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Immunodetection of Cytoplasmatic Membrane-Bound Thrombomodulin in Formalin-Fixed Paraffin-Embedded Human Tissue Microarrays

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Abstract

Background: Loss of thrombomodulin immunoreactivity denotes a poor prognosis for patients presenting with malignant disease.

Objectives: Immunodetection for cytoplasmatic membrane-bound thrombomodulin in formalin-fixed paraffin-embedded (FFPE) tissue microarrays.

Methods: Thrombomodulin immunoreactivity was assessed in 97 FFPE tissue cores representing bladder, kidney, prostate, testis, penis, ovary, cervix, vulva, endometriosis, and myometrium, from 84 patients arrayed on three TMAs (64 (66%) cancers, 22 (23%) normal controls, and 11 (11%) benign pathologies). Human bladder tissue lysate from matched normal urothelium and TCC biopsies were also examined by western blot analysis for thrombomodulin expression.

Results: Thrombomodulin immunoreactivity was strongest in the TCC cores where ≥60% of the tissue sections scored +3, or greater. By contrast, SCCs, adenocarcinomas, CCCs, papillary carcinoma, sarcomas, seminomas, teratoma and all other tissue sections scored ≤ +2. Thrombomodulin expression was also detected by western blot analysis in the human bladder tumour lysate. No signal was detected in adjacent normal control.

Conclusion: Cytoplasmatic membrane-bound thrombomodulin immunostaining is strongest in TCC sections with respect to SCC, adenocarcinoma, sarcoma, seminoma, teratoma, papillary carcinoma, CCC, and is independent of both grade and stage. Thrombomodulin immunostaining in tumour tissue sections are predominately membranous.

Keywords: Bladder; Tumour tissue microarray; Thrombomodulin; Immunostaining; Transitional cell carcinoma; Squamous cell carcinoma; Adenocarcinoma; Urothelial cancer

Abbreviations:

TMA: Tissue Microarray; TCC: Transitional Cell Carcinoma; SCC: Squamous Cell Carcinoma; FFPE: Formalin Fixed Paraffin Embedded; DAB: 3,3'-diaminobenzidine

Introduction

Thrombomodulin, a 75 kDa glycoprotein, is a membrane protein expressed on the surface of endothelial cells where it serves as a cofactor for thrombin. Thrombomodulin reduces blood coagulation by converting thrombin to an anticoagulant from a pro-coagulant enzyme [1]. Recent studies, however, have shown that thrombomodulin also affects physiological and pathophysiological systems which include anti-proliferative and anti-metastatic effects in carcinogenesis, and a loss of thrombomodulin expression has been linked to a poor prognosis [2].

Manning et al., have described an inverse correlation with thrombomodulin expression with cancer progression and metastasis [3]. However, in direct contrast, Ding et al., reported that thrombomodulin was up-regulated in bladder cancer patients and cancer cell lines, but not in normal cells [4]. Furthermore, these authors also claim that thrombomodulin immunostaining in bladder cancer TMAs were more pronounced in high grade and invasive cancers.

Identification of poorly differentiated tumours originating in the renal pelvis can prove challenging due to the numerous histological variants [5]. As such, thrombomodulin has been suggested as a potential biomarker that could be useful in the differential diagnosis of urothelial cancers [5,6].

Materials and Methods

Materials

Human bladder cancer urinary tissue microarrays (TMAs) (TMA2205/2206/2207) and human bladder tissue lysate (normal (T10-003-N-1) and tumour (T10-003-T-1)) were purchased from Protein Biotechnologies, Ramona, CA92065. Tumour grading and staging information accompanied the TMAs and lysate provided by Protein Biotechnologies. Rabbit monoclonal anti-thrombomodulin [EPR4051] antibody (AB109189), HistoReveal (AB103720) and DAB

substrate kit (AB64238) were obtained from Abcam, UK. Clearing reagent Histo-Clear (HS-200) was obtained from National Diagnostics, Atlanta, Georgia, US. Bovine serum albumin (BSA) (A4503), goat anti-rabbit HRP (A0545), absolute ethanol (24103), and counterstain, Harris haematoxylin (HHS32) were obtained from Sigma, Poole, UK. Hydrogen peroxide 30% (95321) was obtained from Fluka, UK. All other reagents, unless otherwise indicated, were obtained from Sigma, Poole, UK.

