Up-Regulation of Endothelial Delta-like 4 Expression Correlates with Vessel Maturation in Bladder Cancer

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Abstract

Purpose: Angiogenesis and vascular endothelial growth factor (VEGF) expression are associated with a poor outcome in bladder cancer. To understand more about the mechanisms, we studied the role of delta-like 4 (DLL4), an endothelial-specific ligand of the Notch signaling pathway, in bladder cancer angiogenesis.

Experimental Design: The expression of DLL4, CD34, and VEGF were studied in a cohort of 60 bladder tumors and 10 normal samples using quantitative PCR. *In situ* hybridization was used to study the pattern of DLL4 expression in 22 tumor and 9 normal samples. Serial sections were also stained for CD34 and α-smooth muscle actin (α-SMA) using conventional immunohistochemistry.

Results: The expression of DLL4 was significantly up-regulated in superficial ($P < 0.01$) and invasive ($P < 0.05$) bladder cancers. DLL4 expression significantly correlated with CD34 ($P < 0.001$) and VEGF ($P < 0.001$) expression. The *in situ* hybridization studies showed that DLL4 was highly expressed within bladder tumor vasculature. Additionally, DLL4 expression significantly correlated with vessel maturation as judged by periendothelial cell expression of α-SMA, 98.7% of DLL4-positive tumor vessels coexpressed α-SMA, compared with 64.5% of DLL4-negative tumor vessels ($P < 0.001$). High DLL4 expression may have prognostic value in superficial and invasive bladder.

Conclusion: DLL4 expression is associated with vascular differentiation in bladder cancer; thus, targeting DLL4 may be a novel antiangiogenic therapy.

Bladder cancer results in over 13,000 deaths per year in the United States alone. Despite advances in the management of bladder cancer, the need for new treatment modalities remains. As with most solid tumors, bladder cancer growth and metastatic progression is dependent on the acquisition of an adequate blood supply through the process of angiogenesis (1). The role of angiogenesis in bladder cancer has been extensively studied ever since Chodak et al. (2) showed that urine from patients with bladder cancer contained proangiogenic growth factors (3).

The Notch signaling pathway has recently been shown to play an important role in angiogenesis (4–6). It is an evolutionarily conserved signaling pathway involved in cell fate determination, cellular differentiation, proliferation, survival, and apoptosis (7–9). In mammalian cells, it comprises five transmembrane Notch ligands (Jagged1, Jagged2, DLL1, DLL3, and DLL4) and four Notch receptors (Notch1-4). Ligand receptor binding leads to the cleavage and subsequent translocation from the cell membrane to the nucleus of the Notch intracellular domain (10, 11). In the nucleus, the Notch intracellular domain interacts with the transcription factor CSL to regulate the transcription of the basic helix-loop-helix proteins hairy/enhancer of split (HES) and HES-related protein (HEY; refs. 12–14).

Delta-like 4 (DLL4) is an endothelial-specific ligand expressed at sites of vascular development and angiogenesis (15, 16). DLL4 expression has previously been shown to be up-regulated within the vasculature of breast and renal tumors. *In utero* DLL4 knockout mice die of severe vascular defects, and, interestingly, haploinsufficiency of DLL4 also results in embryonic lethality from severe vascular defects (15, 17, 18). Similar haploinsufficiency phenotypes for angiogenic pathways have only been previously described for vascular endothelial growth factor (VEGF) knockout mice (19, 20). In human endothelial cells, we have recently shown that DLL4 plays an important role in regulating endothelial cell proliferation, migration, survival, and network formation (21).

In this study, we have investigated the expression of DLL4 in transitional cell carcinoma of the bladder, studied the role of

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DLL4 in vessel maturation, and carried out an initial evaluation of the role of DLL4 in prognosis.

Materials and Methods

Tissue samples. Human bladder tissue was obtained postoperatively from the Department of Urology, Churchill Hospital, Oxford, United Kingdom. All patients gave signed, informed consent for their tissues to be used for scientific research. Ethical approval for the study was obtained from the Clinical Ethics Committee, Oxford Radcliffe Hospitals NHS Trust, Oxford, United Kingdom.

Tumor samples were obtained following either transurethral resection (TURBT) or radical cystectomy. Bladder cancers were staged using the tumor-node-metastasis classification (22). Tumor samples were divided into three groups depending on their stage: T_a tumors, T_1 superficial tumors (T_a and T_1 tumors were collectively designated as superficial tumors), and T_{2-4} invasive tumors. For the quantitative PCR (qPCR) analysis, a cohort of 10 normal bladder samples and 60 bladder tumors were studied. Areas of normal bladder and tumor were identified at cytoscopy, excised, and snap frozen in liquid nitrogen. For the *in situ* hybridization/immunohistochemistry vessel counting study, the series comprised six T_a (TURBT), six T_1 (TURBT), six T_{2-4} (TURBT), and four T_{2-4} (cystectomy) samples. Truly normal full thickness bladder sections were difficult to source, so microscopically normal areas within radical cystectomy specimens (four samples) and normal ureters (five samples) obtained following organ donation were used to represent "normal tissue."

RNA extraction and reverse transcription. Total RNA from frozen tissue samples was extracted after morselizing in liquid nitrogen and homogenizing in TRI-Reagent (Sigma-Aldrich, St. Louis, MO). The quality of RNA extracted was assessed using RNA 6000 Nano Chips and the Agilent 2100 Bio-analyzer (Agilent Technologies, Paulo Alto, CA). cDNA was synthesized by reverse transcribing total RNA using the High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA).

Real-time qPCR. Real-time qPCR and analysis was done using the method previously described by Patel et al. (21). Human *β-actin* was used as a reference gene to normalize for differences in the amount of total RNA in each sample. Primer/probe kits were purchased as Assays-on-Demand from Applied Biosystems: *DLL4*, *VEGF*, and *CD34*. Relative quantitation of gene expression was done using the method described by Pfaffl (23). The normal bladder sample with the median expression of β-actin was designated the comparator.

