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Value of CDX2, villin, and α-methylacyl coenzyme A racemase immunostains in the distinction between primary adenocarcinoma of the bladder and secondary colorectal adenocarcinoma

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Primary adenocarcinoma of the urinary bladder is an uncommon neoplasm that can be indistinguishable morphologically from colorectal adenocarcinoma secondarily involving the bladder by direct extension or metastasis. In the current study, 17 enteric-type primary adenocarcinomas of the bladder were immunohistochemically examined for the expression of CDX2, villin and α-methylacyl coenzyme A racemase (AMACR), immunomarkers preferentially expressed in colorectal adenocarcinoma. For comparison, 17 secondary colorectal adenocarcinomas involving the bladder, 23 primary colorectal adenocarcinomas and 14 conventional urothelial carcinomas were similarly studied. The results show that all 40 (100%) colorectal adenocarcinomas expressed CDX2 and 39 (98%) expressed villin. The expression of these two immunomarkers was less frequent in primary bladder adenocarcinomas, observed in eight (47%) and 11 (65%) cases, respectively ($P<0.0001$ and $P=0.0019$, respectively). The frequency of positive AMACR immunostaining was similar between these two types of tumors, detected in 28 (70%) colorectal adenocarcinomas and 11 (65%) primary bladder adenocarcinomas ($P=0.694$). None of the urothelial carcinomas exhibited CDX2 or villin immunoreactivity; and only two (14%) showed positive staining for AMACR. These results demonstrate that CDX2 and villin are of diagnostic value in aiding in the distinction between primary adenocarcinoma of the bladder and secondary colorectal carcinoma. Lack of CDX2 and villin signals points strongly to a bladder primary.
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Secondary involvement of the urinary bladder by adenocarcinoma from other anatomic sites, either by direct extension or hematogenous/lymphatic metastasis, occurs more frequently than adenocarcinoma originating from the bladder itself.[1] The distinction between primary and secondary adenocarcinomas of the bladder is important to clinical management, but may not always be straightforward to pathologists or clinicians. This is particularly true when the secondary adenocarcinoma is of colorectal origin, which may exhibit histologic features similar to or indistinguishable from primary bladder adenocarcinoma.

We have previously reported that a panel of immunomarkers, including β-catenin, cytokeratin (CK) 7, CK20 and thrombomodulin, is of diagnostic value in helping distinguishing primary bladder adenocarcinoma from secondary adenocarcinoma of the colorectal origin.[2] Primary bladder adenocarcinoma was shown to express a distinct immunophenotype that was intermediate between urothelial carcinoma and colorectal adenocarcinoma. In comparison with colorectal adenocarcinoma, primary

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bladder adenocarcinoma expressed CK7 and thrombomodulin more frequently, CK20 less frequently, and did not exhibit nuclear staining for β-catenin. In this study, we examined additional immunomarkers, CDX2, villin and α-methylacyl coenzyme A racemase (AMACR) that have been shown to be preferentially expressed in colorectal adenocarcinoma, to assess their potential diagnostic value in the distinction between primary adenocarcinoma of the bladder and secondary involvement by colorectal adenocarcinoma.

Materials and methods

Case Selection

A total of 17 enteric-type primary adenocarcinomas of the urinary bladder were retrieved from the 1989–2003 surgical pathology archives at Washington University Barnes-Jewish Hospital and the 1986–2000 surgical pathology archives at the University of Chicago Hospitals. Clinical data were reviewed to ensure that they were indeed bladder primaries. Cases with any known history of adenocarcinoma in other organs were excluded from this study. All the tumors were derived from the bladder proper and morphologically reminiscent of well or moderately differentiated colorectal adenocarcinomas. None of the cases was associated with *Schistosoma haematobium* infestation or exstrophy. Hematoxylin and eosin (H&E)-stained slides were reviewed to confirm the diagnosis and to exclude conventional urothelial carcinomas with focal glandular differentiation.

A total of 40 cases of colorectal adenocarcinoma were selected for the study. This included 17 cases secondarily involving the bladder by direct extension based on clinical history. The remaining 23 cases were colorectal primaries randomly selected from the left and right colon. In addition, 14 conventional urothelial carcinomas (without glandular differentiation) were included for comparison.

Immunohistochemistry

Immunohistochemical studies were performed on 4-μm tissue sections employing the LSAB Plus system obtained from DAKO Corp. (Carpinteria, CA, USA) and the ABC kit obtained from Vector Laboratories (Burlingame, CA, USA), following the manufacturers' instructions with slight modifications. Briefly, deparaffinized tissue sections were first treated with 3% H_2O_2 for 15 min to inhibit endogenous peroxidase followed by antigen retrieval as described below. After incubation with blocking serum for 20 min, sections were incubated with the primary antibodies (described below) for 1 h at room temperature, followed by further incubation with biotinylated link antibody and peroxidase-labeled streptavidin. The staining was developed by reaction with diaminobenzidine substrate–chromogen solution followed by counterstaining with hematoxylin 7211 purchased from Richard-Allan Scientific (Kalamazoo, MI, USA).

The primary antibodies used in this study included mouse anti-CDX2 IgG1 (clone CDX2-88) obtained from BioGenex (San Ramon, CA, USA) used at 1:50 dilution, mouse anti-villin IgG1 (clone ID2C3) obtained from Immunotech (Marseille, France) used at 1:80 dilution, and prediluted rabbit anti-AMACR IgG obtained from Biocare Medical (Walnut Creek, CA, USA). The antigen retrieval conditions were microwave heating in 10 mM citrate buffer (pH 6.0) for 10 min for CDX2, 8 min for villin and 20 min for AMACR. In each experiment, a negative control was included in which the primary antibody was replaced by preimmune mouse or rabbit IgG. Positive controls used in this study were sections from a prostate that contained both adenocarcinoma and benign prostatic glands for AMACR, and a colorectal adenocarcinoma known to be positive for CDX2 and villin.

