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Role of Tumor Suppressor and Angiogenesis Markers in Prediction of Recurrence of Non Muscle Invasive Bladder Cancer

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Abstract Non muscle invasive bladder cancers recur frequently and identification of biomarkers for predicting recurrence are necessary. The present study evaluated the individual and synergistic effects of tumor suppressor (p53/p21waf1) and angiogenesis [vascular endothelial growth factor (VEGF)/endoglin (CD105)] markers. The study included 90 cases of non muscle invasive bladder cancer. Cell spots were stained with primary antibodies and Flourescein isothiocyanate (FITC). Slides were observed under confocal laser scanning microscope for protein expression. The association between the markers individually and synergistically with recurrence were assessed by a $\chi 2$ and Fisher's Exact test. Survival analysis was performed to predict recurrence and test for significant difference in recurrence free survival probability. Recurrence [overall:39(43.3%) and low grade(LG):26(54.2%)] was significant with p53 and VEGF expression and the profiles p53/VEGF, p53/CD105, VEGF/CD105, p53/p21/CD105, p53/VEGF/CD105 and all four were significantly associated with recurrence in both groups. In the multivariable model the [HR (95%CI),p: overall and LG] profiles p21/VEGF [2.195(1.052-4.582),0.036; 3.425(1.332-8.811),0.011], VEGF/CD105 [2.624(1.274-5.403),0.009 and 3.380(1.348-8.472),0.009], p53/p21/CD105 [2.000(0.993-4.027),0.052 and 2.539(1.047-6.157),0.039], p53/VEGF/CD105 [2.360(1.148-4.849),0.020 and 2.738(1.104-6.788),0.030], p21/VEGF/CD105 [2.611 (1.189-5.731),0.017 and 3.946(1.530-10.182),0.005] and all four [2.382(1.021-5.556),0.045 and 3.572(1.287-9.911),0.014] significantly predicted the recurrence along with significant log rank. In the pTa subset (n=33) the profiles p53/p21, p53/CD105, p21/VEGF, VEGF/CD105, p53/VEGF/CD105, p53/p21/CD105 and p21/VEGF/CD105, significantly predicted hazard for recurrence. The present study emphasizes an underlying association between tumor suppressor (p21waf1) and angiogenesis (VEGF/CD105) biomarkers. In addition combination profiles appeared to indicate an aggressive nature with high propensity for recurrence in LG and pTa tumours.

Keywords Non muscle invasive bladder cancer · Biomarkers · Predictive role · Survival analysis · Hazard ratio

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Introduction

The incidence of non muscle invasive bladder cancers in India ranges from 0.38% to 1.02% in females and 1.89% to 5.8% in males as per the National Cancer Registry programme (NCRP) report, 2006 [1]. These cancers constitute more than 80% of bladder cancer and of these more than 70% patients recur within the first 2 years of diagnosis [2]. Hence it is imperative to treat these tumours with Transurethral resection of bladder tumour (TURBT) followed by adjuvant intravesical BCG in the intermediate and high risk groups [3, 4]. Tumour suppressor genes (p53) have been identified as potential predictors for recurrence, but the estimated hazard rate seems variable [5, 6]. Significant correlation between p53 mutations and/or p53 expression with poor survival has been reported [7, 8]. The tumor suppressor gene, p21waf1, is regulated by p53 to mediate cell-cycle control and is also induced by a

p53 independent pathway [9]. p53 gene mutation results in the loss of p21waf1 expression leading to unregulated cell growth [10]. Vascular Endothelial Growth Factor (VEGF) is a family of polypeptides having a central role in angiogenesis and enhanced expression is associated with recurrence and progression [11]. Another angiogenesis marker CD105 (endoglin), independently influences cell morphology, adhesion and angiogenesis [12]. The present study was undertaken to evaluate the individual and synergistic effects of markers in the p53 pathway (p53, p21waf1) and angiogenesis markers (VEGF and CD105).

Materials and Methods

The study included 90 cases (77 males and 13 females) of non muscle invasive transitional cell cancer of bladder followed up for 2–55 months (median 23.5), aged 17–78 years (mean±SD; 54.6±13.85). Informed consent from patients and Institutional ethics committee was obtained. All patients had intermediate and high risk and were treated with TURBT followed by BCG [4]. Resected tumour tissue was divided into two parts, one part for formalin fixed, paraffin embedded tissue and the other part was minced and a cell spot was prepared for immunoflourescent (IF) staining. The slide was air dried, fixed in cold acetone for 10 min and incubated at room temperature for 1 h after adding primary antibody. The antibodies used were p53 & p21Waf1 (DAKO), VEGF & CD105 (Neomarkers) in a dilution of 1:50. Slides were washed well and incubated with FITC (flourescein isothiocyanate) conjugated secondary antibody in the dark for 30 min. Freshly prepared Propidium Iodide (1:10 dilution in

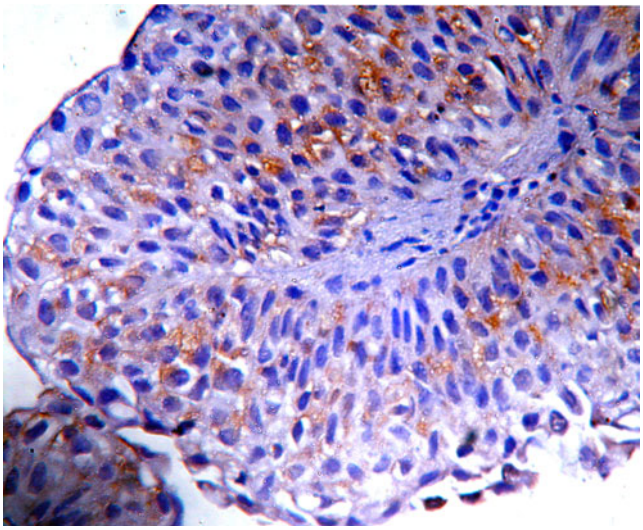

Fig. 2 Immunohistochemical expression of CD105 in tumour cell cytoplasm in a case of papillary transitional cell carcinoma of bladder

