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ORIGINAL ARTICLE

Stem cell marker-positive stellate cells and mast cells are reduced in benign-appearing bladder tissue in patients with urothelial carcinoma

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Abstract Survival after invasive bladder cancer has improved less than that of other common non-skin cancers. In many types of malignancy, treatment failure has been attributed to therapy-resistant stem-like cancer cells. Our aim was therefore to determine identities of stem cell marker-positive cells in bladder cancer tissue and to investigate possible associations between these cells and different forms of bladder neoplasia. We investigated tissue from 52 patients with bladder neoplasia and 18 patients with benign bladder conditions, from a cohort that had been previously described with regard to diagnosis and outcome. The samples were analysed immunohistologically for the stem cell markers aldehyde dehydrogenase 1 A1 (ALDH1) and CD44, and markers of cell differentiation. The majority of stem cell marker-positive cells were located in connective tissue, and a smaller fraction in epithelial tissue. Stem cell marker-positive cells exhibiting possible stem cell characteristics included cells in deeper locations of benign and malignant epithelium, and sub-endothelial cells in patients with or without neoplasia. Stem cell marker-positive cells with non-stem cell character included stellate cells, mast cells, endothelial cells, foamy histiocytes, and neurons. Significantly, ALDH1+ stellate cells and ALDH1+ mast cells were reduced in number in stroma of benign-appearing mucosa of bladder cancer patients. The stem cell markers ALDH1 and CD44 label several types of differentiated cells in bladder tissue. ALDH1+ stellate cells and mast cells appear to be reduced in stroma of normal-appearing mucosa of bladder cancer patients, and may be part of a "field effect" in cancer-near areas.

Keywords Bladder cancer · Tumour microenvironment · Stem cells · Stellate cells · Mast cells · Aldehyde dehydrogenase · CD44

Introduction

Among the five most common forms of non-skin malignant disease, bladder cancer is the one for which least improvement has been achieved with regard to patient

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outcome during the last three decades, at the same time as it is the most expensive cancer to manage on patient basis [1–4]. The most common histological type of bladder cancer is urothelial [5]. Its flat, high-grade, pre-invasive form (carcinoma in situ, CIS) occurs concomitantly with visible tumours in up to 50 % of bladder cancer patients [6–10]. In fact, the majority of invasive bladder cancer foci originate in CIS, and up to 45 % of patients with CIS progress to invasive disease and die within 10 years [8–19]. The patient material in this study was selected from all bladder specimens collected during 24 consecutive months of care at the urology services in Telemark County, Norway (pop. ~170,000), hence population-based [10].

Adult somatic stem cells (SCs) are characterised by their ability to renew tissues and generate different types of terminally differentiated cells [20, 21]. Cancer-associated SCs (CSCs) (alternative and perhaps more appropriate term: stem-like cancer cells), that were first documented in malignancies of haematopoietic tissue, colon, and breast [22–24], are interesting for the fact that in breast they are resistant to chemotherapy and cause late recurrences [21]. The combined immunophenotype $CD44^{+}$ $CD24^{-/low}$ $Lineage^{-/low}$ was the first cellular protein SC marker (SCM) identified for epithelial cells, and aldehyde dehydrogenase 1 A1 (ALDH1) was the first single SCM (thus especially practical for immunohistochemistry (IHC) purposes) [24, 25]. ALDH1 labels a subpopulation of CD44+ cells in epithelial organs, including breast, pancreas, colon, prostate, and urinary bladder, and the combined ALDH1+ CD44+ immunophenotype appears to be particularly potent for identifying stem cells [24, 26–29]. With regard to the comparative efficiency of these markers, the outcome of chemotherapy is better predicted by the tumoural presence of ALDH1+ cells than CD44+ CD24− cells [30]. Considering the urinary bladder, in situ evidence of SCs or CSCs has been lacking in benign tissue, in CIS, and in the stroma of invasive cancer [29]. Most experimental studies have required a population of approximately 100–1,000 SCM+ cells to consistently elicit SC functions, which indicates that the majority of SCM+ cells are indeed not SCs. Accordingly, the present study aimed to establish possible differentiated identities of SCM-positive cells.

ALDH1 is not only a SCM but it is also vital for vitamin A (retinoid) metabolism. Retinoids are essential for cell differentiation and for detoxification of endogenous and exogenous substances [31]. In the intestine, vitamin A is taken up as retinyl esters that are subsequently stored in hepatic stellate cells. The early vitamin A metabolite retinol is formed in hepatocytes and then delivered to retinol-binding proteins in the circulation. Retinol is taken up by extra-hepatic stellate cells, in which it is bound by cellular retinol-binding protein 1 (CRBP1). The aldehyde dehydrogenase 1 family of enzymes transform retinol into the metabolically most active form of retinoid, retinoic acid. Within cells, retinoic acid is bound to cellular retinoic acid-binding protein type 1 (CRABP1), which is expressed in most types of human cells [32]. ALDH1 eliminates aldehydes, which are cytotoxic, and it also aids the removal of chemotherapeutic agents, the latter capacity representing one of the mechanisms that render ALDH1+ SCs resistant to chemotherapy [30]. Thus, the roles of aldehyde dehydrogenases as SCMs and as essential enzymes in retinoid metabolism and chemotherapy resistance may be interrelated through mechanisms of cell differentiation and cell preservation. Therefore, we conducted the current study to examine the concomitant presence of the major retinoid-binding proteins and ALDH1 in cells in the lamina propria of urinary bladder with and without neoplasia.

In light of recent evidence of the importance of the microenvironment in cancer, it is possible that the management of bladder cancer would be improved by increased understanding of cells in the microenvironment, including SCs. We hypothesised that SCM+ cells would exhibit abnormal distribution in bladder cancer tissue.

Materials and methods

Patients

In this retrospective study, the patient material was obtained from our previously published studies of 272 consecutive bladder tissue samples that had been re-diagnosed by uropathologist consensus according to the 2004 World Health Organization classification of neoplasia [10, 33–35]. The material was population-based. All patients with any of the following bladder conditions were included, as defined by the above-mentioned classification: dysplasia, CIS, invasive carcinoma, and non-neoplastic disease. Only specimens with unequivocal diagnoses were considered, and if more than one specimen had been obtained from a patient the earliest specimen was selected. Seventy patients were included in the investigation: 52 had neoplasia, and 23 of those subjects were under follow-up for non-muscle-invasive bladder cancer; the remaining 18 patients had no evidence of neoplasia in the current or previous specimens, or within the follow-up period of a minimum of 35 months [33]. In the neoplastic groups, 26 patients (50 %) had CIS, and all of those individuals were found to have a high-grade papillary or invasive urothelial tumour in the current or a previous specimen. The median age was 76 years among the

Table 1 Patient groups and histopathological diagnoses

Patient groups		Urothelial neoplasia		Patients		Flat intraurothelial neoplasia		Papillary and/or invasive urothelial neoplasia			
Group	*n*	In previous specimen	In current specimen	Age group, years (median)	Gender	Dysplasia	Carcinoma in situ	LMP[a] or LG pTa	HG pTa	HG pT1	HG pT2
I	23	Yes	Yes	56–87 (77)	5 F 18 M	5 (22 %)	12 (52 %)	4 (17 %)	4 (17 %)	8 (35 %)	3 (13 %)
II	29	No	Yes	39–87 (74)	5 F 24 M	8 (28 %)	14 (48 %)	4 (14 %)	3 (10 %)	13 (45 %)	6 (21 %)
III	18	No	No	20–89 (53)	12 F 6 M	NA	NA	NA	NA	NA	NA
Total	70			20–89 (74)	22 F 48 M	13 (19 %)	26 (37 %)	8 (11 %)	7 (10 %)	21 (30 %)	9 (13 %)

Patients groups: *I* under follow-up for urothelial neoplasia; *II* not previously diagnosed with urothelial neoplasia but diagnosed with that disease in current specimen; *III* not diagnosed with urothelial neoplasia before, in, or after the current specimen (i.e., representative of benign urothelium). Groups I and II were pooled for some analyses, as indicated in text

F female, *M* male, *LMP* papillary urothelial neoplasia of low malignant potential, *LG* low grade, *HG* high grade

NA not applicable

[a] LMP was present in three cases (4 % of total)

patients with neoplasia but 56 years for those with non-neoplastic conditions (see Table 1).