In situ *hybridization.* Radioactive *in situ* hybridization was done on paraffin-embedded tissue sections by the *in situ* hybridization service, Cancer Research UK London Research Institute, following a method described by Poulsom et al. (24). The probe used to detect *DLL4* was a 741 bp fragment located from position 1,775 to 2,516 bp (16). After 14 days, sections were visualized using a Nikon Eclipse ME600 dark field microscope, an Optronics digital camera, and Magnafire Version 2.0.

Immunohistochemistry. Paraffin-embedded tissue blocks were serially sectioned 4 μm in thickness, dewaxed, and rehydrated in serial alcohol washes. Endogenous peroxidase activity was blocked with 0.03% hydrogen peroxide (20 minutes). Double immunostaining for CD34 and α-smooth muscle actin (α-SMA) was done by sequentially adding CD34 primary antibody 1:200 for 30 minutes (Novocastra, Newcastle-upon-Tyne, United Kingdom, QBEnd/10), DAKO (Carpinteria, CA) Checkmate EnVision Detection kit (peroxidase/3,3′-diaminobenzidine-rabbit/mouse, K5007) for 30 minutes, 3,3′-diaminobenzidine substrate for 2 minutes, α-SMA primary antibody 1:1,000 for 30 minutes (DAKO-1A4), Envision AP Polymer 30 minutes, and Vector Blue substrate for 6 minutes. Sections were mounted using Aquamount.

Analysis of DLL4, CD34, and α-SMA expression in bladder cancer. *DLL4* (*in situ* hybridization) and CD34/α-SMA (immunohistochemistry) expression in serial sections was quantified. First, the distribution of *DLL4* expression was assessed by two observers (N.P. and M.D.). Twenty random fields within the *DLL4 in situ* sections were scanned using a light microscope at a magnification of ×100 and assessed for the presence of any *DLL4*-positive vessels. Second, tissues were further analyzed by identifying the two to three areas of highest *DLL4* expression. These areas were photographed at ×100 magnification using a Nikon Eclipse ME600 dark field microscope, an Optronics digital camera, and Magnafire version 2.0. The equivalent areas were identified upon the serial sections and captured accordingly. The digital images were then used to quantify the vasculature for CD34, α-SMA, and *DLL4*.

Statistics. Data are presented as mean ± SE. Statistical analysis was done using GraphPad Prism. Tests used included *t* test, one-way ANOVA, Mann-Whitney and χ^2 tests. Statistical significance was concluded when $P < 0.05$ and is denoted in the figures with an asterisk.

Table 1. Patient demographics for the qPCR bladder cancer samples

	Normals	T_a	T_1	T_2	T_{3-4}	Superficials	Invasives	Tumors
Number	10	11	32	11	6	43	17	60
Mean age (y)	76.4	71.0	71.2	69.5	68.3	71.2	69.1	70.57
Age range (y)	68-87	62-82	42-89	56-83	46-91	42-89	46-91	42-91
Sex								
M	10	9	25	10	4	34	14	48
F	0	2	7	1	2	9	3	12
Operation	Cystoscopy and biopsy	TURBT	TURBT	TURBT	T_3 (5) = TURBT; T_4 (1) = CYST	TURBT	TURBT = 16; CYST = 1	TURBT = 59; CYST = 1
Adjuvant intravesical therapy		Nil = 4; MMC = 2; BCG = 2; both = 1; N/A = 2	Nil = 21; MMC = 2; BCG = 6; both = 1; N/A = 2			Nil = 25; MMC = 4; BCG = 8; both = 2; N/A = 4		
Invasive cancer treatment				DXT = 7; CYST = 2; N/A = 1	DXT = 1; CYST = 2; Nil = 3		DXT = 8; CYST = 4; Nil = 1; N/A = 1	

Abbreviations: CYST, cystectomy; DXT, radiotherapy; BCG, bacille Calmette Guérin; MMC, mitomycin C; Nil, no further treatment; N/A, not applicable.

Results

Quantitative analysis of DLL4 expression in bladder cancer using qPCR. Quantitative PCR was used to study gene expression in a cohort of bladder tumors and normal bladder samples (Table 1). Relative gene expression was assessed using the method of Pfaffl (23), a modified method of comparative quantitation. In this technique, the expression of *DLL4* was calculated relative to the reference gene *β-actin*. The expression of *β-actin* was equivalent between the normals and tumors (*β-actin* expression normals: median C_t = 23.0, range 22.1-23.8, n = 10; *β-actin* expression tumors: median C_t = 22.8, range 22.0-23.8, n = 60, P = 0.3).

The relative expression of *DLL4* was significantly elevated in bladder tumors compared with normal bladders (mean *DLL4* expression in tumors = 4.5 ± 0.4, n = 60; mean expression of *DLL4* in normal samples = 2.4 ± 0.2, n = 10, P = 0.01). In comparison to normal tissue, *DLL4* expression was noted to be highest in superficial tumors (mean expression = 4.7 ± 0.6, n = 43, P < 0.05) followed by invasive tumors (mean expression = 3.9 ± 0.6, n = 17, P > 0.05; Fig. 1A).

Quantitative PCR was done for the endothelial marker *CD34* to determine if the differences in *DLL4* expression were simply due to differences in vascular density between normal and tumor samples. The relative expression of *CD34* was highest in the normal samples (mean expression = 1.2 ± 0.2, n = 10) when compared with superficial (mean expression 0.6 ± 0.1, n = 43) and invasive tumors (mean expression = 0.5 ± 0.1, n = 17; Fig. 1B).