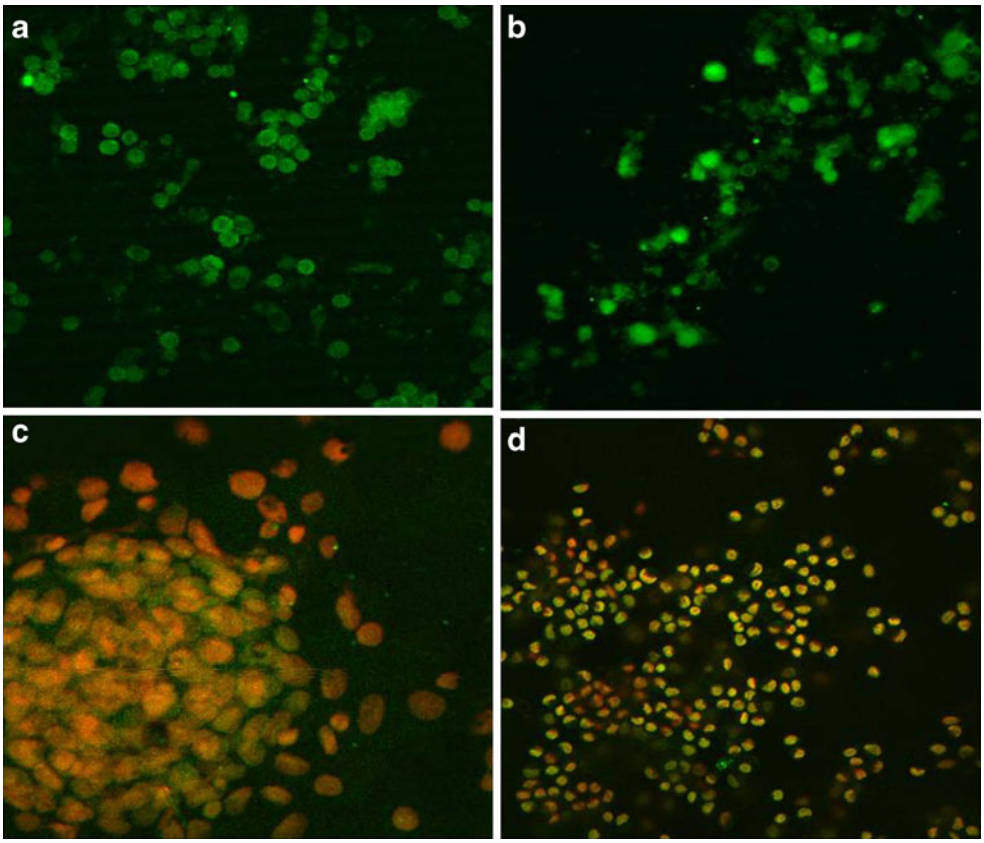

Fig. 1 The expression of the three tumor markers is seen by Immunoflourescence staining. **a** p53 nuclear expression in cell spots of bladder cancer **b** p21waf1 nuclear expression **c** cytoplasmic expression of VEGF with propidium iodide nuclear counterstain, **d** CD105 expression in cytoplasm of bladder cancer cells

PBS) was used as counterstain for cytoplasmic markers (VEGF, CD105) and incubated in the dark for 15–30 min. Slides were mounted in 50% glycerol and observed under Confocal Laser Scanning Microscope. The cut off scores for the expression of markers was obtained through ROC analysis (>20% for p53, p21 and VEGF and >15% for CD105). CD105 was also evaluated on paraffin section by immunohistochemistry to confirm its expression in tumor cells. The association of the IF markers, individually and in combination, with recurrence was assessed by the χ^2 and Fishers' Exact Test. Survival analysis (Cox Regression, Kaplan Meier and Log Rank Test), involved recurrence of non muscle invasive bladder cancer and time to first recurrence as the dependent and time variable. The data was analyzed overall, low grade and tumor stages (pTa and pT1). The data analysis was performed using SPSS 17.0.

Results

Out of 90 patients [low grade: 66 (73.3%), high grade: 24 (26.7%), pTa: 33 (36.7%) and pT1: 57 (63.3%)], 39 (43.3%) patients had recurrence [low grade: 26 (39.4%), high grade: 13 (54.2%), pTa: 13 (39.3%) and pT1: 26 (45.6%)]. Of the 33 pTa

Table 1 Clinicopathological characteristics of the study population and their association with recurrence

Characteristics	Label	Overall				Low grade			
		n	Rec (%)[a]	χ^2	χ^2(Trend)	n	Rec (%)[a]	χ^2	χ^2(Trend)
Age	<40	12	4 (33.3)			10	3(30.0)		
	41–60	40	17 (42.5)	0.687	0.400	29	11(37.9)	0.71	0.413
	>61	38	18 (47.4)			27	12(44.4)		
Gender	Female	13	4 (30.8)	0.379	0.326	10	2(20.0)	0.293	0.176
	Male	77	35 (45.5)			56	24(42.8)		
p53	−	36	8 (22.2)	0.001*	0.001*	34	8(23.5)	0.007*	0.007*
	+	54	31 (57.4)			32	18(56.3)		
p21Waf1	−	35	15 (42.9)	0.942	0.942	25	10(40.0)	0.937	0.938
	+	55	24 (43.6)			41	16(39.0)		
VEGF	−	58	19 (32.8)	0.006*	0.007*	46	13(28.3)	0.005*	0.005*
	+	32	20 (62.5)			20	13(65.0)		
CD105	−	17	8 (47.1)	0.731	0.790	8	2(25.0)	0.464	0.378
	+	73	31 (42.5)			58	24(41.4)		
p53p21	Others	30	22 (36.7)	0.070	0.070	49	17 (34.7)	0.185	0.188
	(++)	60	17 (56.7)			17	9 (52.9)		
p53vegf	Others	64	21 (32.8)	0.002*	0.002*	53	15 (28.8)	0.001*	0.001*
	(++)	26	18 (69.2)			14	11 (78.6)		
p53cd105	Others	53	16 (30.2)	0.003*	0.005*	42	10 (23.8)	0.001*	0.001*
	(++)	37	23 (62.2)			24	16 (66.7)		
p21vegf	Others	72	28 (38.9)	0.089	0.019*	55	19 (34.5)	0.071	0.095
	(++)	18	11 (61.1)			11	7 (63.6)		
p21cd105	Others	46	20 (43.5)	0.977	0.977	30	11 (36.7)	0.679	0.681
	(++)	44	19 (43.2)			36	15 (41.7)		
vegfcd105	Others	67	23 (34.3)	0.003*	0.003*	49	14 (28.6)	0.004*	0.002*
	(++)	23	16 (39.6)			17	12 (70.6)		
p53p21vegf	Others	76	30 (39.5)	0.085	0.140	59	21 (35.6)	0.067	0.069
	(+++)	14	9 (64.3)			7	5 (71.4)		
p53p21cd105	Others	71	27 (38.0)	0.050*	0.051	54	18 (33.3)	0.050	0.034
	(+++)	19	12 (63.2)			12	8 (66.7)		
p53vegfcd105	Others	73	25 (34.2)	<0.001*	<0.001*	55	16 (29.1)	<0.001*	<0.001*
	(+++)	17	14 (82.4)			11	10 (90.9)		
p21vegfcd105	Others	77	30 (39.0)	0.067	0.043*	56	19 (33.3)	0.041	0.033
	(+++)	13	9 (69.2)			10	7 (70.0)		
p53p21vegfcd105	Others	81	32 (39.5)	0.037*	0.029*	60	21 (35.0)	0.031	0.022
	(++++)	9	7 (77.8)			6	5 (83.3)		