Biological material and immunohistochemistry

Formalin-fixed paraffin-embedded tissue was used for analysis. From each specimen, one tissue block was selected according to the features upon which the consensus diagnosis had been made, and it was sectioned for histological staining and immunohistochemistry (IHC) as described previously [10, 33].

ALDH1 IHC was performed as single-antibody reactions for all selected tissue material. Double IHC using ALDH1 combined with CD44, with CRABP1, or with CRBP1 was performed on six randomly selected specimens from each of these three patient categories: CIS, invasive tumour, and benign. To determine cell differentiation in selected cases, single- and double-antibody reactions were carried out using S-100, CD68, cytokeratin AE1/AE3, vimentin, and CD117. Single-antibody reactions were conducted in a Dako Autostainer with PT Link (Dako Denmark A/S, Glostrup, Denmark) according to the manufacturer's instructions. Double-antibody reactions were done using a Benchmark XT system (Ventana Medical Systems, Inc., Tucson, Arizona, USA). For double reactions, ALDH1 was labelled red with an UltraView

Table 2 Antibodies and immunohistochemistry conditions used

Target protein	Manufacturer	Product code/clone	Host and clonality	Pre-treatment	Dilution
ALDH1A1	BD Transduction Laboratories, San Jose, CA, USA	44/ALDH	Mouse, mono-	pH 6.0, PTLink (Dako Denmark A/S, Glostrup, Denmark)	1:100
CD44 (isoforms 1, 6, 7, 8, and 9)	Atlas Antibodies, Stockholm, Sweden	HPA005785	Rabbit, poly-	pH 6.0, 30 min 95 °C	1:50
CD68	Dako Denmark A/S, Glostrup, Denmark	PG-M1	Mouse, mono-	pH 9.0, PTLink	1:100
CD117	Dako	A4502	Rabbit, poly-	pH 9.0, PTLink	1:200
CRABP1	Atlas Antibodies	HPA017203	Rabbit, poly-	pH8.5, 30 min 95 °C, mild protease 4 min	1:50
CRBP1 (FL-135)	Santa Cruz Biotechnology, Dallas, TX, USA	Sc-30106	Rabbit, poly-	pH8.5, 30 min 95 °C, mild protease 4 min	1:50
Cytokeratin	Dako	AE1 & AE3	Mouse, mono-	pH 9.0, PTLink	1:50
S-100	Dako	Z0311	Rabbit, poly-	pH 9.0, PTLink	1:2000
Vimentin	Dako	V9	Mouse, mono-	pH 9.0, PTLink	1:500

Universal Alkaline Phosphatase Red Detection Kit, and the other antibody was labelled brown with an UltraView DAB Detection Kit (both from Ventana Medical Systems Inc.). Alcian blue staining was performed in a standard fashion at pH 0.5 to visualise mast cells. The antibodies and IHC conditions applied are listed in Table 2.

Microscopy

Two pathologists (BLI and CB) examined haematoxylin and eosin (H&E) and IHC tissue sections primarily in a Nikon Eclipse N*i* microscope (Nikon Instruments Europe BV, Amsterdam, the Netherlands). Histological and IHC findings were photographed using a Nikon Digital Sight DS-Fi2

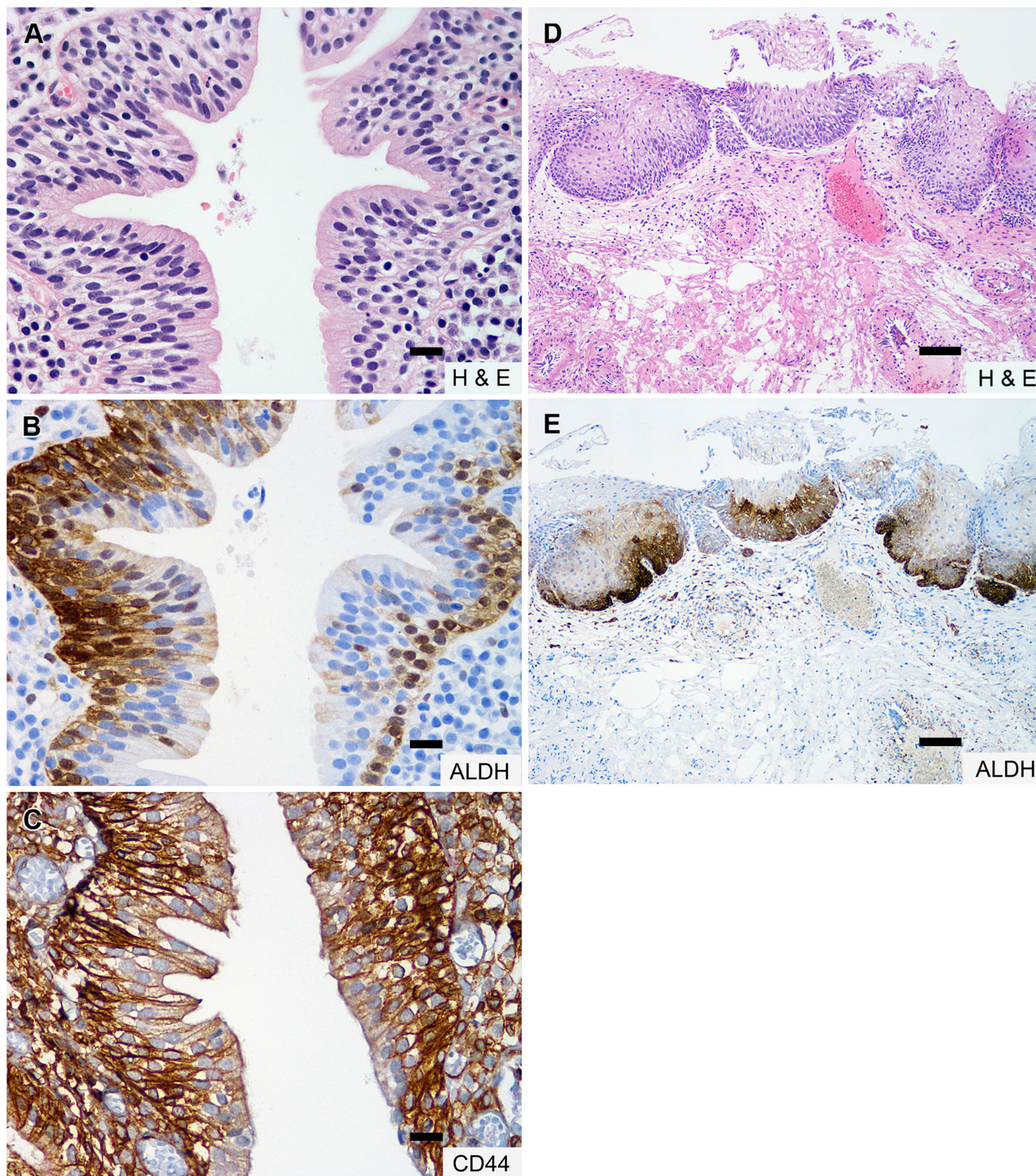


Fig. 1 Areas of basally and intermediately located epithelial cells in benign epithelium of the bladder neck exhibiting stem cell immunophenotype ALDH1+ CD44+. **a**,**b**,**c**, The three images show sections from a 64-year-old man with no malignancy. **d**,**e**, Basally located ALDH1+ cells in reactive squamous epithelium of the bladder trigone. Such cells were frequently found in this location, suggesting stem cell status. The images are from a 48-year-old woman with no malignancy. *Scale bars* 20 μm

camera and NIS Elements 4.00.00 imaging software (Nikon). IHC results for each specimen were recorded simply as the presence or absence of positive (or double positive) cells, for each morphological cell type and location. For stromal findings, only observations from the soft tissue between the epithelium and muscularis propria were included.