Study population

We investigated thrombomodulin immunoreactivity in 97 FFPE tissue samples including bladder, kidney, prostate, penis, testis, cervix, vulva, endometrium and ovary from 84 patients. Fifty-two were males (61.9%) (Age 54.8 ± 17.2, median age 56) and 32 females (38.1%) (Age 47.5 ± 12.6, median age 49.5). We assessed samples from 22 patients (22/97 (22.7%)) with histologically "normal" tissue; 11 patients (11/97 (11.3%)) with benign pathologies consisting of 2/97 (2.1%) chronic cystitis bladder, 2/97 (2.1%) benign prostate hyperplasia (BPH), 5/97 (5.1%) benign pathologies of the kidney, 1/97 (1%) cervical polyps, and 1/97 (1%) with leiomyoma myometrium. The remaining 64/97 (66%) were transitional cell carcinoma of the bladder 35/97 (36.1%); squamous cell carcinoma 7/97 (7.2%) of the bladder (n=2), penis (n=1), cervix (n=3) and vulva (n=1); adenocarcinoma 14/97 (14.4%) of the bladder (n=6), prostate (n=2), ovary (n=3) and endometriosis (n=3); seminoma testis 2/97 (2.1%); sarcoma 2/97 (2.1%) of the bladder (n=1) and myometrium (n=1); kidney clear cell carcinoma 2/97 (2.1%); ovarian teratoma 1/97 (1%) and papillary carcinoma kidney 1/97 (1%).

Immunohistochemistry for cytoplasmatic membrane-bound thrombomodulin

Thrombomodulin immunostaining was performed on 4 μm FFPE sections cut from three TMAs containing cores across a range of malignant and benign tissues. To demonstrate the distribution of cytoplasmatic membrane-bound thrombomodulin, we selected TMAs which covered the urinary bladder and the renal pelvis. Tissue sections were deparaffinised in Histo-Clear and rehydrated in descending concentrations (100% to 50%) of ethanol. The slide sections were kept in tap water until ready to perform antigen retrieval. Excess water was removed from the slides, without allowing the tissue section to dry, and one or two drops of HistoReveal were applied to the TMA. The slides were then incubated for 5 min at RT followed by 2×5 min washes in TBS/Tween 20 (0.1%), with gentle agitation. The slides were then blocked for 2 h at RT in 10% normal serum (Randox, UK) prepared in TBS/Tween 20 (0.1%). The tissue sections were drained and wiped with tissue paper prior to incubation overnight at 4°C with gentle rocking with the primary antibody (rabbit monoclonal anti-thrombomodulin) diluted 1:100 in TBS/Tween 20 (0.1%), 1% BSA. The sections from the TMAs were then rinsed three times in TBS/Tween 20 (0.1%) for 5 min with gentle agitation prior to incubation in 0.3% hydrogen peroxide/TBS for 15 min. Following two further washes using TBS/Tween 20 (0.1%), the TMAs were incubated with enzyme-conjugated goat anti-rabbit HRP secondary antibody, (1:2000) prepared in TBS/Tween 20 (0.1%), and incubated for 1 h at RT and then washed in TBS/Tween 20 (0.1%). Chromogen was prepared according to manufacturer's instructions and the sections from the TMAs were incubated with 3,3'-diaminobenzidine (DAB) reagent (0.5 ml) for 10 min at RT, then rinsed in running tap water for 5 min and counterstained with Harris haematoxylin. Following washing, the slides were dehydrated through the alcohols (50% to 100%) up to Histo-Clear. A microscope cover glass (Erie Scientific, Portsmouth, N.H., US) (24×40 mm) was then applied to the TMA using Eukitt© media as the mountant. Immunoreactivity scoring for cytoplasmatic membrane-bound thrombomodulin was undertaken by two observers (MWR and CNR).