[a] Recurrence

*p significant at <0.05

tumours, 30(90.9%) were of low grade. The association of grade with recurrence (p=0.211) and the distribution of tumor stage with recurrence, both overall (p=0.566) as well as in low grade (p=0.679) were not found statistically significant.

The markers p53, p21waf1, VEGF and CD105 were expressed in 60%, 61.1%, 35.5% and 81.1% respectively (Fig. 1). Localization of CD105 was observed in the tumour cells by immunohistochemistry on paraffin sections (Fig. 2). Individually the association of p53 and VEGF with recurrence was statistically significant (p=0.001, p=0.006). Analysis of combination profiles was positive for expression of markers compared to all other possible forms. Profiles with simultaneous expression of two markers viz. p53/VEGF (0.002), p53/CD105 (0.003), VEGF/CD105 (0.003); three markers p53/p21/CD105 (0.050), p53/VEGF/CD105 (<0.001); and all four markers p53/p21/VEGF/CD105 (p= 0.037) were significantly associated with recurrence. In low grade (LG) cases the same two marker profiles and in addition the three marker profile p21/VEGF/CD105 revealed statistically significant (p=0.041) association (Table 1).

The evaluation of markers on tumor stages revealed that the profiles p21; p53/CD105 and p53/VEGF/CD105 were

Table 2 Clinicopathological characteristics of the study population and the association with recurrence in various subgroups

Characteristics	Category	Overall (90)						Low grade (66)					
		pT_a (33)			pT_1 (57)			pT_a (30)			pT_1 (36)		
		n	Recurrence (%)	χ^2 χ^2 (trend)	n	Recurrence (%)	χ^2 χ^2 (trend)	n	Recurrence (%)	χ^2 χ^2 (trend)	n	Recurrence (%)	χ^2 χ^2 (trend)
Age	<40	4	1 (25.0)	0.772	8	3 (37.5)	0.568	3	0 (0.0)	0.349	7	3 (42.9)	0.530
	41–60	11	5 (45.5)	0.807	29	12 (41.4)	0.319	11	5 (45.5)	0.491	18	6 (33.3)	0.524
	>61	18	7 (38.9)		20	11(55.0)		16	6 (37.5)		11	6 (54.5)	
Gender	Female	6	1 (16.7)	0.208	7	3 (42.9)	0.876	6	1 (16.7)	0.372	4	1 (25.0)	0.626
	Male	27	12 (44.4)	0.215	50	23 (46.0)	0.877	24	10 (41.7)	0.264	32	14 (43.8)	0.480
p53	−	19	5 (26.3)	0.073	17	3 (17.6)	0.006*	19	5 (26.3)	0.238	15	3 (20.0)	0.041*
	+	14	8 (57.1)	0.078	40	23 (57.5)	0.006*	11	6 (54.5)	0.128	21	12 (57.1)	0.028*
p21Waf1	−	13	1 (7.7)	0.003*	22	14 (63.6)	0.030*	12	1 (8.3)	0.018*	13	9 (69.2)	0.017*
	+	20	12 (60.0)	0.003*	35	12 (34.3)	0.032*	18	10 (55.6)	0.010*	23	6 (26.1)	0.013*
VEGF	−	26	9 (34.6)	0.279	32	10 (31.3)	0.014*	24	7 (29.2)	0.094	22	6 (27.3)	0.028*
	+	7	4 (57.1)	0.286	25	16 (64.0)	0.015*	6	4 (66.7)	0.156	14	9 (64.3)	0.030*
CD105	−	4	1 (25.0)	0.530	13	7 (53.8)	0.498	2	0 (0.0)	0.520	6	2 (33.3)	0.650
	+	29	12 (41.4)	0.536	44	19 (43.2)	0.501	28	11 (39.3)	0.273	30	13 (43.3)	0.655
p53p21	Others	25	6 (24.0)	0.001*	35	16 (45.7)	0.985	24	6 (25.0)	0.008*	25	11 (44.0)	0.669
	(++)	8	7 (87.5)	0.002*	22	10 (45.5)	0.985	6	5 (83.3)	0.016*	11	4 (36.4)	0.673
p53vegf	Others	29	10 (34.5)	0.120	35	11 (31.4)	0.007*	27	8 (29.6)	0.041*	25	7 (28.0)	0.025*
	(++)	4	3 (75.0)	0.126	22	15 (68.2)	0.007*	3	3 (100.0)	0.041*	11	8 (72.7)	0.013*
p53cd105	Others	23	6 (26.1)	0.026*	30	10 (33.3)	0.050	21	5 (23.8)	0.042*	21	5 (23.8)	0.017*
	(++)	10	7 (70.0)	0.011*	27	16 (59.3)	0.052	9	6 (66.7)	0.028*	15	10 (66.7)	0.011*
p21vegf	Others	30	10 (33.3)	0.052*	42	18 (42.9)	0.484	27	8 (29.6)	0.041*	28	11 (39.3)	0.694
	(++)	3	3 (100.0)	0.027*	15	8 (53.3)	0.488	3	3 (100.0)	0.018*	8	4 (50.0)	0.593
p21cd105	Others	15	2 (13.3)	0.011*	31	18 (58.1)	0.039*	13	1 (7.7)	0.007*	17	10 (58.8)	0.090
	(++)	18	11 (61.1)	0.006*	26	8 (30.8)	0.041*	17	10 (58.8)	0.005*	19	5 (26.3)	0.051*
vegfcd105	Others	27	9 (33.3)	0.182	40	14 (35.0)	0.020	24	7 (29.2)	0.156	25	7 (28.0)	0.025*
	(++)	6	4 (66.7)	0.137	17	12 (70.6)	0.014	6	4 (66.7)	0.094	11	8 (72.7)	0.013*
p53p21vegf	Others	31	11 (35.5)	0.148	45	19 (45.2)	0.349	28	9 (32.1)	0.126	31	12 (38.7)	0.630
	(+++)	2	2 (100.0)	0.075	12	7 (58.2)	0.324	2	2 (100.0)	0.059	5	3 (60.0)	0.377
p53p21cd105	Others	27	7 (25.9)	0.002*	44	20 (45.5)	0.965	25	6 (24.0)	0.003*	29	12 (41.4)	0.942
	(+++)	6	6 (100.0)	0.001*	13	6 (46.2)	0..965	5	5 (100.0)	0.002*	7	3 (42.9)	0.944
p53vegfcd105	Others	30	10 (33.3)	0.052*	43	15 (34.9)	0.006*	27	8 (29.6)	0.041*	28	8 (28.6)	0.005*
	(+++)	3	3 (100.0)	0.027*	14	11 (78.6)	0.005*	3	3 (100.0)	0.018*	8	7 (87.5)	0.003*
p21vegfcd105	Others	30	10 (33.3)	0.052*	47	20 (42.6)	0.486	27	8 (29.6)	0.041*	29	11 (37.9)	0.418
	(+++)	3	3 (100.0)	0.027*	10	6 (60.0)	0.319	3	3 (100.0)	0.018*	7	4 (57.1)	0.362
p53p21vegfcd105	Others	31	11 (35.5)	0.148	50	21 (42.0)	0.228	28	9 (32.1)	0.126	32	12 (47.5)	0.287
	(++++)	2	2 (100.0)	0.075	7	5 (71.4)	0.147	2	2 (100.0)	0.059	4	3 (75.7)	0.157