A ratio of *DLL4* expression to *CD34* expression (*DLL4*/*CD34* ratio) was calculated by dividing the relative expression of *DLL4* by that of *CD34*. The mean *DLL4*/*CD34* ratio for normal samples was 3.2 ± 1.2, for superficial tumors was 10.6 ± 1.0 (P < 0.001), and for invasive tumors was 9.1 ± 4.0 (P < 0.001; Fig. 1C). The expression of *DLL4* correlated significantly with the expression of *CD34* in tumors (Spearman rank correlation coefficients: all tumors = 0.6, n = 60, P < 0.001; superficial tumors = 0.6, n = 47, P < 0.001; invasive tumors = 0.6, n = 13, P < 0.05), but not in normal bladder tissue controls (normal samples = 0.2, P > 0.05; Fig. 1D).

The expression of *VEGF* was also assessed using qPCR. The mean expression of *VEGF* in both superficial and invasive tumors was significantly higher than in normal bladder tissue

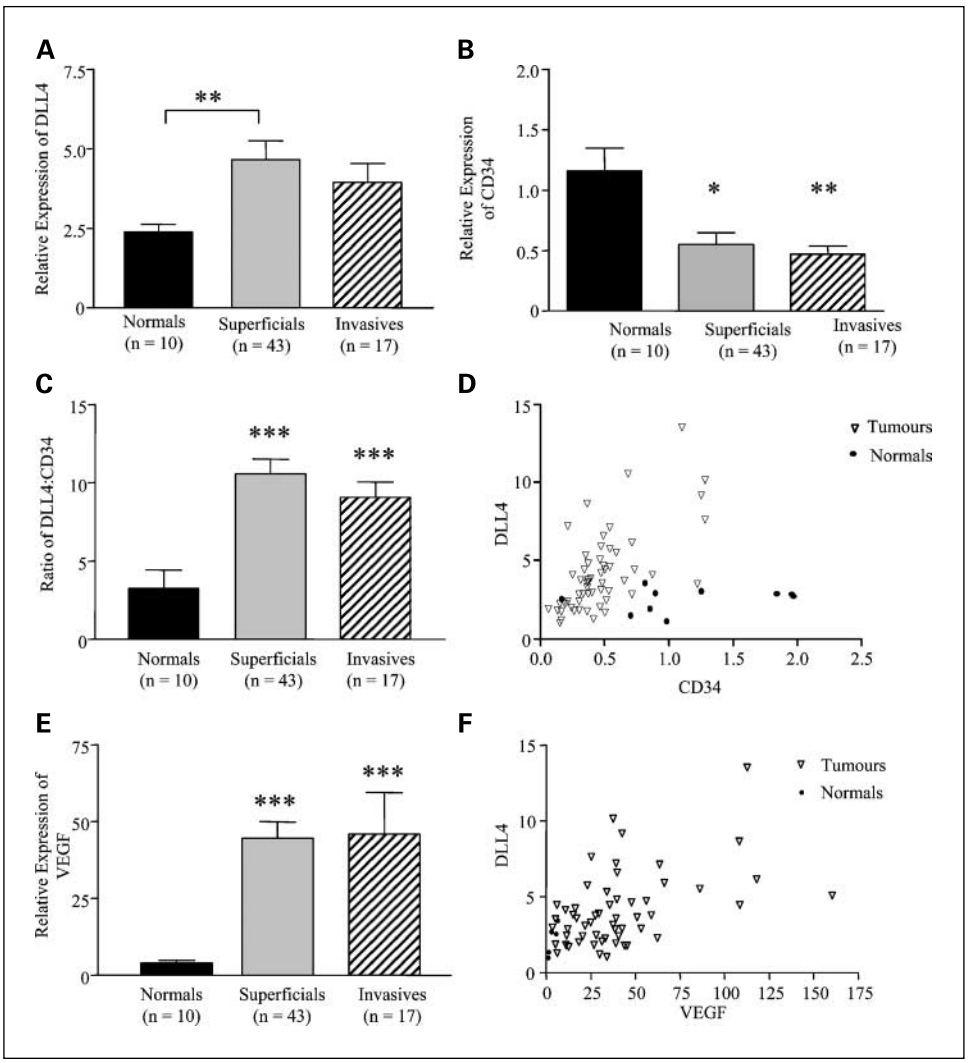

Fig. 1. Analysis of DLL4, CD34, and VEGF expression using qPCR. *A,* DLL4 expression was significantly up-regulated in superficial bladder cancer compared with normal bladder tissue. *B,* the expression of CD34 was significantly higher in the normal tissues than in the bladder tumors. *C,* the DLL4/CD34 ratio was significantly higher in superficial and invasive bladder cancer compared with normal tissue. *D,* DLL4 expression was significantly correlated with CD34 expression in bladder tumors (Spearman rank correlation coefficient = 0.6, P < 0.001, n = 60). *E,* VEGF expression was significantly elevated in both superficial and invasive bladder cancer. *F,* the expression of DLL4 significantly correlated with VEGF expression (Spearman rank correlation coefficient = 0.4, P = 0.001, n = 60. *, P < 0.05; **, P < 0.01; ***, P < 0.001). Bars, SE.