Western blot analysis for the detection of thrombomodulin expression in normal and tumour tissue lysate

Lysates from human transitional cell carcinoma, grade 2, stage II (T2NxM0)) tissue and the corresponding histologically "normal" matched urothelium from a male, aged 64 were subjected to Western blot analysis. Protein (10 μg) from the matched normal and tumour lysate (1 mg/ml, respectively) were resolved on 12.5% SDS PAGE gel (300 V, 1 hour) under reducing conditions. Proteins were then transferred to nitrocellulose (BioRad, US) (120 V, 1.5 h). The membrane was blocked in 3% BSA prepared in TBS/Triton X-100 (0.025%) at RT for 1 h with gentle rocking. Primary anti-thrombomodulin antibody (1:1000) prepared in TBS/Triton X-100 (0.025%), 1% BSA (10 ml) was applied to the membrane which was then incubated overnight at 4°C with gentle rocking after which the membrane was then washed with TBS/Triton X-100 (0.025%) (3×15 minutes). A secondary antibody (goat anti-rabbit HRP 1:5000) prepared in TBS/Triton X-100 (0.025%), 1% BSA (10 ml) was applied to the membrane which was then incubated for a further 1 h at RT with gentle rocking. The membrane was washed with TBS/Triton X-100 (0.025%) (3×15 minutes) and protein bands were visualised using enhanced chemiluminescence (ECL) solution (Roche, France).

Results

Tumour grade

The grades of the TCC (n=35) were grade I (n=6), grade I - II (n=3), grade II (n=10), grade II - III (n=3), grade III (n=10), tumour in situ (Tis) (n=1), and 2 ungraded tissue samples. The SCC (n=7), were grade I (n=3), grade II (n=2), grade III (n=1) and one tissue section was ungraded. For the adenocarcinoma (n=14), grade I (n=1), grade II (n=3), grade II - III (n=2), grade III (n=3) and five tissues were ungraded. Eight further tissue samples were also ungraded: seminoma (n=2), sarcoma (n=2), CCC of the kidney (n=2), teratoma (n=1) and papillary carcinoma of the kidney (n=1).

Tumour staging

Tumour staging for the tissue samples were as follows: TCC (n=35): T1N0M0 (n=18), T2N0M0 (n=11), T2NXM0 (n=1), T2N0M1 (n=1), T3N2M1 (n=1), T4N2MX (n=2) and TisN0M0 (n=1); SCC (n=7): T1N0M0 (n=3), T1N1M0 (n=2) and T2N0M0 (n=2); adenocarcinoma (n=14): T1N0M0 (n=7) and T2N0M0 (n=7); seminoma (n=2): T2N0M0 (n=1) and one section was not staged; kidney clear cell carcinoma (n=2) T1N0M0, teratoma [1] not staged; kidney papillary carcinoma (n=1) T1N0M0.

Immunodetection for cytoplasmatic membrane-bound thrombomodulin in human TMAs

Thrombomodulin immunoreactivity scores are summarized in Table 1.

Transitional cell carcinoma						
Cases		0	1+	2+	3+	4+
CIS	1					1
I	6	1		2	1	2
I-II	3			1		2
II	10		4	1	3	2
II-III	3				3	
III	10		3	1	4	2
Ungraded	2			1		1
% cases		2.8	20	17.2	31.4	28.6
Squamous cell carcinoma						
I	3		3			
I-II	0					
II	2		1	1		
II-III	0					
III	1		1			
Ungraded	1		1			
% cases		0	85.7	14.3	0	0
Adenocarcinoma						
I	1			1		
I-II	0					
II	3		1	2		
II-III	2		1			1
III	3		2	1		
Ungraded	5	3	1	1		
% cases		21.4	35.7	35.7	0	7.2
Normal Tissue						
Normal	22	3	11	7	1	
% cases		13.7	50	31.8	4.5	0
Benign pathologies						
Benign	11	2	6	3		
% cases		18.2	54.5	27.3	0	0
Seminomas (n=2) 1+, sarcomas (n=2) 0, 3+, clear cell carcinomas (n=2) 1+, teratoma (n=1) 1+, and papillary carcinoma (n=1) 1+.						

Table 1: Immunostaining for cytoplasmatic membrane-bound thrombomodulin in human TMAs.

Thrombomodulin immunostaining on all tissue sections was undertaken by DMR. Two independent observers (MWR and CNR) scored the tissue sections for thrombomodulin immunostaining using the EORTC-GCCG scoring system [7]. Briefly, tissue sections that were negative (score 0), weakly positive sections (score 1), positive sections (score 2), strongly positive sections (score 3) and very strongly positive sections (score 4). Representative micrographs for thrombomodulin immunostained TCC, SCC and adenocarcinoma, and their complementary H&E stained sections are shown in Figure 1.