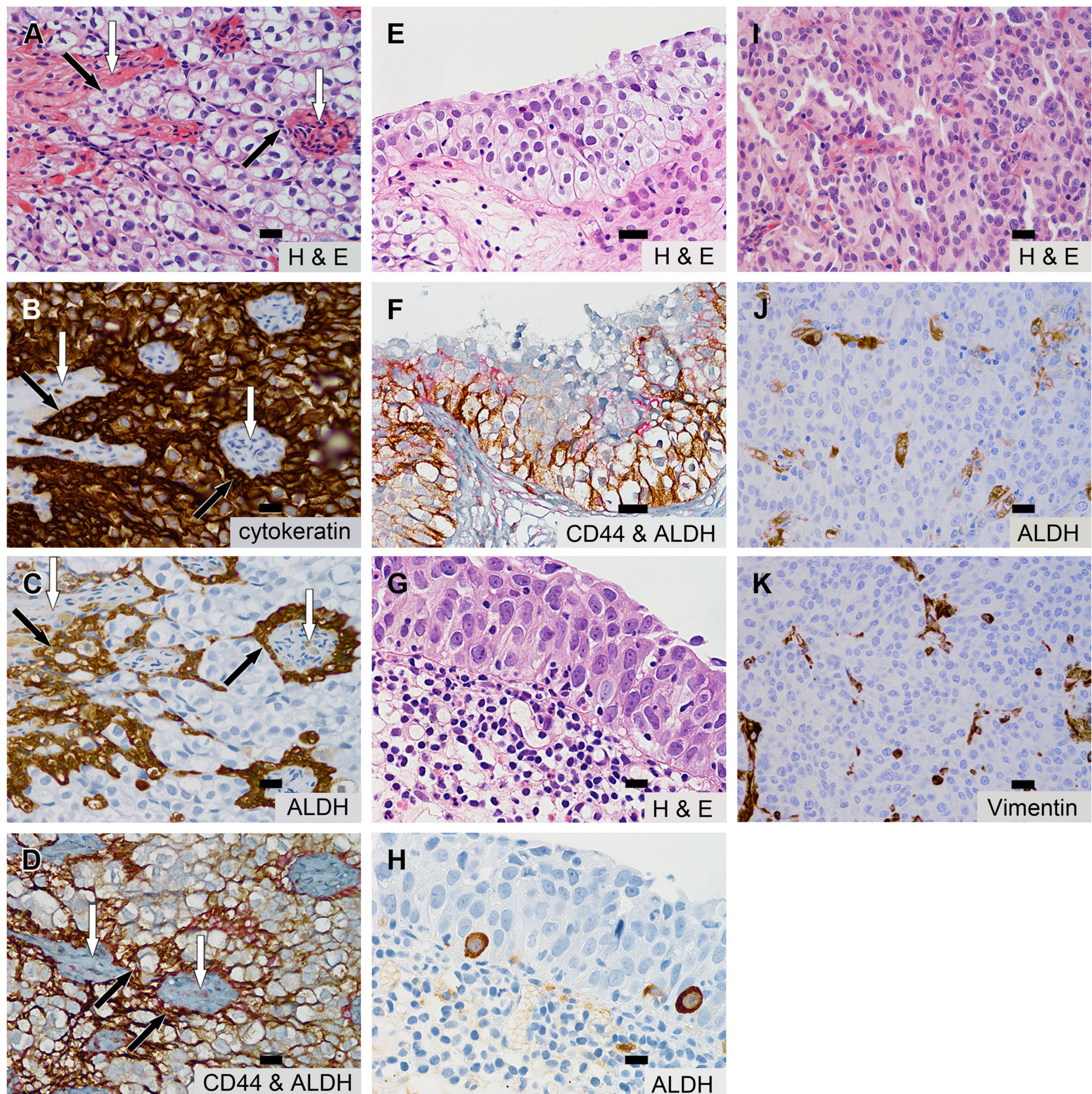


Fig. 2 **a**–**d** Urothelial carcinoma invasive into the lamina propria, with small tumour front cells (*black arrows*) located adjacent to stroma (*white arrows*) and showing regular nuclei and cytokeratin AE1/AE3+ ALDH1+ CD44+ immunophenotype, suggestive of cancer stem cells. From a 68-year-old man with no previous bladder diagnosis. **e**,**f** An area of carcinoma in situ in von Brunn's nests with most basally and intermediately located cells staining positive for ALDH1 (*red*) and/or CD44 (*brown*), representative for samples from many carcinoma in situ patients, particularly when cells were "clear" indicating a high glycogen content. The cellular location and immunophenotype are consistent with cancer stem cells. From an 83-year-old man debuting with a high-grade carcinoma invading the lamina propria. **g**,**h** Carcinoma in situ containing few, scattered intraepithelial ALDH1+ cells that were morphologically indistinguishable from surrounding malignant epithelial cells and thus seemed to be cancer stem cells. From a 79-year-old man with a recurrent high-grade bladder carcinoma without invasion. **i**, **j**, **k** An area of a urothelial tumour invasive into the muscularis propria showing ALDH1+ cells in the vimentin-positive stroma between tumour cell sheets (i.e., close to but not in the carcinoma itself), representative for the vast majority of invasive tumour samples in this study. From a 62-year-old man never before diagnosed with bladder neoplasia. *Scale bars* 20 μm

Statistical analysis

Comparisons between groups were performed using the fraction of specimens that contained any positive cells for the relevant marker(s) in each group. Fisher's exact test of probability was used to evaluate associations, and $p<0.05$ (two-tailed) was considered to be statistically significant. For some statistical analyses, the total study population was divided into age groups as indicated in the text below.

Ethics

The regional ethics committee (REK sør-øst) approved the study plan (2012/1730/REK sør-øst A), and waived need for informing patients or obtaining patient consent. The study included consecutive hospital patients not selected for gender, race, or age. The authors declare no conflicts of interest.

Results

ALDH1+ cell morphology by location

Within benign urothelium and squamous epithelium (bladder trigonum), most ALDH1+ cells were of epithelial type, confluent and integrated into the architecture (Fig. 1a–e). Epithelial-type ALDH1+ cells were also observed in urothelial carcinoma cell sheets (Fig. 2a–f). Less often, in both benign and malignant tissue, ALDH1+ cells in epithelium occurred as single cells that were morphologically indistinguishable from adjoining cells (Fig. 2g–h). Most ALDH1+ cells were however located in the stroma (Fig. 2i–k). Scattered invasive tumour areas contained ALDH1+ cells that resembled foamy macrophages and were located in the stroma; these cells were positive for CD68 (Fig. 3a–c). In the lamina propria of neoplastic and non-neoplastic tissue, the most common type of ALDH1+ cells had a spindle-shaped or angular cell body and cytoplasmic extensions, and the cytoplasm was positive for ALDH1 and often contained small vacuoles. Many of these ALDH1+ cells were also CRBP1 positive (see below), and ALDH1− CRBP1+ cells with the same morphology were observed as well (Fig. 4a–l). The second most common ALDH1+ cell variant in the stroma was mononuclear leukocyte-like, round or oval, with a small regular nucleus and cytoplasm with a granular appearance that was recapitulated in granular ALDH1 positivity; these cells were positive for CD117 (c-kit) and Alcian blue staining performed at pH 0.5 (Fig. 5a–h). A less common location for ALDH1+ cells in stroma was underneath the endothelium of occasional capillaries (Fig. 6a–b). Also, endothelial cells in sporadic capillaries were ALDH1+ (Fig. 7a–b). Furthermore, all nerves in benign and malignant specimens were consistently positive for ALDH1, CD44, and S-100 (Fig. 8a–d).