*p significant at <0.05

statistically significantly associated with recurrence in all the four subsets [p: overall/pTa, overall/pT1, LG/pTa, LG/pT_1], [p21(0.003, 0.030, 0.018, 0.017)]; [p53/CD105 (0.026,0.050, 0.042,0.017)] and [p53/VEGF/CD105 (0.052,0.006,0.041,0.005)]. Among the pTa tumours a statistically significant (p:overall,low grade) association was found with p21(0.003,0.018) ; p53/p21(0.001,0.008), p53/CD105(0.026,0.042), p21/VEGF(0.052,0.041), p21/CD105 (0.011,0.007), p53/p21/CD105(0.002,0.003), p53/VEGF/CD105(0.052,0.041), p21/VEGF/CD105(0.052,0.041) and in addition, the profile p53/VEGF (0.041) in LG/pTa only. As pTa/high grade constitute only 3 cases no conclusion can be drawn. The profiles p53(0.006,0.041), p21(0.030,0.017), VEGF(0.014,0.028), p53/VEGF(0.007,0.025), p53/CD105 (0.050,0.017), VEGF/CD105(0.020,0.025), p53/VEGF/CD105(0.006,0.005) in pT1 (p:overall, low grade) and in addition the profile p21/CD105(0.039) in overall/pT1 were significant (Table 2). The association of concomitant expression of all four markers with recurrence was significant overall (p=0.037) and in low grade cases (p=0.031) (Table 1) but not in pTa (p=0.148) cases (Table 2). The proportion of recurrent cases increased with increase in number of altered biomarkers in both overall [p (trend):0.001) and pTa [p(trend):<0.001)] subset (Fig. 3).

The results of univariate and multivariate Cox regression analysis are shown in Table 3. The multivariable analysis (adjusted for age and gender) revealed marginally significant hazard [HR (95% C.I.), p] for recurrence in VEGF positive cases both overall [1.807 (0.909, 3.593), 0.092] and in low grade [2.420 (0.977, 5.990), 0.056] cases in comparison to VEGF negative cases. Tumors expressing the profiles p21/VEGF, VEGF/CD105, p53/p21/CD105, p53/VEGF/CD105, p21/VEGF/CD105 and p53/p21/VEGF/CD105 had significantly greater hazard for recurrence in the entire study group and low grade subset. Additionally, the profile p53/p21/VEGF was also found predictive of significant hazard in the low-grade cases. The associated Kaplan Meier plot for these profiles exhibited significantly (Log Rank: p<0.05) decreased recurrence free survival probability (Figures not shown) and the median time for first recurrence was lower when these markers were expressed (Table 3).

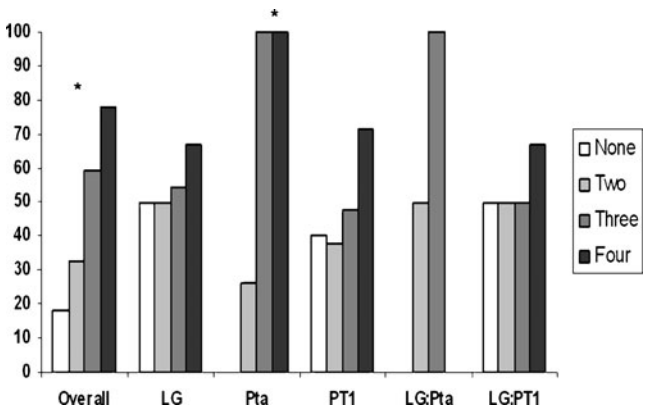

Fig. 3 Bar diagram representing increasing trend of recurrent proportion with increasing number of altered biomarkers in overall and pTa tumours

The profiles that were found to predict significant hazard in pTa overall and in low grade were viz., p53/p21, p53/CD105, p21/VEGF, VEGF/CD105, p53/p21/CD105, p53/VEGF/CD105 and p21/VEGF/CD105. In addition, VEGF and p53/VEGF in the pTa low grade group predicted statistically significant hazard for recurrence along with significantly decreased recurrence free probability (Table 4). The results were statistically not significant in the tumor stage pT1 (Table 4).