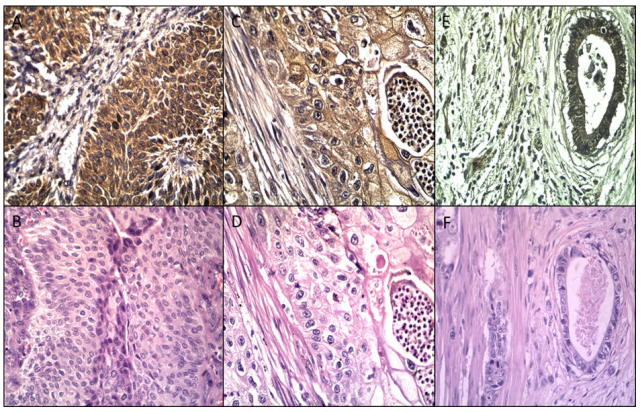

Figure 1: Immunostaining for cytoplasmatic membrane-bound thrombomodulin and an H&E stain in human bladder tissue (i) TCC (grade 2, T3N0M0) (panel A and B,respectively), (ii) SCC (grade 2, TN0M0) (panel C and D, respectively), and (iii) adenocarcinoma (grade 2, T3N0M0) (panel E and F, respectively) TMAs. Transitional cell and SCC of the bladder (panel A and C) demonstrate strong immunoreactivity for cytoplasmatic membrane-bound thromobomodulin (scoring +3 and +2, respectively). However, adenocarcinoma of the bladder shows weak immunoreactivity for thrombomodulin (scoring +1). Micrograph images were captured by an Optika B-800 series microscope using an Optika PRO3 Vision PRO digital camera (magnification x 400).

Transitional cell carcinomas 34/35 (97%), SCC 7/7 (100%), adenocarcinoma 11/14 (78.6%), seminomas 2/2 (100%), sarcomas 1/2 (50%), CCCs 2/2 (100%), teratoma 1/1 (100%), papillary carcinoma 1/1 (100%), normal urothelium 19/22 (86.4%) and benign pathologies 9/11 (81.8%) all displayed prominent thrombomodulin immunoreactivity. Tissue sections from TCCs displayed the strongest thrombomodulin immunoreactivity with 21/35 (60%) of the cores scoring >3+, 11/35 (31.4%) 3+ and 10/35 (28.6%) scoring 4+. By contrast, thrombomodulin immunoreactivity for SCC cores were all <2+ with 1/7 (14.3%) 2+ and 6/7 (85.7%) scoring 1+. Immunoreactivity within the cores representing adenocarcinoma displayed similar thrombomodulin immunoreactivity to that of SCCs with 5/14 (35.7%) 2+, 5/14 (35.7%) 1+, 3/14 (21.4%) 0 and 1/14 (7.2%) scoring 4+. Benign pathologies also scored <2+ for thrombomodulin immunoreactivity with 2/11 (18.2%) scoring 0, 6/11 (54.5%) 1+ and 3/11 (27.3%) 2+. Normal tissue sections also immunostained positive for thrombomodulin in a similar fashion to that observed for the benign pathologies with 3/22 (13.7%) scoring 0, 11/22 (50%) 1+, 7/22 (31.8%) 2+ and 1/22 (4.5%) scoring 3+. In agreement with previous data [5], we did not observe any correlation between thrombomodulin immunoreactivity and the histological grade or stage of the tumour sections.

Western blot analysis

Normal (T10-003-N-1) and tumour (T10-003-T-1) tissue lysate from human bladder was subjected to western blot analysis, as described in the materials and methods section. A positive signal for thrombomodulin (approximately 100 kDa) was observed on the blot for the tumour lysate sample (Figure 2).