Histologically benign tissue in cancer versus non-cancer patients

Table 3 outlines the distribution and morphology of ALDH1+ cells in histologically benign mucosal areas in bladder tissue

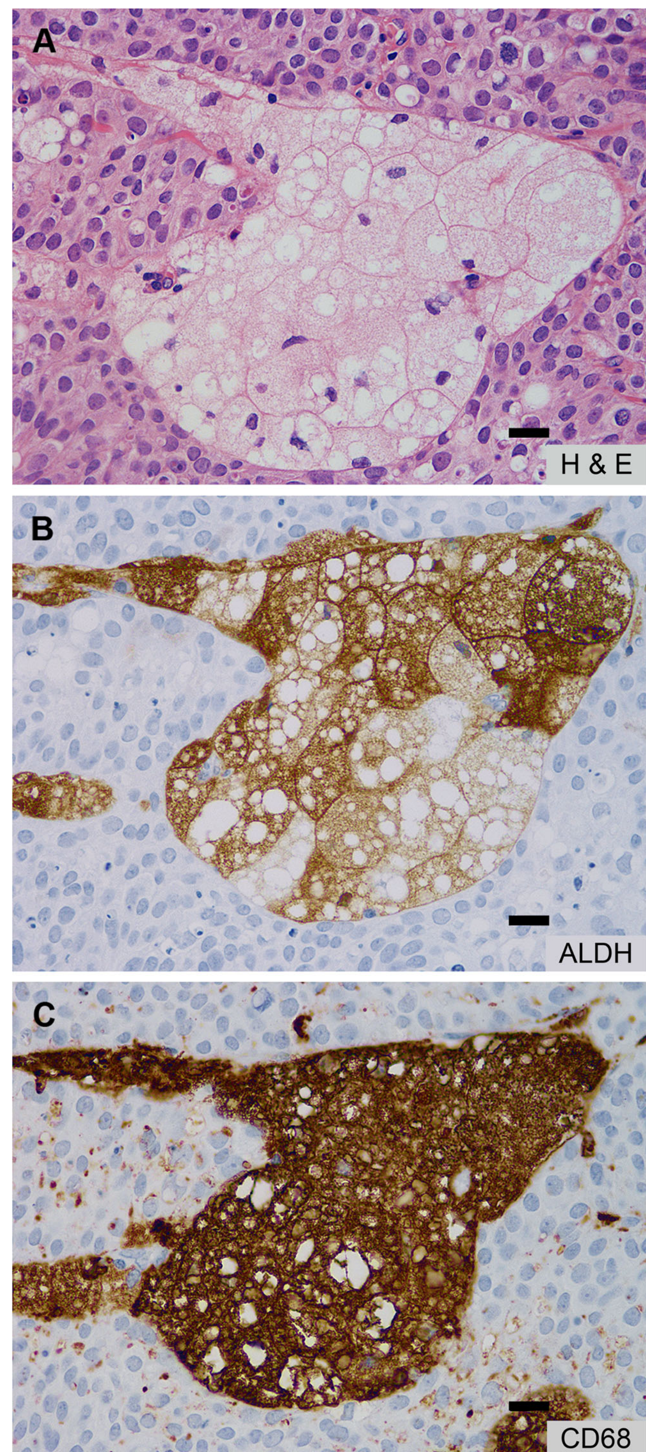


Fig. 3 **a**, **b**, **c** Foamy histiocyte-like ALDH1+ cells found in the stroma of an urothelial carcinoma that invaded the muscularis propria. These cells were CD68+ and thus identified as macrophages. From a 52-year-old woman never before diagnosed with bladder neoplasia. *Scale bars* 20 μm

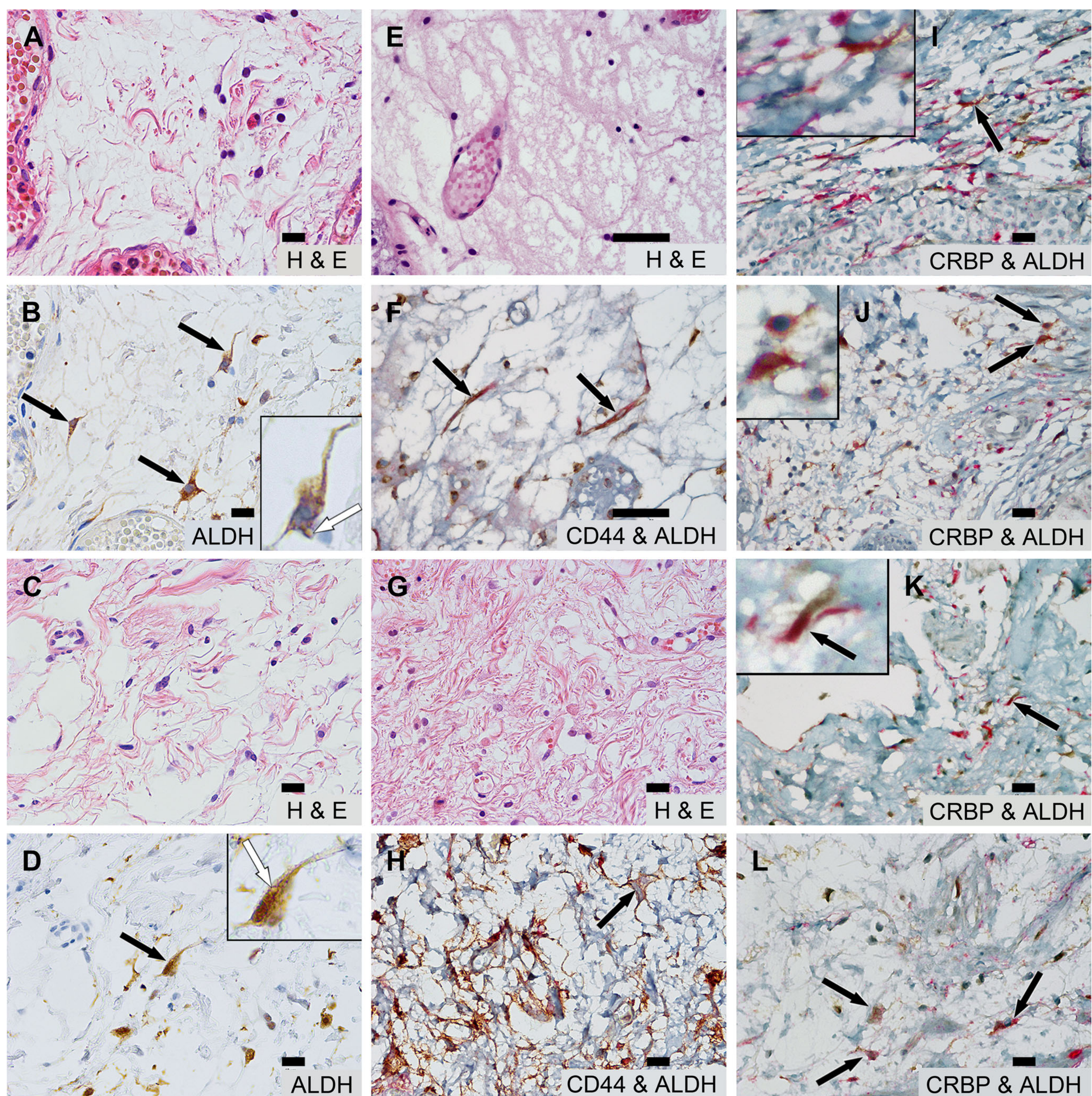
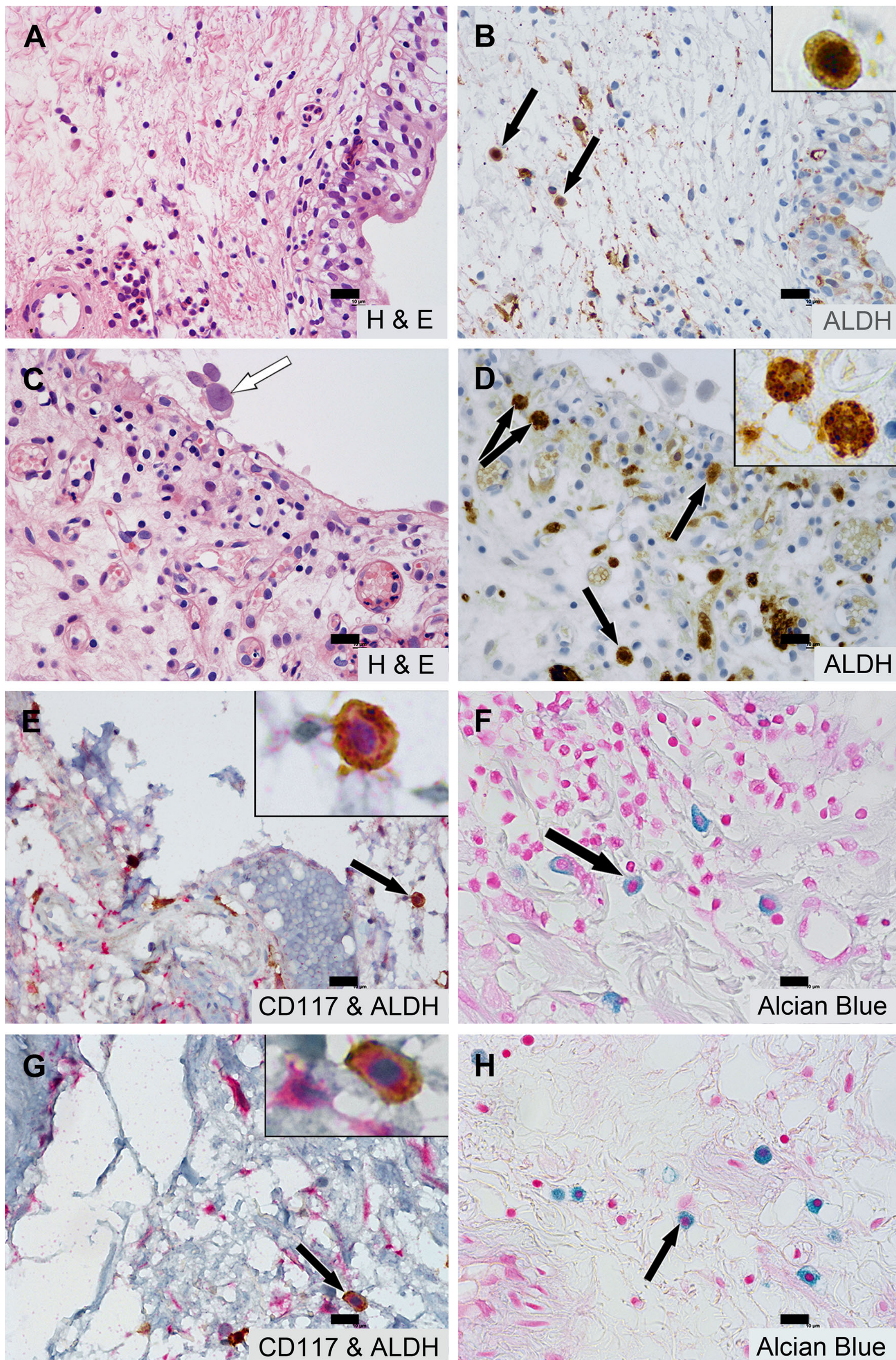