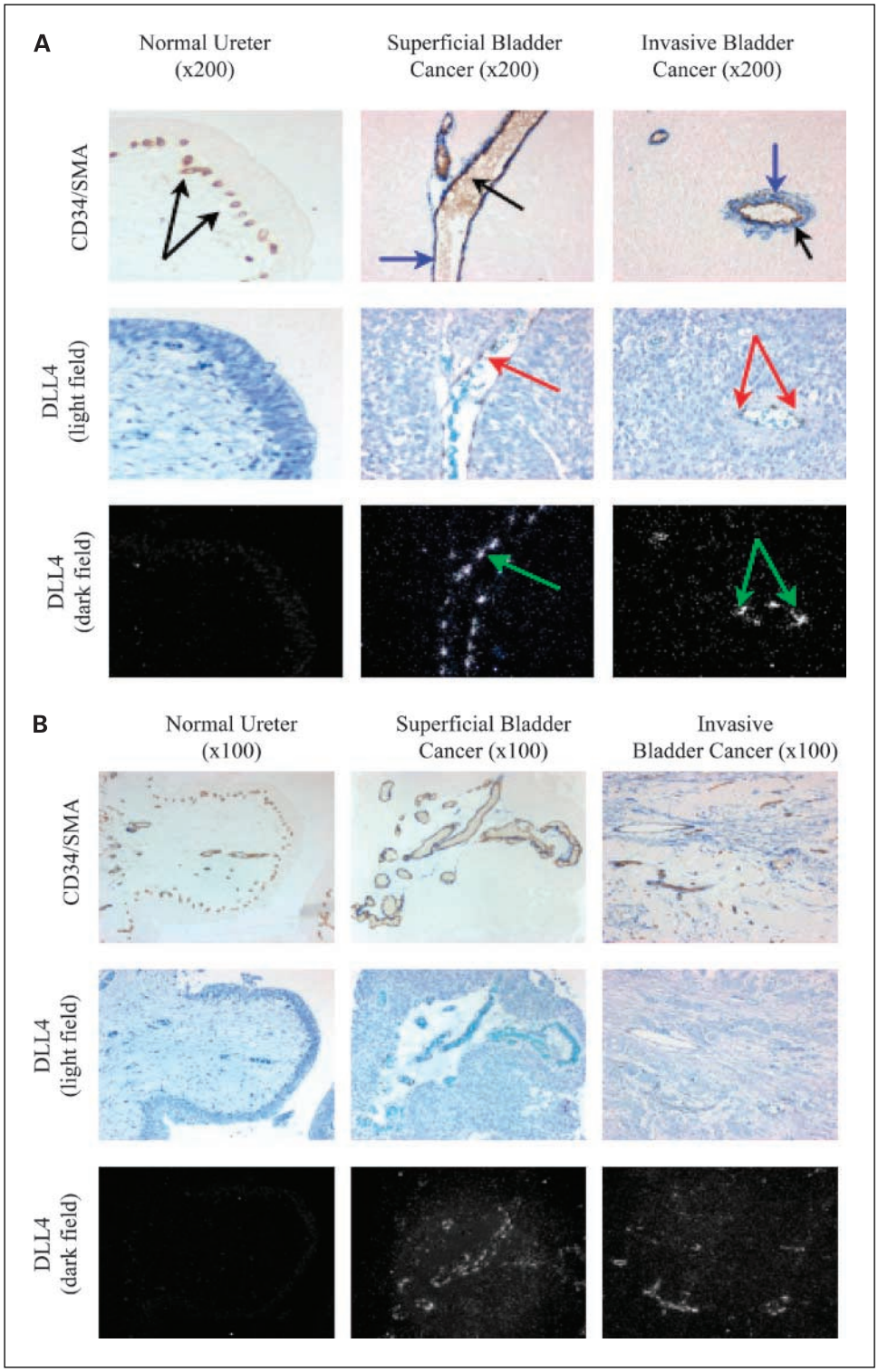

Fig. 2. DLL4, CD34, and α-SMA expression in bladder cancer. *A, in situ* hybridization was used to study the pattern of DLL4 expression in bladder cancer. Serial sections were also stained for CD34 and α-SMA using conventional double immunohistochemistry. Vessels in the suburothelial layer of normal ureter were predominantly microvessels staining only for CD34 (*black arrows*). Vessels within superficial and invasive bladder tumors stained positive for CD34 (*black arrow*) and for α-SMA (*blue arrows*). DLL4 expression was confined to the vascular endothelium. Endothelial expression of DLL4 is shown in the light (*red arrows*) and dark field (*green arrows*) *in situ* hybridization. Quantitation studies of the expression of DLL4, CD34, and α-SMA were done at ×100 magnification. *B,* representative fields of view. DLL4 light fields, black autoradiographic silver grains; DLL4 dark field, white reflections; CD34, brown staining; α-SMA, blue staining.

(44.6 ± 5.3 and 45.9 ± 13.6 versus 4.0 ± 1.0; $P < 0.001$; Fig. 1E). The Spearman rank correlation coefficient of *VEGF* and *DLL4* expression was significant only for superficial tumors (Spearman rank correlation coefficients all tumors = 0.4, $P = 0.001$, $n = 60$; superficial tumors = 0.4, $P < 0.01$, $n = 43$; invasive tumors = 0.3, $P = 0.3$, $n = 17$; normals = 0.4, $P > 0.3$, $n = 10$; Fig. 1F).

***DLL4* mRNA expression pattern.** *In situ* hybridization was used to assess the pattern of *DLL4* mRNA expression in a series of 22 bladder cancers, 4 normal bladders, and 5 normal ureters. The expression of *DLL4* was compared with that of the pan-endothelial marker *CD34*. Like *CD34*, *DLL4* expression was confined to the vascular endothelium, unlike *CD34*; however, *DLL4* expression was only noted within a subset of vessels (Fig. 2A).

Twenty random fields within each of the 9 normal (180 fields), 12 superficial bladder tumor (240 fields), and 10 invasive bladder tumor (200 fields) sections were scored for the presence or absence of *DLL4*-positive vessels (Fig. 2B). The presence of a single *DLL4*-positive vessel was sufficient for a field to be regarded as *DLL4* positive (maximum score: 20 of 20 fields, minimum score: 0 of 20). Compared with normal tissue (positive random fields 1.4 ± 0.7), *DLL4*-positive vessels were most abundant in superficial tumors (positive random fields 14.3 ±1.2, $P < 0.001$) followed by invasive tumors (positive random fields 8.0 ±1.4, $P < 0.05$; Fig. 3A). The differences are likely to be greater as eight of the nine normal cases had less than two *DLL4*-positive random fields, with the remaining normal case having seven *DLL4*-positive fields. The number of positive cases and their distributions for each tissue group are shown in Fig. 3B.