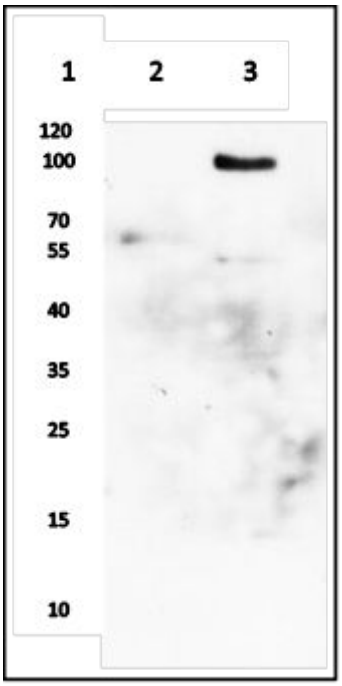

Figure 2: Immunoblot for the detection of thrombomodulin in human bladder clarified tissue lysate (normal tissue (lane 2) and tumour tissue (lane 3)) from a 64 year old male with the following pathology: TCC, grade 2, stage II, T2NxM0. Protein (10 µg) from normal and bladder tumour tissue lysate (1 mg/ml, respectively) was electrophoresed on a 12.5% SDS PAGE under reducing conditions, and then transferred to PVDF membrane. The membrane was blocked with 3% BSA in TBS-T and then probed with a rabbit monoclonal anti-thrombomodulin antibody (1:1000), as described in the materials and methods section. A single band at approximately 100 kDa was observed in lane 3, tumour tissue lysate. However, no signal was observed for thrombomodulin in the normal bladder lysate, lane 2. Molecular mass markers (kDa) are indicated in lane 1.

No signal was observed for the normal bladder control lysate. The predicted molecular weight for thrombomodulin is approximately 75 kDa. However, as the thrombomodulin is heavily glycosylated, the protein runs on the gel at <100 kDa.

Discussion

Thrombomodulin is a surface glycoprotein that has been shown to be an important biomarker in patients presenting with malignant disease [5,6]. Decreased thrombomodulin expression is associated with cell proliferation and an increase in malignancy. Moreover, the loss of thrombomodulin expression is associated with a poor prognosis [2].

Thrombomodulin exists in two forms, a high molecular weight form which is bound to cytoplasmic membranes and a lower molecular weight form found in both plasma and urine [8]. Elevated plasma levels of thrombomodulin have been correlated with the number of years of tobacco smoking, a known risk factor for bladder cancer, which results in vascular endothelial dysfunction and shedding [9-11]. Endothelial dysfuction and shedding are common pathologies reported for patients presenting with hypertension or diabetes [12].

An increase in thrombomodulin expression in the transitional epithelium has also been reported with a worsening pathological status of cystitis [13]. Interestingly, thrombomodulin levels have been shown to differ significantly between blood groups (AB0) [7].

In this study, thrombomodulin immunostaining was observed in 88/97 (90.7%) of the FFPE tissue sections examined. Thrombomodulin immunostaining was greater in the TCC sections with 60% of the sections scoring ≥ 3+. However, tumour grading and staging in the TCC sections did not correlate with the intensity of thrombomodulin immunostaining, as previously reported [5]. Weak thrombomodulin immunostaining was observed in both SCCs (6/7 (85.7%) scoring 1+) and adenocarcinoma (10/14 (71.4%) scoring ≤ 2+) tissue sections, and again thrombomodulin immunostaining was independent of either tumour grade or stage. Four tissue sections, 3/14 (21.4%) adenocarcinoma and 1/35 (2.8%) TCC were negative for thrombomodulin immunostaining. Normal and benign pathologies also demonstrated weak thrombomodulin immunoreactivity with most of the tissue sections scoring either 1+ or 2+. Three normal 3/22 (13.7%) and 2/11 (18.2%) benign pathologies were negative for thrombomodulin immunoreactivity.

Our data is in agreement with previous studies that suggest that thrombomodulin immunostaining may have clinical utility in differentiating between tumours arising in the renal pelvis [5,6]. However, in contrast to Ding et al., we did not observe an increase in thrombomodulin immunostaining with respect to either tumour grade or staging. Moreover, if the loss of thrombomodulin expression correlates with an increase in malignancy and poor prognosis, then it seems counterintuitive that thrombomodulin expression would be augmented in higher grade and higher stage tumours as suggested [4]. A recent paper by Wu et al. clearly demonstrates that thrombomodulin expression in bladder cancer cell lines is inversely correlated with invasive ability.

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