Fig. 4 Stellate-shaped ALDH1+ cells (*black arrows*) with small cytoplasmic vacuoles (*white arrows*) were found in the lamina propria of all specimen categories. **a**,**b** Sections from a lamina propria area of a 71-year-old man debuting with high-grade carcinoma invading the lamina propria. **c**,**d** Sections from a lamina propria area of a 63-year-old woman with a benign bladder condition. **e**,**f** and **g**,**h** Image sets from lamina propria areas from an 83-year-old man debuting with a carcinoma invading the lamina propria and a 73-year-old man with a carcinoma invading the muscularis propria, showing spindle- or stellate-shaped ALDH1+ (*red*) and CD44+ (*brown*) cells with small intracytoplasmic vacuoles. Such cells exhibited various combinations of ALDH1 and CD44 positivity in the lamina propria of benign and malignant specimens. **i**–**l** Four different lamina propria areas containing CRBP1+ (*brown*) and ALDH1+ (*red*) spindle- or stellate-shaped cells (*black arrows*) as detected in nearly all benign and malignant specimens. The images also show similar cells that were positive for only one of the two proteins. Epithelial cells were CRBP1 negative. **i** Benign-appearing lamina propria in between invasive carcinoma areas in a 62-year-old man debuting with carcinoma invading the muscularis propria. **j** From an 83-year-old man debuting with high-grade carcinoma invading the lamina propria and concomitant carcinoma in situ. **k** From an 89-year-old man with a benign bladder condition. **l** From the specimen shown in **g**–**h**. *Scale bars* 20 μm

from patients with and without bladder cancer. Only 19 of 52 patients with neoplasia had such tissue represented in their specimen; this lack of benign urothelium in tumour resection material has been commented on before [10]. Confluent ALDH1+ epithelial cells were detected in benign-appearing urothelium in 74 % of cancer patients and 39 % of non-cancer


A
H & E
B
ALDH
C
H & E
D
ALDH
E
CD117 & ALDH
F
Alcian Blue
G
CD117 & ALDH
H
Alcian Blue

◀ **Fig. 5** Stromally located ALDH1+ round/oval leukocyte-like cells with granular cytoplasm (*black arrows*). **a**,**b** From a lamina propria area of a 31-year-old man with a benign bladder condition. **c**,**d** From a lamina propria area of a 78-year-old man with recurrent high-grade bladder cancer; images show clinging type of carcinoma in situ (*white arrow*). **e-h** These cells were positive for ALDH1 (*red*), CD117 (*brown*), and Alcian blue staining at pH 0.5 (*blue*) and were thus identified as mast cells. **e**,**f** Sections from a 62-year-old man debuting with a high-grade carcinoma invading the muscularis propria. **g**,**h** Sections from a 68-year-old man debuting with a high-grade carcinoma invading the lamina propria. *Scale bars* 20 μm

patients (p=0.049). However, the presence of these cells was more closely associated with patients being below median age (p=0.018), and no statistically significant associations were found between ALDH1+ cells and cancer status in separately analysed age groups. ALDH1+ mast cells were detected in the lamina propria of benign-appearing urothelium in 47 % of bladder cancer patients and 100 % of non-cancer patients (p= 0.023), and when this association was analysed separately for patients being below or above median age, it remained statistically significant for low age (p=0.032). Similarly, ALDH1+ stellate cells were detected in the lamina propria of benign-appearing urothelium in 53 % of patients with bladder cancer and 94 % of those without such disease (p=0.008), and no significant association with age was found for this variable. Patients with CIS lacked ALDH+ stellate cells underneath normal epithelium in 7/11 (64 %) cases, compared with 2/8 (25 %) cases for non-neoplastic patients, although this difference was not statistically significant.

Neoplastic tissue

Results regarding ALDH1 in neoplastic bladder tissue are given in Table 4. In neoplastic urothelium, ALDH1+ cells were present in a similar proportion of dysplastic and CIS lesions, and in slightly higher numbers in invasive cancer lesions, but none of these levels were significantly different internally or from what was observed in urothelium of non-cancer patients. Notwithstanding, in invasive tumour areas, ALDH1+ cells were found much more frequently in the stroma than in the tumour cell sheets (Fig. 2i–k). In the lamina propria, ALDH1+ mast cells and ALDH1+ stellate cells were present in almost all neoplastic and non-neoplastic specimens. No trends or significant differences were found for different grades or stages of neoplasia or all neoplasia compared with specimens from non-cancer patients, and the same was noted for all the different types of ALDH1+ cells and locations that were analysed.

CD44+ cells

In benign urothelium, cytoplasmic CD44 positivity with accentuated membranous positivity was observed in basally and intermediately located cells, whereas umbrella cells were generally negative for CD44. Meanwhile, glycogen-rich urothelial and squamous cells were strongly CD44+. Tumour cell sheets were at least focally CD44+ in the great majority of tested specimens, and this cell positivity tended to be in a basal location (i.e., near the stroma). In CIS urothelium, there was CD44 positivity at least basally in most cases. In both benign and malignant tissue, double IHC analysis against ALDH1 and CD44 revealed that essentially all cell types addressed in this study occurred with all possible permutations of positivity for these two protein epitopes, except that neurons occurred only as ALDH1+ CD44+. For the ALDH1+ CD44+ immunophenotypes, no statistically significant differences were found between benign and malignant tissue specimens (see Table 5).