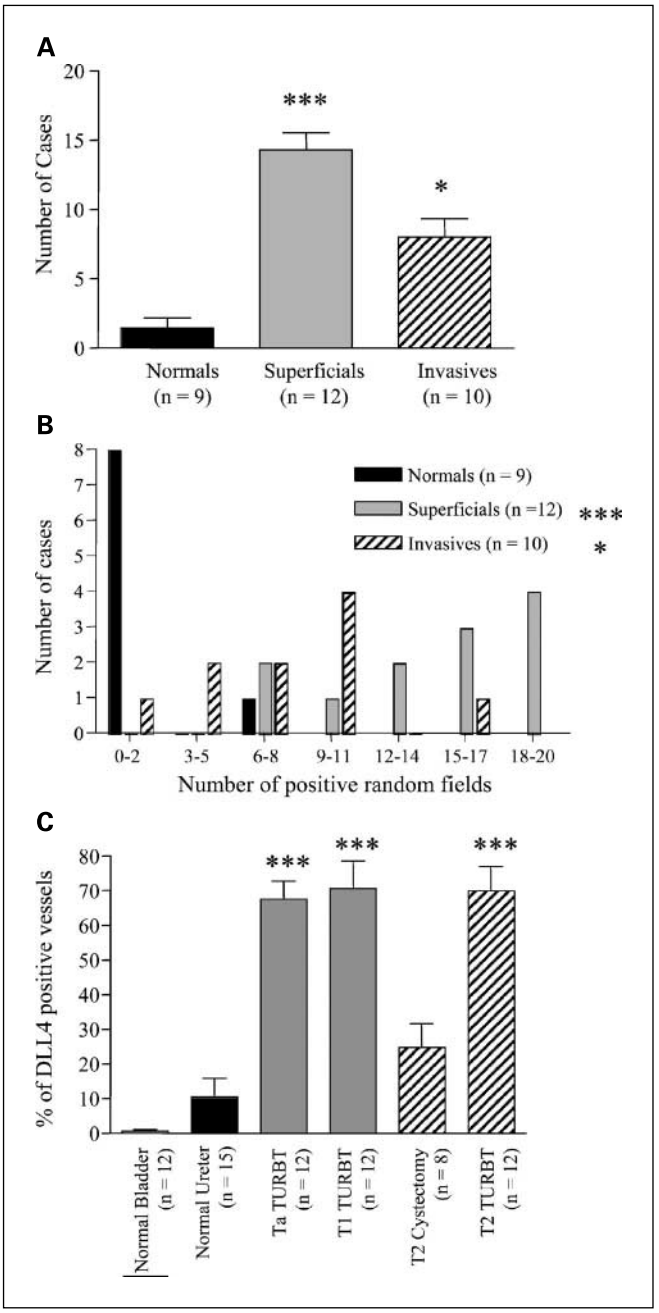

Fig. 3. Quantitation of DLL4 *in situ* hybridization studies. The expression of DLL4 was studied in 20 random fields per case (10 normal, 12 superficial bladder tumors, and 10 invasive bladder tumors) using *in situ* hybridization. *A,* a significantly higher number of random fields had DLL4-positive vessels in superficial ($P < 0.001$) and invasive tumors ($P < 0.05$) compared with normal tissue. *B,* the expression of DLL4 was uncommon within the normal vasculature. Eight of nine normal cases had less than two DLL4-positive random fields; this compares to 0 of 12 superficial tumors and 1 of 10 invasive tumors. *C,* the percentage of DLL4-positive vessels within DLL4 hotspot areas was significantly higher in superficial tumors and invasive tumors compared with both normal bladder and ureteric tissue. Expression of DLL4 was noted to be higher in invasive TURBT specimens than in invasive cystectomy specimens. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Bars, SE.

The expression of *DLL4* was further studied by identifying *DLL4* "hotspots." For each tumor, the two areas with the highest concentration of *DLL4*-positive vessels were identified and photographed using a light and dark field microscope. In the case of normal tissue, the three most positive *DLL4* areas were photographed; in the absence of *DLL4* expression, representative photographs were taken to include both sub-urothelial microvessels and mature stromal vessels.

The proportion of vessels positive for *DLL4* within the hotspots was higher in superficial and invasive tumor samples compared with both normal bladder tissue and normal ureter (mean percentage of positive hotspot vessels: normal bladder = 0.6 ± 0.4, $n = 12$; normal ureter = 10.5 ± 5.3, $n = 15$; T_a TURBT = 67.5 ± 5.2, $n = 12$, $P < 0.001$, compared with normal ureter and bladder; T_1 TURBT = 70.6 ± 8.0, $n = 12$, $P < 0.001$, compared with normal ureter and bladder; $T_{2\text{-}4}$ cystectomy = 24.7 ± 6.7, $n = 8$, compared with normal ureter and bladder; $T_{2\text{-}4}$ TURBT = 70.0 ± 6.8, $n = 12$, $P < 0.001$, compared with normal ureter and bladder; Fig. 3C). Within the invasive tumor group, *DLL4* expression was noted to be higher in the TURBT specimens than in the cystectomy specimens although this was not statistically significant.

Coexpression of DLL4 and α-SMA. Serial sections of each case were also stained for CD34 and α-SMA using conventional double immunohistochemistry (Fig. 2B). The *DLL4* hotspot areas were identified within these serial sections and corresponding matched photographs were taken.

DLL4 hotspots were studied within normal (27 fields, 9 cases) and tumor tissue (44 fields, 22 cases). Similar numbers of vessels were present within the different tissue types (normal bladder = 41.7 ± 8.9, normal ureter 31.5 ± 7.2, T_a TURBT = 52.3 ± 8.0, T_1 TURBT = 47.2 ± 9.5, $T_{2\text{-}4}$ cystectomy = 41.8 ± 6.1, $T_{2\text{-}4}$ TURBT = 37.6 ± 4.9).

