular parameters (NVES & VSD) with prognostic factors (grade, pathologic stage, lymph node metastases, vascular/ lymphatic invasion, perineural invasion and presence of carcinoma in situ) were assesed with independent samples t-test and one way analysis of variance. The relationship between pathologic stage and other clinicopathologic parameters were evaluated by chi-square tests.

Results

Fifty - one patients (50 men and 1 woman) with UC of bladder were studied. The median age was 60 (range 40 to 79). The distribution of cases among pathologic stages were as follows; 7 (14%) pTa, 8(16%) pT1, 11(22%)pT2 all of which were pT2b, 17 (33%) pT3 and 8 (16%)pT4. Thirty – five (%69) of the tumors were grade III and 16 (%31) were grade II. Thirty seven percent of the cases had carcinoma - insitu adjacent to the tumor. Only 32 of the patients had lymph nodes dissected and 34% of these patients noted to have lymph node metastases histologically. Of the lymph node positive patients 2 were classified as pT4, 7 were pT3, 2 were pT2b. The presence of vascular or lymphatic invasion was assesed in %51 of the cases (6 cases T2b, 14 cases T3 and 6cases T4), where the perineural invasion was detected only in 5 patients (2cases T2b, 1case T3, 2 cases T4). NVES and VSD values of tumors associated with carcinoma in situ had significantly higher vessel parameters than the tumors without carcinoma in situ. (VSD; p=0,01 and NVES; p=0,03 (p values were calculated according to independent samples t-test)). Mean VSD and NVES value of the cases with carcinoma in situ in order was 202,84mm^{-1} and 93,94mm^{-2} and without carcinoma in situ was; 142,2 mm^{-1} and 69,76 mm^{-2} respectively. Tumor pathologic stage, grade, presence of lymphovascular invasion, perineural invasion and lymph node metastases did not show any relation with vessel parameters. Also, when NVES and VSD values of superficial tumours and invasive tumors were analyzed seperately, no relation of significance was found between the two groups. Correlation between tumor pathologic stage and grade with vascular parameters are given in table 1 and 2. In comparison of histopathologic values with each other, tumor stage and lymph node metastases showed a significant relationship with tumour grade and with the presence of vascular/ lymphatic invasion (Table 3 - 4).

Table 1. Vessel parameters in different pathologic stages

	Pathologic Stage					P*
	pTa	**pT1**	**pT2**	**pT3**	**pT4**	
VSD(mm^{-1})	167,8±65,6	161,6±60,7	164±52,6	163,2±70,4	170,1±96,2	0,999
NVES(mm^{-2})	72,9±22	76,6±28,6	88,1±28,3	74,1±22,3	83±47,4	0,744

Data presented as the mean ± SD

Key: VSD= vascular surface density, NVES= number of vessels per square millimeterof stroma

**p values calculated according to one-way Anowa test*

Table 2. Vessel parameters in different grades

	Grade		P*
	II	**III**	
VSD(mm^{-1})	160,8±60,4	165,7±70,5	0,889
NVES(mm^{-2})	79,2±26,4	78,5±30,6	0,939

Data presented as the mean ± SD

Key: VSD= Vascular surface density, NVES= number of vessels per square millimeter of stroma

**p values calculated according to independent samples t-test*

Table 3. Relationship of lymph node metastases with grade and lymphovascular invasion

	Lymph Node Metastases		P**
Grade	**Negative**	**Positive**	
II	9	0	0,010
III	12	11	
LV* invasion			
Absent	14	2	0,026
Present	7	9	

**LV= lypmhovascular*

***p values calculated according to chi-square test*

Table 4. Relationship of pathologic stage with grade and lymphovascular invasion.

	Pathologic Stage					P**
Grade	**Ta**	**T1**	**T2**	**T3**	**T4**	
II	7	7	2			0,000
III		1	9	17	8	
LV* invasion						
Absent	7	8	5	3	2	0,000
Present			6	14	6	

**LV= lynphovascular*

***p values calculated according to chi-square test*

Discussion

Angiogenesis is the name given to development of new capillary blood vessels from pre-existing blood vessels and is mandotory for tumor growth (9). Angiogenesis is not only fundamental for healing, reproduction and embryonic development but also an important process that plays an important role in tumor growth, progression and metastases (9, 10) In fluorescein angiography, in contrast to normal epithelium, papillary tumors and carcinoma in situ demonstrated increased vascularity verifying that neovascularity is acquired relatively early during bladder tumorigenesis (11).