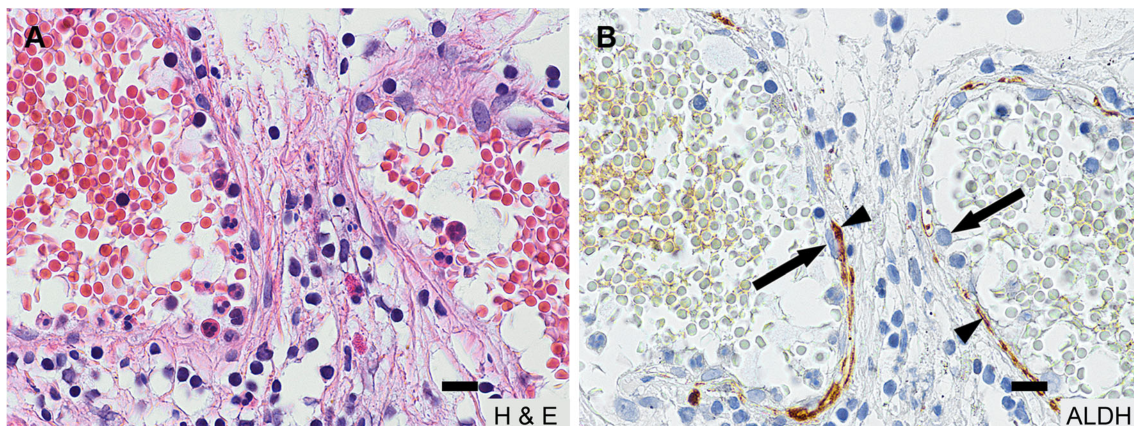


Fig. 6 **a**,**b** Benign-appearing lamina propria containing capillaries with ALDH1+ cells (*arrowheads*) located underneath ALDH1− endothelial cells (*arrows*), representative for benign and malignant specimens. From a 72-year-old man with recurrent high-grade bladder cancer and invasion into the lamina propria. *Scale bars* 20 μm

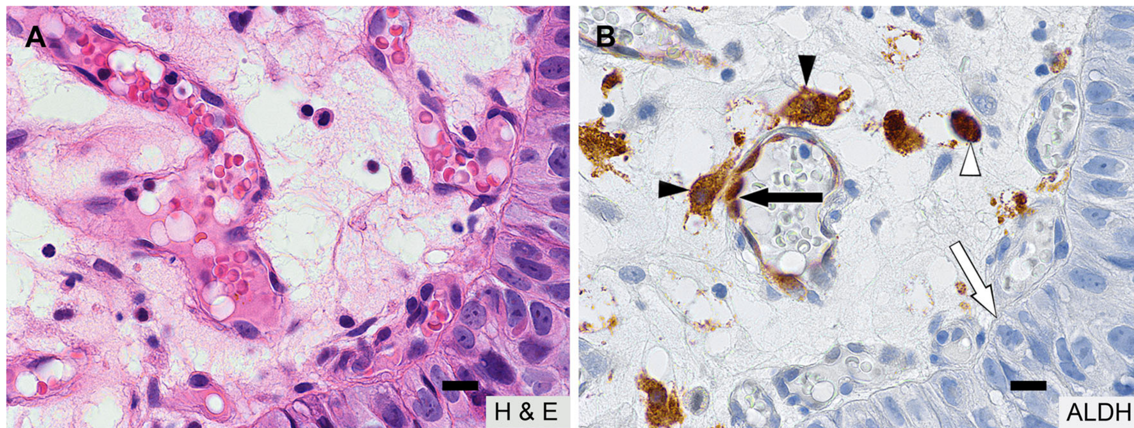


Fig. 7 **a**,**b** Lamina propria just underneath carcinoma in situ (*white arrow*) containing capillaries with ALDH1+ endothelial cells (*black arrow*), representative for nearly all benign and malignant specimens. Also, notice ALDH1+ stellate-shaped cells with small cytoplasmic vacuoles (*black arrowheads*), and ALDH1+ round/oval leukocyte-like cells (*white arrowhead*). From a 78-year-old man debuting with a non-invasive high-grade papillary carcinoma. *Scale bars* 20 μm

CRBP1+ cells

CRBP1 positivity in urothelium, although present in the majority of cases, occurred only focally and was very weak in both malignant and benign specimens. Differently, in the lamina propria, CRBP1 positivity and CRBP1−ALDH1 co-positivity were widespread and was observed in the following cell types in neoplasia- and non-neoplasia patients: stellate cells (primarily immunophenotype ALDH1+ CRBP1+), mast cells (mostly ALDH1+ CRBP1+ and ALDH1−CRBP1+),

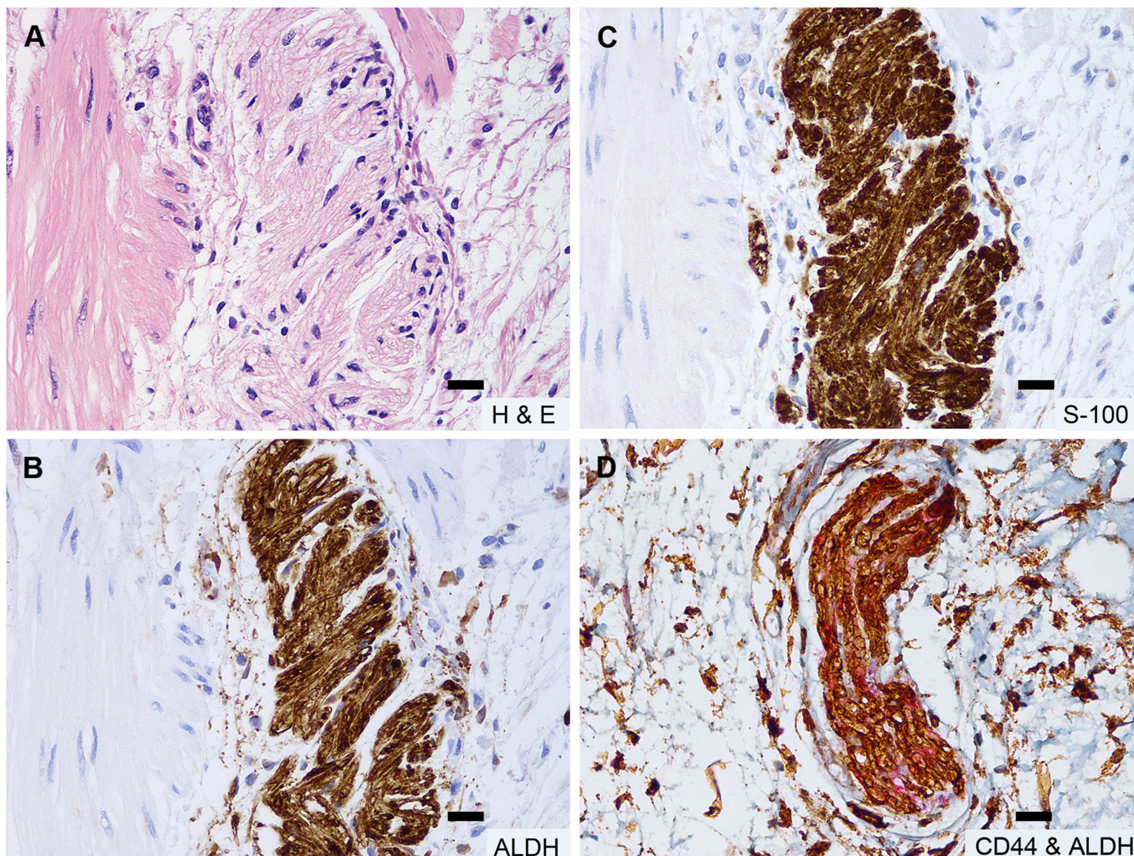


Fig. 8 **a**,**b**,**c** A submucosal area with an ALDH1+ and S-100 positive nerve, representative of benign and malignant specimens. **d** A submucosal nerve from a different patient, positive for the putative stem cell markers ALDH1 (*red*) and CD44 (*brown*), as detected in all nerves visualised in benign and malignant specimens. *Scale bars* 20 μm

Table 3 Comparison of patients with and without bladder cancer regarding aldehyde dehydrogenase 1 A1-positive (ALDH1+) cells in areas of benign-appearing mucosa in the bladder

	Histomorphologically benign epithelium			Histomorphologically benign lamina propria	
	ALDH1+ confluent cells, no. of cases (%)	ALDH1+ single cells, no. of cases (%)	ALDH1+ Spindle-/stellate-shaped cells[a], no. of cases (%)	ALDH1+ Leukocyte-like cells[b], no. of cases (%)	ALDH1+ Spindle-/stellate-shaped cells[a], no. of cases (%)
Patients with no bladder cancer (18 cases)	7 (39)	6 (33)	2 (11)	18 (100)	17 (94)
Patients with bladder cancer (19 cases)	14 (74)	6 (32)	2 (11)	9 (47)	10 (53)

[a] Identified as stellate cells in this study

[b] Identified as mast cells in this study

and endothelial cells (chiefly immunophenotype ALDH1+ CRBP1+). Statistically significant differences between benign and malignant specimens were noted for CRBP1+ mast cells in the lamina propria, which occurred less frequently in malignant specimens than in benign specimens ($p=0.009$; see Table 5).