In the course of the hotspot study, a total of 3,008 vessels were identified as staining positive for CD34. Of these vessels, 2,025 were within tumor sections (22 cases) and 983 in normal tissue sections (9 cases). Taken as a whole, 2,403 (79.9%) hotspot vessels showed periendothelial expression of α-SMA, whereas 1,226 (40.8%) hotspot vessels expressed *DLL4* and 589 (19.6%) vessels expressed neither ($n = 31$ cases). Further evaluation showed that 1,210 of 1,226 (98.7%) of *DLL4* positive vessels were also positive for α-SMA; in comparison, only 1,193 of 1,782 (66.9%) of *DLL4*-negative vessels coexpressed α-SMA ($n = 31$ cases; $P < 0.001$, χ^2 test; Fig. 4A).

These data were then analyzed after subclassifying into normal and tumor vasculature (9 normal, 22 tumor cases). Within the normal tissue, 687 of 983 (69.9%) vessels were positive for α-SMA. Of the normal hotspot vessels, 26 of 983 (2.6%) were *DLL4* positive, 100% of which coexpressed α-SMA. Nine hundred fifty-seven of 983 vessels were *DLL4*

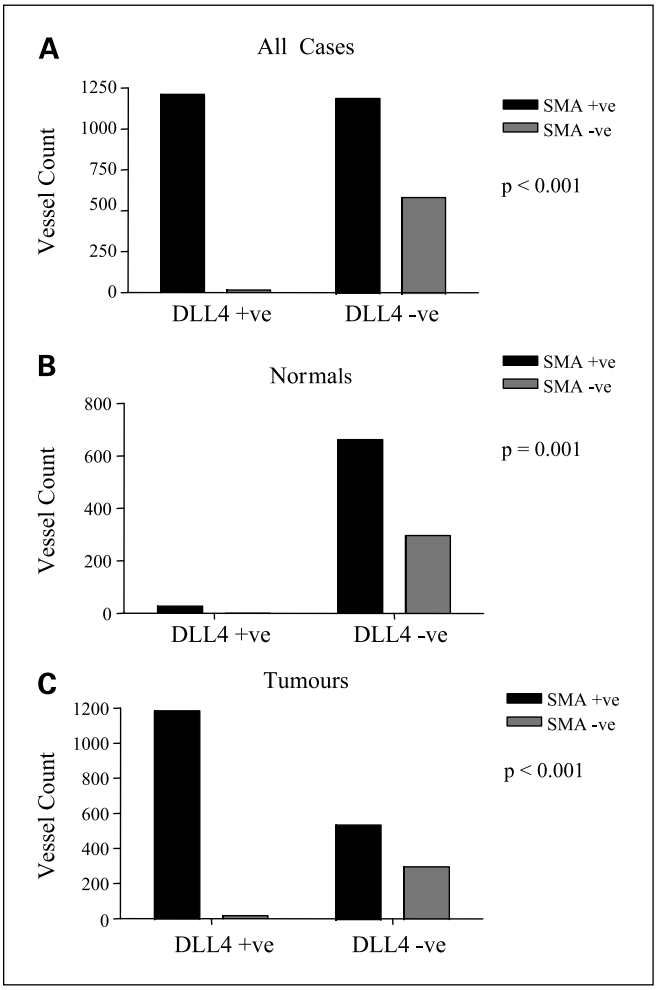

Fig. 4. DLL4 and vessel maturation. DLL4 and α-SMA expression was analyzed by creating χ^2 contingency tables. DLL4 expression was significantly associated with α-SMA expression ($P < 0.001$).

negative of which 661 (69.1%) also expressed perivascular α-SMA ($P = 0.001$, χ^2 test; Fig. 4B).

Analysis of the tumor vasculature showed 1,716 of 2,025 (84.7%) vessels were α-SMA positive and 1,200 of 2,025 (59.3%) vessels were *DLL4* positive. Of the 1,200 DLL4-positive vessels, 1,184 (98.7%) also expressed α-SMA, whereas only 532 of 825 (64.5%) *DLL4*-negative tumor vessels stained positive for α-SMA ($P < 0.001$, χ^2 test; Fig. 4C). These results indicate that *DLL4* is significantly up-regulated and colocalized together with α-SMA in the tumor vasculature.

Relation of DLL4 expression to outcome. Follow-up was available for 39 of the 43 superficial tumors (91%) and 16 of the 17 invasive tumors (94%). The mean duration of follow-up for the superficial cases was 12 months (range 1-36 months, median = 6) and for the invasive cases was 33 months (range 5-84 months, median = 23.5).

In the case of superficial cancers, the disease-free period was measured by determining the time to first recurrence (Fig. 5A). Kaplan-Meier curves were used to show that the median time to recurrence for patients in whom the expression of *DLL4* was above the median was 6 months, whereas that for patients with low levels of *DLL4* was 18 months, but this was not statistically significant.

Superficial bladder cancer is often treated with adjuvant intravesical chemotharpy. The tumor samples used in this study were collected when the administration of mitomycin C immediately after TURBT was not routine practice. Of the 39 patients with superficial tumors for whom follow-up was available, 14 subsequently received adjuvant courses of intravesical therapy; 4 received mitomycin C, 8 bacille Calmette Guérin, and 2 both. Of these 14 patients, 10 were noted to have high levels of *DLL4* expression.