Discussion

Tumor grade and stage are well established independent risk factors for recurrence of non-muscle invasive bladder tumors. Even then, 50% to 70% cases of low grade/low stage tumors recur [13]. The stages of non muscle invasive bladder tumors are: carcinoma in situ (TiS), papillary tumor confined to epithelium (pTa) and invading lamina propria (pT1). Ta, low grade papillary tumors progress in grade or become invasive less than 5% of the time, and are high in grade in only 1% to 3% of cases. In contrast, stage T1 tumors may be high grade in 30% to 50% of cases and are rarely low grade [14]. Studies have shown that the prognosis for recurrence, progression and survival is considerably worse for patients with pT1 than for patients with pTa tumours and our study also showed similar results with higher recurrence in pT_1 (45.6%) tumors as compared to pTa (39.3%). The cystoscopic follow-up of superficial bladder cancer, while increasing the workload of the urologist is also an invasive procedure with high costs. In such a scenario the identification of additional parameters as marker/s of aggressive behavior in lower tumor grades and stages is of importance for instituting early therapy.

The analysis in our study revealed that the combined expression of angiogenesis markers VEGF and CD105 showed significant association with recurrence, increased hazard for recurrence and decreased recurrence free survival both in overall and low grade patients. Addition of p53 or p21 to this profile showed significant association with recurrence, predicted significant hazard for recurrence and decreased recurrence free survival probability in LG and pTa groups. The profile p53/CD105 showed significant association with recurrence, increased hazard and shorter recurrence free survival in overall, LG, and pTa tumors (Table 5). These results suggest that angiogenesis markers VEGF and CD105 have significant potential for prediction of hazard for recurrence, and hence can serve as targets for therapy. In this study, p53, p21 and VEGF are observed to be individually

Table 3 Survival analysis of the tumor subgroups in relation to the expression of biomarkers individually and in combination

Biomarkers	Expression	Overall (n=90)				Low grade (n=66)				Log rank			
		Unadjusted HR (95% C.I.)	P	Adjusted[a] HR (95% C.I.)	P	Unadjusted HR (95% C.I.)	P	Adjusted[a] HR (95% C.I.)	P	Overall		Low grade	
										Median	P	Median	P
P53	−	1.00		1.00		1.00		1.00		30		30	0.349
	+	1.979(0.903,4.338)	0.088	1.793(0.807,3.986)	0.152	1.487(0.632,3.497)	0.364	1.338(0.557,3.215)	0.515	19	0.076	21	
P21waf1	−	1.00		1.00		1.00		1.00		22		34	0.430
	+	1.239(0.630,2.436)	0.535	1.123(0.553,2.280)	0.748	1.395(0.596,3.266)	0.443	1.194(0.489,2.915)	0.698	23	0.526	30	
VEGF	−	1.00		1.00		1.00		1.00		30		#	0.080
	+	1.702(0.891,3.252)	0.107	1.807(0.909,3.593)	0.092	1.990(0.890,4.451)	0.094	2.420(0.977,5.990)	0.056	19	0.096	18	
CD105	−	1.00		1.00		1.00		1.00		23		49	0.048*
	+	1.555(0.680,3.554)	0.295	1.593(0.673,3.772)	0.290	5.868(0.780,44.151)	0.086	6.002(0.757,47.609)	0.090	21	0.282	22	
P53/P21waf1	Others	1.00		1.00		1.00		1.00		30		34	0.155
	++	1.819(0.951,3.480)	0.071	1.775(0.868,3.627)	0.116	1.784(0.780,4.081)	0.170	1.674(0.654,4.285)	0.282	18	0.061	17	
P53/VEGF	Others	1.00		1.00		1.00		1.00		30		#	0.192
	++	1.552(0.809,2.981)	0.186	1.619(0.817,3.206)	0.167	1.704(0.744,3.905)	0.208	1.987(0.802,4.924)	0.138	19	0.174	18	
P53/CD105	Others	1.00		1.00		1.00		1.00		30		49	0.014*
	++	2.321(1.209,4.453)	0.011*	2.132(1.091,4.168)	0.027*	2.628(1.159,5.956)	0.021*	2.253(0.966,5.252)	0.060	17	0.008*	18	
P21waf1/Vegf	Others	1.00		1.00		1.00		1.00		30		44	0.005*
	++	2.278(1.099,4.720)	0.027*	2.195(1.052,4.582)	0.036*	3.349(1.340,8.369)	0.010*	3.425(1.332,8.811)	0.011*	17	0.020*	17	
P21waf1/CD105	Others	1.00		1.00		1.00		1.00		22		44	0.181
	++	1.230(0.644,2.348)	0.531	1.172(0.611,2.250)	0.633	1.730(0.753,3.976)	0.197	1.578(0.680,3.662)	0.288	20	0.522	20	
Vegf/CD105	Others	1.00		1.00		1.00		1.00		30		39	0.003*
	++	2.434(1.269,4.669)	0.007*	2.624(1.274,5.403)	0.009*	3.041(1.373,6.735)	0.006*	3.380(1.348,8.472)	0.009*	18	0.005*	18	
P53/P21waf1/VEGF	Others	1.00		1.00		1.00		1.00		30		44	0.031*
	+++	1.998(0.926,4.311)	0.078	1.957(0.902,4.246)	0.089	2.812(1.028,7.693)	0.044*	3.001(1.078,8.353)	0.035*	18	0.066	17	
P53/P21waf1/CD105	Others	1.00		1.00		1.00		1.00		30		44	0.013*
	+++	2.162(1.081,4.324)	0.029*	2.000(0.993,4.027)	0.052*	2.752(1.171,6.468)	0.020*	2.539(1.047,6.157)	0.039*	17	0.023*	12	
P53/VEGF/CD105	Others	1.00		1.00		1.00		1.00		30		49	0.010*
	+++	2.290(1.177,4.453)	0.015	2.360(1.148,4.849)	0.020*	2.710(1.206,6.092)	0.016*	2.738(1.104,6.788)	0.030*	18	0.011*	18	
P21waf1/VEGF/CD105	Others	1.00		1.00		1.00		1.00		23		44	0.002*
	+++	2.431(1.132,5.224)	0.023*	2.611(1.189,5.731)	0.017*	3.684(1.480,9.173)	0.005*	3.946(1.530,10.182)	0.005*	17	0.016*	12	
P53/P21waf1/VEGF/CD105	Others	1.00		1.00		1.00		1.00		23		44	0.014*
	++++	2.158(0.939,4.958)	0.070	2.382(1.021,5.556)	0.045*	3.188(1.170,8.690)	0.023*	3.572(1.287,9.911)	0.014*	17	0.058	12	