CRABP1+ cells

Confluent areas of CRABP1+ epithelial cells were present in all specimens (see Fig. 9a–d). The general impression was that even more extensive CRABP1 positivity was present in malignant urothelium than in benign urothelium (not quantified). For the lamina propria, specimens showed weak and focal CRABP1 positivity for the following cell types in both benign and malignant tissue: stellate cells, mast cells, and endothelial cells. There were no significant differences regarding the frequency of CRABP1+ or ALDH1+ CRABP1+ cells in malignant tissue as compared to benign specimens (see Table 5).

Discussion

In the neoplastic epithelium, only one ALDH1+ cell type was identified: epithelial. The frequency of this cell type in neoplastic epithelium did not differ significantly from benign epithelium. Differently, sub-epithelial stroma of neoplastic lesions exhibited significant reduction in the frequencies of ALDH1+ stellate cells and ALDH1+ mast cells. This suggests that future stem cell marker studies should focus on the stroma rather than the lesion as a whole.

A large number of cell types addressed in this study exhibited all possible variations of positivity for ALDH1 and CD44

Table 4 Occurrence of aldehyde dehydrogenase 1 A1-positive (ALDH1+) cells in neoplastic areas of different types of urothelial neoplasia

	Epithelium			Lamina propria	
	ALDH1+ Confluent cells, no. of cases (%)	ALDH1+ Single cells, no. of cases (%)	ALDH1+ Spindle-/stellate- shaped cells[a], no. of cases (%)	ALDH1+ Leukocyte-like cells[b], no. of cases (%)	ALDH1+ Spindle-/stellate- shaped cells[a], no. of cases (%)
Dysplasia (13 cases)	5 (38)	4 (31)	2 (15)	13 (100)	12 (92)
Carcinoma in situ (26 cases)	10 (38)	9 (35)	7 (27)	25 (96)	24 (92)
Invasive tumour (45 cases)	18 (40)	25 (56)	16 (36)	44 (98)	43 (96)

Note: many cases of invasive carcinoma had concomitant carcinoma in situ or dysplasia, and hence the number of lesions examined exceeded the total number of patients included in the study

[a] Identified as stellate cells in this study

[b] Identified as mast cells in this study

Table 5 Number of cases in which cells positive for CRBP1 and CRABP1 were detected; comparison between malignant and benign tissue

Tissue compartment	Cell type	Bladder condition	CRBP1+	CRABP1+
Epithelium[a]	Epithelial	Malignant	9/12	12/12
		Benign	5/6	6/6
Lamina propria	Spindle-/stellate shaped[b]	Malignant	12/12	*6/12*
		Benign	6/6	*5/6* (p=0.061)
	Leukocyte-like[c]	Malignant	*3/12*	7/12
		Benign	*5/5* (p=0.009)	3/5
	Endothelial	Malignant	6/12	12/12
		Benign	2/5	5/5
	Sub-endothelial	Malignant	0/12	0/12
		Benign	0/4	1/4
	Nerve	Malignant	0/3	0/12
		Benign	0/3	0/3

Data from 18 randomly selected patients with (n=12) and without (n=6) urothelial carcinoma. The denominators indicate the numbers of cases in which the mentioned histological structures or cells were present in the examined tissue specimens, and the nominators indicate the number of cases in which immunohistochemical positivity (of any intensity) was detected for those structures and cells

[a] Benign in non-cancer patients and malignant in cancer patients

[b] Identified as stellate cells in this study

[c] Identified as mast cells in this study

Results with statistical significance or trend are indicated with p values (two-tailed)

in double IHC analysis, which is in itself consistent with earlier studies showing that both of these markers can serve as SCMs, especially when they are present simultaneously. Notwithstanding, a number of cell types that were positive for both these markers did show features of specific non-stem cells. For example, the CD44+ and ALDH1+ spindle- or stellate-shaped cells exhibited small intracytoplasmic vacuoles and were positive for CRBP1, characteristics that concur with the morphological appearance and the retinoid transport role of previously described stellate cells. The CD44+ and ALDH1+ round/oval leukocyte cell type was positive for CD117 and Alcian blue staining at pH 0.5, consistent with mast cells. The CD44+ and ALDH1+ cytoplasm-rich foamy cells were also positive for CD68, thus identified as histiocytes. Neurons were consistently CD44+/ALDH1+, and also endothelial cells of some capillaries showed such positivity. Together, these constitute the five types of SMC+ cells that were identified as differentiated cells in this study.

With regard to benign and malignant urothelium, single and confluent CD44+ ALDH1+ cells were detected predominantly in basal/near-basal or tumour front locations, thus suggesting that some of those may be urothelial SCs [36]. Sub-endothelially located cells with a CD44+ and/or ALDH1+ phenotype had a distribution that suggests pericytes, but, based on recent data indicating that sub-endothelial or peri-vascular sites are preferential locations for SCs [37], it is possible that they are instead SCs. We perceived these two types of CD44+ ALDH1+ cells, deeply located epithelial cells and sub-endothelial cells, as being consistent with SCs.

ALDH1+ mast cells occurred in abnormally low numbers in the lamina propria of benign-appearing urothelium of the patients with bladder cancer, which, when considering the multifocal and recurrent nature of bladder cancer (implying field effect), suggests that ALDH1+ mast cells may play a protective role against cancer. This is congruent with earlier data that the presence of mast cells is reduced in tissue of various cancers, as described below. Also, CRBP1+ mast cells in the lamina propria occurred less commonly in malignant specimens compared with specimens from non-cancer patients, this novel finding also supporting the idea that mast cells play a cancer-preventive role. Similar to the present study, other investigations have shown that SCs in myocardium include CD117 positive mast cells [38, 39]. Our results suggest an inverse relationship between SCM+ mast cells and cancer, which supports earlier reports concerning the low occurrence of mast cells in cancers of the uterine cervix, breast, colon, prostate, and lung [40–44].