Kaplan-Meier curves were used to assess the time to cancer-specific death in the invasive cancers (Fig. 5B). Of the 16 patients with available follow-up, 8 received radical radiotherapy (high *DLL4* = 4, low *DLL4* = 4), 4 underwent radical cystectomies (high *DLL4* = 3, low *DLL4* = 1), and 4 were treated palliatively (high *DLL4* =1, low *DLL4* = 3). Patients with high *DLL4* levels had a median survival of 7.5 months compared with 44.5 months for those with low *DLL4* levels (hazard ratio = 2.40). Because of the small numbers in this study, no statistical significance was shown upon comparison of the curves.

Discussion

The aim of this study was to evaluate *DLL4* as a potential novel antiangiogenic target in bladder cancer. Using quantitative PCR, we showed that *DLL4* expression is up-regulated in transitional cell carcinoma of the bladder. Additionally, quantitation of *CD34* expression was done using qPCR as a means of assessing sample vascular density. Interestingly, the expression of *CD34* was higher in the normal samples than in

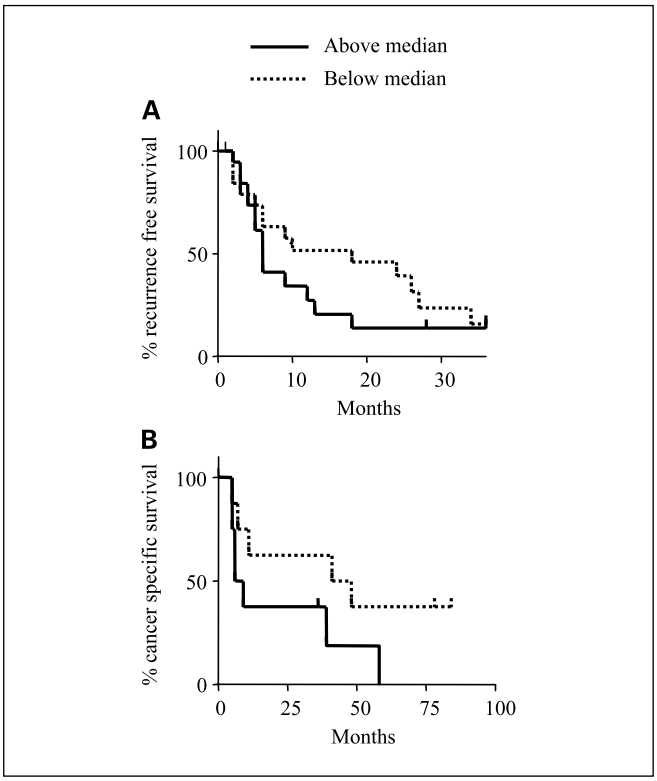

Fig. 5. DLL4 as a prognostic markers. High DLL4 expression was associated with an earlier median time to recurrence in superficial bladder cancer (*A*; $P > 0.05$) and earlier median time to cancer-specific death in invasive cancer (*B*; $P > 0.05$).

the tumor samples. These normal bladder samples were obtained as cold cup biopsies at cytoscopy, and so contained a high number of suburothelial microvessels. The presence of these vessels within the normal tissues is the most likely explanation for the high *CD34* expression levels seen in the normal samples.

In keeping with previous work (25), we showed that *VEGF* mRNA expression was up-regulated in bladder cancer. In our study, the expression of *DLL4* also significantly correlated with the expression of *CD34* and *VEGF* in bladder cancers. These results matched our previous findings in clear-cell renal cell carcinoma that *DLL4* expression correlated with *VEGF* expression (21) and also supported *in vitro* studies that have shown the regulation of *DLL4* in endothelial cells by *VEGF* (21, 26).

In situ hybridization was used for the first time to show that *DLL4* expression is up-regulated in bladder cancer and has an endothelial cell–specific pattern of expression. Quantitation of *DLL4* expression was done by assessing expression within both random fields and *DLL4* hotspots. *DLL4* expression within random fields was more widespread within bladder cancer samples than normal tissues. Further subclassification of the tumors showed that *DLL4* expression was most widespread within the superficial cases. O'Brien et al. (27) reported that tumor vessel formation in superficial and invasive bladder cancers was driven by differing angiogenic pathways. Angiogenesis in papillary-type superficial bladder tumors is associated more strongly with VEGF and results in characteristic mature, well-defined, functional fibrovascular cores that do not penetrate the underlying muscle (28). In invasive cancers, new vessel formation can also arise as a result of interactions between tumor cells and native vessels within the muscularis layer. The production of metalloproteases disrupts the extracellular matrix and liberates growth factors like basic fibroblast growth factor (29). Vessels formed under the influence of these varying angiogenic pathways may show differences in their characteristics.

It is interesting to note that *DLL4* expression was highest in the TURBT samples whether superficial or invasive. The majority of tumor tissue obtained at TURBT is from exophytic tumors. The vessels within these areas of tumor are neoangiogenic tumor vessels, and most likely arise from the suburothelial microvessels. The vasculature within the cystectomy specimens, however, is composed of a combination of neoangiogenic vessels and preexisting bladder vessels. The difference in *DLL4* expression between the cystectomy and TURBT specimens suggests that *DLL4* expression is enhanced in neoangiogenic vessels, of which there may be more in the TURBT samples.

Angiogenic vessels mature upon the recruitment of pericytes (30). To understand if *DLL4* played a role in vessel maturation in human cancer, serial sections were immunostained for *CD34* and α-SMA, and an assessment was made of vessel maturation within the *DLL4* hotspot areas. This is the first time that vessel maturity has been assessed in bladder cancer. α-SMA expression was found on nearly 70% of normal vessels and 85% of tumor vessels studied. The vessels that lacked α-SMA expression were most often microvascular in nature.

Vessel maturation has previously been studied in a number of different tumor types. Eberhard et al. (31) showed that the degree of vessel maturation was highly variable between different tumors, in breast cancer nearly 70% of vessels were α-SMA positive, whereas only 10% to 20% of glioblastoma and renal cancer vessels were α-SMA positive. More recently, confocal microscopy was used by Morikawa et al. (32) to show that >97% of pancreatic tumor vessels expressed α-SMA compared with 22% of normal pancreatic vessels. They also found that α-SMA expression was absent from a significant proportion of capillaries and microvessels within the normal vasculature; these vessels did, however, stain for desmin, another marker of periendothelial cells.