[a] Adjusted for age and gender, # not estimated

*p significant at <0.05

Table 4 Survival analysis of the pTa and pT1 tumours in relation to the expression of biomarkers and combination profiles

Biomarkers	Exp	Overall		Low grade		Overall				Low grade			
		PT_a	PT_1	PT_a	PT_1	PT_a		PT_1		PT_a		PT_1	
		Unadjusted(95%CI),*p* Adjusted(95%CI),*p*	Unadjusted(95%CI),*p* Adjusted(95%CI),*p*	Unadjusted(95%CI),*p* Adjusted(95%CI),*p*	Unadjusted(95%CI),*p* Adjusted(95%CI),*p*	Md	p	Md	P	Md	p	Md	P
P53	−	1.000	1.000	1.000	1.000	30	0.143	#	0.353	30	0.205	#	0.822
	+	2.188(0.715,6.695),0.170	1.761(0.521,5.955),0.363	2.082(0.633,6.846),0.227	1.161(0.313,4.305),0.824	17		20		17		24	
		2.210(0.688,7.098),0.183	1.387(0.393,4.888),0.611	1.895(0.549,6.538),0.312	0.884(0.222,3.514),0.860								
P21waf1	−	1.000	1.000	1.000	1.000	#	0.030*	20	0.416	#	0.075	22	0.566
	+	6.580(0.853,50.764),0.071	0.719(0.322,1.607),0.422	5.156(0.656,40.502),0.119	0.729(0.243,2.185),0.572	17		24		20		#	
		6.789(0.817,56.445),0.076	0.618(0.259,1.474),0.278	3.955(0.479,23.658),0.202	0.631(0.184,2.161),0.463								
VEGF	−	1.000	1.000	1.000	1.000	#	0.077	23	0.399	#	0.003*	#	0.554
	+	2.757(0.815,9.325),0.103	1.414(0.625,3.199),0.406	6.025(1.531,23.712),0.010*	1.384(0.463,4.141),0.561	17		20		11		21	
		2.598(0.748,9.022),0.133	1.564(0.589,4.152),0.369	5.521(1.312,23.227),0.020*	1.889(0.397,8.983),0.424								
CD105	−	1.000	1.000	1.000	1.000	#	0.451	23	0.344	#	0.238	49	0.106
	+	2.086(0.270,16.090),0.481	1.553(0.615,3.919),0.352	Inflated estimate	4.645(0.594,36.341),0.143	20		21		#		22	
		2.655(0.331,21.305),0.358	1.363(0.508,3.661),0.539	Inflated estimate	4.609(0.477,44.556),0.187								
P53/P21waf1	Others	1.000	1.000	1.000	1.000	#	0.001*	21	0.951	#	0.005*	34	0.825
	++	5.167(1.716,15.558),0.003*	0.975(0.431,2.204),0.951	4.606(1.395,15.210),0.012*	0.877(0.269,2.857),0.828	9		23		9		#	
		5.161(1.527,17.439),0.008*	0.795(0.315,2.005),0.626	4.550(1.127,18.369),0.033*	0.705(0.177,2.813),0.620								
P53/VEGF	Others	1.000	1.000	1.000	1.000	30	0.133	23	0.518	#	0.004*	#	0.786
	++	2.525(0.683,9.338),0.165	1.301(0.580,2.921),0.523	5.740(1.425,23.131),0.014*	1.163(0.386,3.498),0.789	11		20		11		34	
		2.189(0.570,8.408),0.254	1.403(0.543,3.627),0.484	5.775(1.134,29.416),0.035*	1.376(0.308,6.142),0.676								
P53/CD105	Others	1.000	1.000	1.000	1.000	#	0.026*	23	0.109	#	0.027*	49	0.117
	++	3.115(1.039,9.333),0.042*	1.919(0.847,4.346),0.118	3.456(1.045,11.433),0.042*	2.438(0.760,7.816),0.134	12		18		12		18	
		3.779(1.161,12.301),0.027*	1.502(0.631,3.575),0.357	3.511(1.047,12.046),0.042*	1.878(0.517,6.822),0.338								
P21waf1/Vegf	Others	1.000	1.000	1.000	1.000	#	0.005*	23	0.210	#	0.002*	44	0.130
	++	5.096(1.340,19.379),0.017*	1.734(0.719,4.184),0.220	6.224(1.541,25.133),0.010*	2.477(0.726,8.455),0.148	9		18		9		18	
		4.740(1.146,19.606),0.032*	1.627(0.661,4.000),0.289	5.134(1.192,22.107),0.028*	2.849(0.760,10.681),0.120								
P21waf1/CD105	Others	1.000	1.000	1.000	1.000	#	0.046*	20	0.575	#	0.031*	34	0.798
	++	3.932(0.869,17.792),0.075	0.751(0.320,1.761),0.510	6.744(0.861,52.821),0.069	0.865(0.280,2.672),0.801	17		24		17		#	
		4.233(0.839,21.369),0.081	0.743(0.316,1.747),0.496	5.705(0.709,45.916),0.102	0.853(0.273,2.666),0.784								
Vegf/CD105	Others	1.000	1.000	1.000	1.000	#	0.007*	23	0.091	#	0.003*	49	0.063
	++	4.680(1.304,16.803),0.018*	1.941(0.882,4.273),0.099	6.025(1.531,23.712),0.010*	2.618(0.901,7.609),0.077	11		18		11		18	
		5.022(1.267,19.912),0.022*	2.211(0.722,6.767),0.165	5.521(1.312,23.227),0.020*	6.775(1.259,36.459),0.026								
P53/P21waf1/VEGF	Others	1.000	1.000	1.000	1.000	30	0.048*	23	0.296	#	0.030*	44	0.242
	+++	3.916(0.844,18.175),0.081	1.606(0.649,3.974),0.306	4.629(0.957,22.383),0.057	2.140(0.570,8.043),0.260	3		18		3		18	
		3.722(0.701,19.766),0.123	1.530(0.610,3.838),0.365	3.999(0.731,21.885),0.110	2.566(0.638,10.329),0.185								
P53/P21waf1/CD105	Others	1.000	1.000	1.000	1.000	#	<0.001*	21	0.918	#	<0.001*	34	0.892
	+++	7.058(2.140,23.277),0.001*	1.049(0.416,2.649),0.919	10.649(2.787,40.689),0.001*	1.092(0.300,3.982),0.894	9		24		9		18	
		6.745(1.880,24.196),0.003*	0.946(0.373,2.400),0.906	9.171(2.211,38.042),0.002*	0.986(0.261,3.732),0.984								
P53/VEGF/CD105	Others	1.000	1.000	1.000	1.000	#	0.008*	23	0.130	#	0.004*	49	0.116
	+++	4.776(1.258,18.131),0.022*	1.822(0.823,4.034),0.139	5.740(1.425,23.131),0.014*	2.266(0.785,6.541),0.130	11		18		11		18	
		5.318(1.097,25.788),0.038*	1.952(0.645,5.908),0.237	5.775(1.134,29.416),0.035*	4.703(0.825,26.809),0.081								
P21waf1/VEGF/CD105	Others	1.000	1.000	1.000	1.000	#	0.005*	22	0.234	#	0.002*	44	0.080
	+++	5.096(1.340, 19.379),0.017*	1.744(0.683,4.452),0.245	6.224 (1.541, 25.133), 0.010*	2.803(0.829, 9.475), 0.097	9		18		9		18	