With an even higher level of statistical significance, the number of ALDH1+ stellate cells in the lamina propria of benign-appearing urothelium was abnormally low in patients with bladder cancer, which suggests that also ALDH1+ stellate cells are involved in suppression of carcinogenesis. As a supplement to our investigation in relation to the identity of stellate cells, we also performed experiments focused on CRABP1. As expected, we found strong positivity for

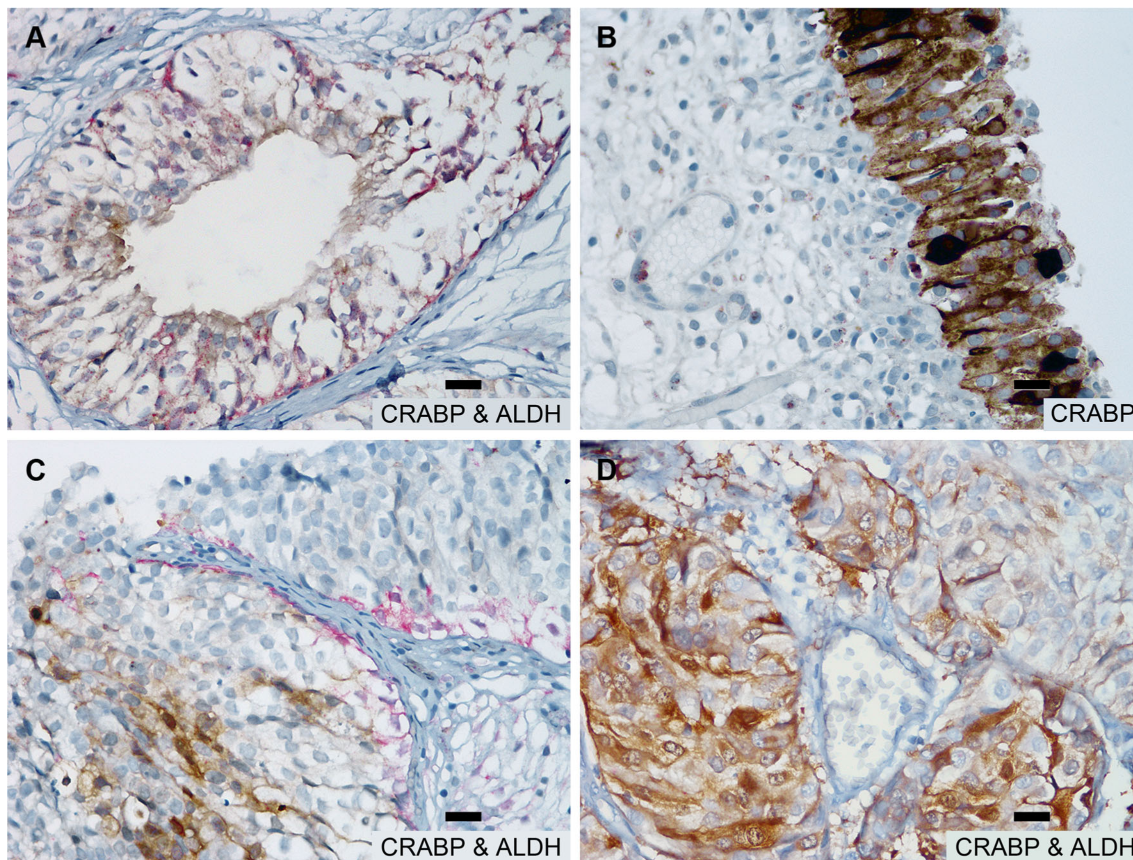


Fig. 9 **a**–**d** Epithelium of four different benign and malignant specimens showing numerous and widespread CRABP1+ cells. Epithelial cells positive for both ALDH1 (*red*) and CRABP1 (*brown*) occurred in less than half of the benign specimens but were more common in carcinomas (for epithelial CRABP1 positivity see Table 5). Stromal cells were largely CRABP1 negative. **a** A benign von Brunn's nest with ALDH1+ cells occurring mostly at basal and intermediate levels, and CRABP1+ cells closer to the surface. A section from an 89-year-old man with a benign bladder condition. **b** Carcinoma in situ showing strong CRABP1 positivity in all cells except for focal cells in the top layer; a section from a 62-year-old man debuting with a carcinoma invading the muscularis propria. **c** A CRABP1 and ALDH1 pattern similar to that shown in **a**, here in a dysplastic von Brunn's nest from an 83-year-old man debuting with high-grade carcinoma invading the lamina propria. **d**, CRABP1+ ALDH1− invasive carcinoma cells in a 76-year-old man with muscle-invasive cancer. *Scale bars* 20 μm

CRABP1 in cancerous urothelium, tissue that can be assumed to be a significant "end-user" of retinoic acid. By comparison, cellular expression of this protein was lower in benign urothelium. In the present material, stellate cells were in fact the most common SCM+ cell type in superficial layers of benign bladder tissue. The scientist von Kupffer discovered stellate cells in 1876 and called them *Stern-Zellen*, and his pupil Rothe later reported observing small cytoplasmic vacuoles in the same cells [45, 46]. Stellate cells have been referred to by different names, such as vitamin A-storing cells and Ito cells [47–53]. In 1963, it was shown that the cytoplasmic vacuoles contain retinoids [54]. Stellate cells have been identified in most organs, including the urinary bladder [55, 56], and are strongly positive for smooth muscle actin in their cytoplasm [57]. Stellate cells are major providers of retinoic acid for other cells. Thus, although the reduced presence of stellate cells in the benign-appearing bladder mucosa of cancer patients has not yet been explained, it can be suggested that a decrease in the numbers of such cells diminishes the supply of retinoic acid and thereby increases the susceptibility to carcinogenesis.

ALDH1+ cells were much more numerous in the stroma than within the carcinomatous epithelium. This observation is noteworthy, because the presence of ALDH1+ putative stem or stem-like cells in bladder cancer tissue has previously been described without any detailed specification of the histological location of these cells [29]. In our study, ALDH1+ cells were found in similar numbers in dysplastic and CIS lesions, and were somewhat more common in invasive cancer lesions, but this did not differ significantly from what we observed in benign tissue from non-cancer patients. The lack of statistically significant differences between carcinoma tissue and tissue from non-cancer patients is difficult to explain, in light of the fact that the frequency of certain SCM+ cells was significantly decreased in normal-appearing mucosa of bladder cancer patients. We can offer a possible explanation that the microenvironment of cancerous tissue is radically altered with respect to cell composition, intercellular matrix, and metabolism,

which may cause multiple factors to influence the occurrence of SCM+ cells, making it difficult to isolate individual factors [58]. Perhaps surprisingly, CIS tissue showed no statistically significant associations with regard to any examined immunophenotypes; hence, this must be investigated in a larger set of specimens.

With regard to aldehyde dehydrogenases as SC markers, a case can be made that retinoic acid is necessary for the initiation of differentiation, and that it contributes to cell preservation and resistance to toxic substances; qualities that are important for long-term quiescent cells that "await the call of duty" to generate differentiated cell progenies. Due to the abundance of evidence indicating that aldehyde dehydrogenase 1 A1 was the most efficient established epithelial organ stem cell marker to date, this was selected as the principal stem cell marker.

To some degree, sectioning of tissue blocks resulted in a shift of distinctive structures between near-consecutive slides. Therefore, we used double IHC to demonstrate cellular co-presence of markers, and we also ensured antibody specificity by employing monoclonal antibodies, and purified polyclonal antibodies that have been characterised extensively in human tissues. This study was essentially qualitative in nature, including a large number of observed features, and hence quantification was kept as simple as possible: presence or absence of the relevant cell type in each specimen. Image analysis was not used, because such evaluation depends on guidance of the investigator and is thus prone to subjectivity. Combined staining for two antibodies was done on only a sub-set of the entire material, which may have reduced the statistical power of the evaluation and consequently given a false impression of non-significant results for some of the features that were examined. Also, it should be kept in mind that the majority of benign-appearing mucosal areas examined in this study were derived from tumour resection specimens, raising possibilities that the frequencies of stromal cells could be directly influenced by the close proximity to tumour.

By means of IHC, our study identified several types of SCM+ differentiated cells in the microenvironment of benign and malignant urinary bladder mucosa. Meanwhile, it must be borne in mind that SCs are defined through functional studies in vivo that identify cell populations characterised by longevity, pluripotency, and tumour initiation. Such investigations have pinpointed protein cell markers that enrich mixed cell populations for SCs. The markers can be used in IHC studies to label cells in situ and thereby histologically visualise cell populations that include SCs. To date, this results in cell populations that contain only about 1 functional SC in 100 to 1,000 SCM+ cells. The results of our study demonstrate that non-specificity is a major explanation for the low efficiency of SCMs, which encourages the search for more specific epithelial SC markers.

Conclusion

Whereas previous studies have firmly established ALDH1 and CD44 as useful markers for SCs, the present results show that these markers also label several types of differentiated cells, including stellate cells and mast cells. Both of these two cell types were significantly reduced in number in the micro-environment of benign-appearing bladder epithelium in patients with bladder cancer, suggesting that this may be a part of a field effect that causes tumour multifocality and recurrence, and that these particular cells may play a tumour-suppressive role.

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