A highly significant association was noted between *DLL4* expression and α-SMA expression in both normal and tumor vessels. The expression of α-SMA in normal specimens was lower in *DLL4*-negative vessels (normal 69%, tumor 65%) compared with *DLL4*-positive vessels (normal 100%, tumor 99%). This suggests that *DLL4* may play a role in regulating vessel maturation by interacting with Notch receptors upon pericytes.

The mechanism by which endothelial expressed DLL4 regulates vessel maturation needs further investigation. DLL4 has been shown to signal through both Notch1 and Notch4 between endothelial cells (33–35), but the effect of endothelial *DLL4* on pericytes is not reported. However, evidence that Notch plays a role in vessel maturation comes from a number of sources. Vessels within mice deficient for *DLL4* have an absence of smooth muscle/pericyte coverings (15). This phenotype is similar to that of platelet-derived growth factor-B or platelet-derived growth factor receptor-B–deficient mice (36, 37).

The human syndrome CADASIL is a vascular disease characterized by mutations of the Notch3 receptor leading to abnormalities of vascular smooth muscle (4). A recent *in vitro* study by Sweeney et al. (38) showed that vascular smooth muscle cells express Notch 1, Notch 3, HES1, HES5, HEY1, HEY2, and HEYL. They showed that inhibition of RBP-Jκ activity led to decreased proliferation, increased apoptosis, and increased migration in vascular smooth muscle cells. Clearly, our clinical study provides justification for more extensive *in vitro* work.

Tumor vessels typically show disorganized vascular patterning, which in conjunction with their abnormal ultrastructures results in chaotic blood flow, high interstitial pressures, and increased vessel leakiness (30). Tumor vasculature consists of a mix of immature vessels devoid of pericyte coverings and more mature vessels with pericyte/smooth muscle cell coats. Endothelial cell proliferation and sprouting have been to shown to be most prevalent within the immature vessels, suggesting that it is these vessels that are angiogenic (32, 39, 40). These vessels are also the ones most susceptible to antiangiogenic therapies. Benjamin et al. (41) studied vessel maturation in prostate cancer. They showed that androgen blockade decreased production of VEGF, which, in turn, led to the regression only of immature vessels lacking pericyte coverings in prostate cancer. They showed that almost 40% of tumor vessels were positive for α-SMA; however, after androgen blockade, the proportion of vessels coated with α-SMA rose to 79%.

Gee et al. (39) also found that vessels covered with α-SMA were protected from the antiangiogenic therapy, which in their case was interleukin-12. These findings have important implications for the development of future antiangiogenic therapies. Tumors that have a high proportion mature vessels

are less likely to respond to for example anti-VEGF therapies, as the pericytes seem to offer protection against growth factor deprivation. We have recently shown in human endothelial cells that overexpression of DLL4 inhibits proliferation by down-regulating VEGF receptor 2 and NRP1 expression (42), providing an additional mechanism for resistance to such therapies. The association shown between DLL4 expression and α-SMA expression suggests that targeting DLL4 in addition to VEGF may, by targeting both mature and immature vessels, prove to be of greater therapeutic benefit and we are currently investigating this in xenograft models.

The biological function of DLL4 is not confined to vessel maturation. Knockout studies have shown that haploinsufficiency of DLL4 results in embryonic lethality from severe vascular defects (15, 17, 18). The demonstration of vascular defects in heterozygous mice is uncommon and highlights the importance of DLL4 in vascular development. Similar phenotypes have only been described for VEGF knockout mice (19, 20). We have recently shown that down-regulating DLL4 expression *in vitro* using RNA interference significantly inhibits endothelial cell proliferation, migration, and network formation (21). Similarly, we have shown up-regulation of Delta 4 expression disrupts endothelial function (42), a phenotype previously found for Notch4, where *in vivo* up-regulation or down-regulation disrupts angiogenesis (43, 44). This implies that a narrow range of notch signaling is needed for optimum angiogenesis. Thus, targeting DLL4 may inhibit tumor angiogenesis by blocking multiple endothelial cell functions.

Endothelial expression of DLL4 may represent an intermediate step toward final differentiation by promoting maturation and functionality; thus, it may be an important target for tumors resistant to anti-VEGF therapy.

Exploratory analysis of the value of *DLL4* as a prognostic marker was assessed by relating *DLL4* expression with clinical follow-up data. In superficial and invasive disease, high levels of *DLL4* conferred an increased risk of tumor recurrence or cancer-specific death, respectively. We additionally noted that 10 of the 14 patients that received adjuvant intravesical therapy had high levels of *DLL4* expression. Adjuvant mitomycin C or bacille Calmette Guérin is commonly used in superficial bladder cancers thought to be at high risk of recurrence and progression. The risk stratification of superficial bladder tumors is based on the number of tumors at first presentation, number and frequency of recurrences, and the tumor histology. In this study, we observed for the first time that conventionally classified high-risk tumors seemed to express high levels of *DLL4*. The intensive nature of the microscopic studies meant that we could not examine a larger cohort; however, we are currently trying to develop antibodies to investigate this in much larger numbers.

Here, for the first time, we have identified *DLL4* as a novel angiogenic target in bladder cancer. We have shown that *DLL4* (*a*) is preferentially expressed in tumor vasculature, (*b*) is associated with vessel maturation, and (*c*) may have prognostic value in superficial and invasive cancer. Taken with our recent findings that an optimal level of *DLL4* is necessary for endothelial cell function (21, 42), targeting *DLL4* either by inhibition or overexpression may be an approach worthy of further investigation in the management of superficial and invasive bladder cancer.

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