Table 4 (continued)

Biomarkers	Exp	Overall			Low grade			Overall				Low grade			
		PT_a	PT_1		PT_a	PT_1		PT_a		PT_1		PT_a		PT_1	
		Unadjusted(95%CI),*p* Adjusted(95%CI),*p*	Unadjusted(95%CI),*p* Adjusted(95%CI),*p*		Unadjusted(95%CI),*p* Adjusted(95%CI),*p*	Unadjusted(95%CI),*p* Adjusted(95%CI),*p*		Md	p	Md	P	Md	p	Md	P
		4.740(1.146, 19.606),0.032*	1.863(0.668,5.198),0.234		5.134 (1.192, 22.107), 0.028*	3.621(0.928, 14.120), 0.064									
P53/P21waf1/VEGF/CD105	Others	1.000	1.000		1.000	1.000		30	0.048*	22	0.330	#	0.030*	44	0.151
	++++	3.916 (0.844, 18.175), 0.081	1.624(0.600,4.394),0.340		4.629 (0.957, 22.383), 0.057	2.503 (0.671, 9.327), 0.172		3		18		3		12	
		3.722 (0.701, 19.766), 0.123	1.777(0.602,5.244),0.298		3.999 (0.731, 21.885), 0.110	3.423 (0.816, 14.364),0.095									

Md: Median Time (recurrence free survival), p: Log rank, # not estimated

*p significant at <0.05

associated significantly with recurrence in pT1 tumors but the marker/s did not predict significant hazard for recurrence implying that in an already invasive tumor the biomarkers studied did not have a role to play in predicting recurrence. Contrarily in pTa tumors many profiles predicted significant hazard for recurrence (Table 4). The profile p53/p21/CD105 predicted maximum hazard for recurrence in pTa tumors and may serve as a valuable biological variable to identify a subgroup of Ta bladder cancer patients at high risk for the development of recurrent disease.

P53, a tumour suppressor gene, is mutated in various malignancies and is reported to be associated with high tumour grade and stage, and thus phenotypically considered aggressive [5, 7]. Numerous studies have confirmed the role of p53 mutation and overexpression in aggressive behavior of non muscle invasive bladder cancer [7, 15]. P53 also acts as a downstream regulator of another tumor suppressor marker p21waf1. Mutation of p53 gene results in the loss of p21waf1 expression and function leading to unregulated cell growth [9]. Furthermore, it has also been observed that a p53 independent pathway can induce p21waf1 and one of the factors implicated is TGF-β [16]. The role of p21waf1 both in the p53 dependent and independent pathways has been documented to be positively associated with recurrence, increased grade and stage [17, 18]. Our study shows significant association of recurrence with p53, but not with p21waf1 (+/−) individually or in combination with p53 [p53(+)/p21(−) & p53(+)/p21(+)]. The available literature reports the positive as well as negative association of p21waf1 (+/−) with regard to grade, stage and recurrence in superficial TCC [17–20]. Owing to these divergent views the exact cause and effect relationship of p21waf1 with recurrence obviously needs to be further investigated. These results may indicate the dual pathway of p21waf1 induction and also imply the role of other factors, suggesting that study of these two markers alone is insufficient for predicting hazard for recurrence.

Angiogenesis markers mainly VEGF and CD105 constitute the propelling forces behind tumour growth and metastasis, showing enhanced expression in malignancies [11, 12]. VEGF is modulated by p53, which positively regulates thrombospondin-1, an inhibitor of angiogenesis. P53 mutations resulting in loss of its function lead to overexpression of VEGF with enhanced angiogenesis and tumor progression [21]. As p53 and VEGF were significantly associated with recurrence individually, their combined expression was expected to predict the significant hazard for recurrence but the results were not supportive of an independent role of VEGF.