Bochner and his colleagues using the hotspot method in 164 radical cyctectomy specimens found that invasive bladder tumors with higher microvessel counts were strongly associated with disease recurrence and survival, but on the other hand, however failed to demonstrate a correlation of microvessel density with grade and stage (3). Using the hotspot method Jager et al. measured microvessel density in 41 patients undergoing cystectomy and pelvic lymphadenectomy, and determined that increased microvessel count was corralated with the presence of lymph node metastases in invasive bladder cancers (2). In a series of 81 patiens undergoing cystectomy, Chaudrey et al. using Chlalkey point graticule with the hotspot method demonstrated that high microvessel counts were associated with lymph node metastases, pelvic reccurences and poor prognosis (12). Dickonson et al. stated that it was not possible to assess microvessel density in papillary UC as it was hard to decide which part of the tumor vasculature to be counted therefore only included the solid invasive bladder carcinomas in their study in which they measured tumor vascularity using the hotspot method with Chalkey point graticule. Consequently tumor vascularity was found to be significantly related to survival (13) .

High microvessel counts are not always associated with worsening prognosis, some of the studies found no correlation with microvessel density and outcome (14-16) while others stated that high microvessel counts is correlated with better prognosis (17, 18). Concerning bladder tumors, the positive correlation of tumor angiogenesis and prognosis detected in invasive bladder cancers, hasn't been shown in superficial bladder cancers satisfactorilly (14, 19, 20). Methodology of microvessel count assessment has been blamed either for the discrepencies between results from different studies or the controversial data obtained on superficial bladder cancers (21).

Mostly used methods for assesing microvessel count is in conflict with the morphology of the SBC which shows heterogenity in histological architecture as these methods require tissue homogenicity (22). An adequate solid area of tumor growth pattern must be present to define the tumor vasculature (13). To compansate the fronded papillary architecture and fragmentary nature of the tumor, streotactic grid was used to evaluate vesssel density (22). Sagol et al. using this method in superficial urothelial carcinomas found no association between microvessel density count and outcome (19).

In human urological tumors, 41 different methods have been used in 48 studies for assesing microvessel density many of which didn't give the field size to allow microvessel counts to be expressed per unit area. Although NVES expresses the number of microvessels per mm^2 stroma, it does not consider the volume portion of stroma or epithelium. It is confidental to perform NVES in tumors with a high stromal component; but in tumors composed of solid and/or papillary areas as in SBC, the comparison of values is a challenging issue. In contrast to NVES, VSD is highly reproducible and precisely quantifies the vessel area per volume tumor tissue (mm^2 /mm^3) solving the heterogenity problem in different areas of the tumor (8, 23).

In our study, tumors associated with carcinoma in situ were found to have higher microvessel counts than the tumors without carcinoma in situ. This is not an unexpected result since urothelial carcinomas of bladder comprising carcinoma in situ are associated with a poor prognosis. The rest of the studied variables ; tumour

grade, pathologic stage, presence of lymphovascular invasion and lymph node metastases, didn't show any association with the vascular parameters. Results didn't change when NVES and VSD values of superficial tumors and invasive tumors were analyzed separetely. Different kinds of methods used for assessing microvessel counts may affect the reliability of the conclusions drawn between reports in literature. Variety of staining techniques and selection of the primary anti-endothelial antibody can also affect visualisation of blood vessels, causing interobserver bias (12). It has been suggested that comparison of these studies would not be reliable untill a unique method for measuring microvessel density is put forward (21). The discordence of our data with the literature may be related to this great heterogenity in methodology. In contrast to the studies which only considered the microvessel density, current study seems to have methodological advantage not only as we chose to evaluate angiogenesis by means of steorology but as we also measured VSD values which helps to avoid the heterogenity problem of stroma/epithelium ratio in SBC.

Our study suggested that quantitative assessment of vascularization is not in relation with the established prognostic factors in urothelial carcinoma of bladder. We think that a safe, simple and reproducible method should be standardized for evaluation of angiogenesis. The availability of such a standardized technique would provide opportunity to make more reliable comparisons between studies conducted in different centres and therefore enables investigators to make more accurate comments about the usefulness of angiogenesis as a prognostic factor.

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