CD105 (endoglin) is a hypoxia-inducible protein acting as a receptor for the TGFβ family of growth factors and also associated with angiogenesis and proliferation. Only a small percentage of CD105 expressed on the cell surface is

Table 5 Comparison of the results of the association and survival analysis for the biomarkers individually and in combination

Evaluation		Biomarkers														
		p53	p21	VEGF	CD105	p53p21	p53vegf	p53cd105	p21vegf	p21cd105	vegfcd105	p53p21vegf	p53p21cd105	p53vegfcd105	p21vegfcd105	p53p21vegfcd105
Overall	Association	S	NS	S	NS	NS	S	S	NS	NS	S	NS	S	S	NS	S
	Univariate	NS	NS	NS	NS	NS	NS	S	S	NS	S	NS	S	S	S	NS
	Multivariate	NS	NS	NS	NS	NS	NS	S	S	NS	S	NS	S	S	S	S
	KM	NS	NS	NS	NS	NS	NS	S	S	NS	S	NS	S	S	S	NS
Low grade	Association	S	NS	S	NS	NS	S	S	NS	NS	S	NS	S	S	S	S
	Univariate	NS	NS	NS	NS	NS	NS	S	S	NS	S	S	S	S	S	S
	Multivariate	NS	NS	NS	NS	NS	NS	NS	S	NS	S	S	S	S	S	S
	KM	NS	NS	NS	S	NS	NS	S	S	NS	S	S	S	S	S	S
Overall (pT_a)	Association	NS	S	NS	NS	S	NS	S	S	S	NS	NS	S	S	S	NS
	Univariate	NS	NS	NS	NS	S	NS	S	S	NS	S	NS	S	S	S	NS
	Multivariate	NS	NS	NS	NS	S	NS	S	S	NS	S	NS	S	S	S	NS
	KM	NS	S	NS	NS	S	NS	S	S	S	S	S	S	S	S	S
Overall (pT_1)	Association	S	S	S	NS	NS	S	S	NS	S	S	NS	NS	S	NS	NS
	Univariate	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
	Multivariate	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
	KM	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Low grade (pT_a)	Association	NS	S	NS	NS	S	S	S	S	S	NS	NS	S	S	S	NS
	Univariate	NS	NS	S	NS	S	S	S	S	NS	S	NS	S	S	S	NS
	Multivariate	NS	NS	S	NS	S	S	S	S	NS	S	NS	S	S	S	NS
	KM	NS	NS	S	NS	S	S	S	S	S	S	S	S	S	S	S
Low grade (pT_1)	Association	S	S	S	NS	NS	S	S	NS	NS	S	NS	NS	S	NS	NS
	Univariate	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
	Multivariate	NS	NS	NS	NS	NS	NS	NS	NS	NS	S	NS	NS	NS	NS	NS
	KM	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS

Inflated estimate

S statistically significant, *NS* not significant

associated with TGFβ receptors and the remaining acts independently and its function is not well understood [12]. CD105 has been found to be associated more with proliferating vessels in neoplastic tissue and not in non-neoplastic tissue [22], but in the present study the association with recurrence was not significant. Though neither angiogenesis marker was able to predict significant hazard independently, their combined expression showed significant association with recurrence, prediction of hazard and shorter recurrence free survival. Furthermore, the median time for recurrence was also significantly lower among patients showing positive expression of these tumor markers simultaneously. Similar results have been reported in lung cancer by Mineo et al. [23], in prostatic cancer by El-Gohary et al. [24] and in colorectal cancer by Saad et al [22], breast by Kumar et al. [25]. In addition, as the increased vascular supply may carry the tumor cells to adjacent areas resulting in recurrence post-TURBT, future strategic therapeutic approach could include targeting CD105 [26] and VEGF [27].

The alteration of p53 and CD105 was observed to be significantly associated with recurrence. As p53 is associated with an aggressive phenotype [8] the clinical relevance of this association in low grade/stage patients demands further investigation on a larger group of non muscle invasive bladder cancer. While the coexpression of p53 with VEGF/CD105 predicted statistical significance hazard for recurrence overall [2.360(1.148, 4.89), 0.020] and LG [2.738(1.104, 6.788), 0.030], this estimated hazard rate was lower in comparison with the profile VEGF/CD105 overall [2.624 (1.274, 5.403), 0.009] and LG [3.380(1.348, 8.472), 0.009]. Similarly the combination of p21waf1/VEGF/CD105 predicted statistically significant hazard, decreased recurrence free survival probability, both overall [2.611(1.189, 5.731), 0.017] and in LG cases [3.946(1.530, 10.182), 0.005]. The phenotype p21/VEGF/CD105 (all three positive) is found to be the most significant profile for prediction of hazard rate (HR) for recurrence in low grade and pTa subset. In the tumor stage pTa the addition of either of tumor suppressor markers (p53, p21) to the angiogenesis profile (VEGF/CD105) increased the hazard for recurrence significantly. The p53/p21/CD105 predicted the maximum hazard in pTa subset (overall:6.745, low grade:9.171).

An improved predictive accuracy of tumor recurrence and cancer specific mortality has been reported with increase in number of altered biomarkers [28]. The coexpression of all the four markers was associated with recurrence both in overall and LG group and also predicted significant hazard. The Log Rank Test with this profile was found significant in low grade and pTa groups only. Although, the proportion of recurrent cases increased with the number of altered biomarkers (Fig. 3), the estimated hazard was less than those observed by considering the addition of either of the tumour markers to the angiogenesis markers. This leads us to believe that complex cross-talks are occurring between the markers in which the effects of one are being modified by another. Studies conducted on a larger cohort can help in exploring all possible combinations of expression of the markers and their associated HR for recurrence. In this context, identification of biomarker profiles which can reliably predict tumor recurrence are of critical importance for the efficacious management of non-muscle invasive bladder cancer.

Conclusion

The study emphasizes mutually modulating effect of tumor suppressor and angiogenesis markers, especially in low grade pTa non muscle invasive bladder carcinoma, with implication on change in therapeutic strategies. Hence it is important to study the interactions between the biomarkers along with the number of altered biomarkers. In addition it is equally important to evaluate the markers in various tumor grades and stages. Concomitant expression of various markers in low grade pTa tumors could possibly indicate the need for stringent follow-up and efficient therapeutic management, in this subset of tumors which are clinically accepted to be less aggressive. Focus on the multiple pathways and their interactions may help in identification of a combined profile and contribute significantly in enriching our knowledge regarding the biological behavior of non muscle invasive bladder cancer in general and low grade/pTa cases in particular.

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