A tumor was recorded positive if greater than 5% of the tumor cells exhibited nuclear staining for CDX2, apical and/or cytoplasmic staining for villin, and cytoplasmic staining for AMACR. The positivity was stratified as diffuse (greater than 75% of the tumor cells stained) and focal (5–75%) which was further divided into three subgroups (5–25, 26–50 and 51–75%).

Statistical Analysis

Statistical analysis was performed using the Statistica software for windows (Tulsa, OK, USA). A *P*-value of <0.05, as determined by two-tailed Fisher's exact test or the χ^2-test with Yates continuity correction, was considered statistically significant.

Results

Table 1 compares the immunohistochemical findings in bladder and colorectal carcinomas. Specifically, all 40 colorectal adenocarcinomas (primary and secondary) exhibited positive nuclear staining for CDX2 (100%), with a strong and diffuse staining pattern (Figure 1a) seen in 37 cases (93%). This was in contrast to primary bladder adenocarcinomas where only eight of 17 cases (47%) showed nuclear CDX2 immunoreactivity ($P<0.0001$). Among them, four cases showed a focal staining pattern (Figure 1b); diffuse positivity was observed in only four cases (24%). None of the 14 urothelial carcinomas exhibited CDX2 immunoreactivity.

Similar to CDX2, villin was also universally expressed in colorectal adenocarcinomas (Figure 2a), with only one exception of a secondary tumor where positive staining was not detected. In total, 11 cases (65%) of primary bladder adenocarcinoma exhibited a variable degree of villin immunoreactivity (Figure 2b). Although the difference did not

Table 1 Comparison of CDX2, villin and AMACR expression among primary bladder adenocarcinoma (PBA), secondary colorectal adenocarcinoma (SCA), primary colorectal adenocarcinoma (PCA), and urothelial carcinoma (UC)

Marker	*Immunoreactivity*	*No. (%) positive*			
		PBA (n = 17)	*SCA (n = 17)*	*PCA (n = 23)*	*UC (n = 14)*
CDX2	Negative (<5%)	9 (53)	0	0	14 (100)
	5–25%	0	0	0	0
	26–50%	3 (18)	0	1 (4)	0
	51–75%	1 (6)	1 (6)	1 (4)	0
	>75%	4 (24)	16 (94)	21 (91)	0
Villin	Negative (<5%)	6 (35)	1 (6)	0	14 (100)
	5–25%	1 (6)	2 (12)	0	0
	26–50%	2 (12)	1 (6)	0	0
	51–75%	5 (29)	4 (24)	1 (4)	0
	>75%	3 (18)	9 (53)	22 (96)	0
AMACR	Negative (<5%)	6 (35)	4 (24)	8 (35)	12 (86)
	5–25%	2 (12)	3 (18)	1 (4)	1 (7)
	26–50%	3 (18)	1 (6)	1 (4)	0
	51–75%	4 (24)	4 (24)	3 (13)	0
	>75%	2 (12)	5 (29)	10 (44)	1 (7)

The % denotes the percentage of tumor cells positively stained.

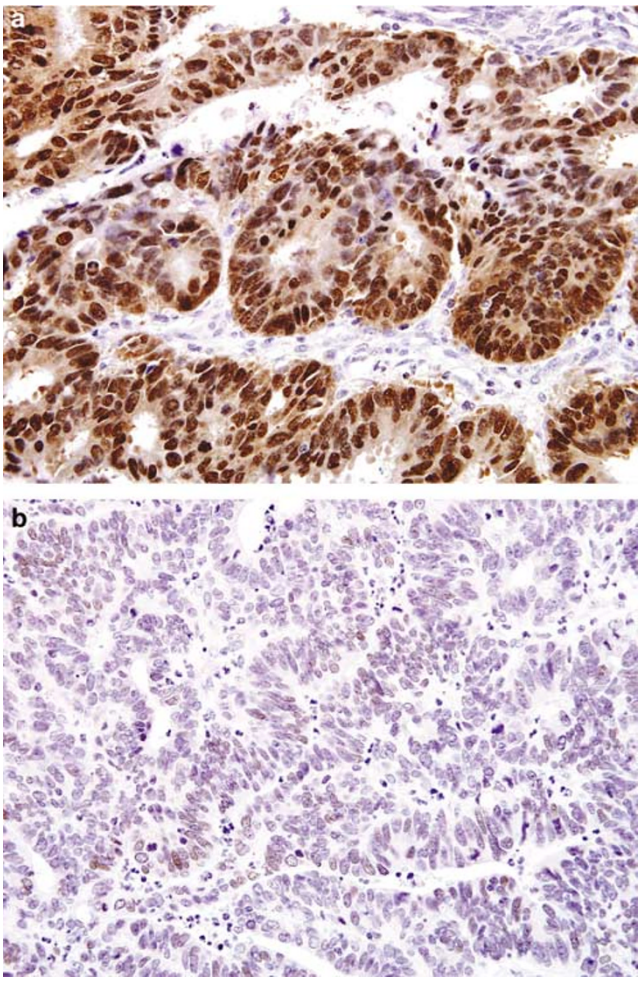

Figure 1 Strong and diffuse nuclear staining (with some cytoplasmic positivity) for CDX2 seen in a secondary colorectal adenocarcinoma (**a**). Note that essentially every tumor cell was positively stained in this case. The staining was focal and weaker in some of the primary bladder adenocarcinomas (**b**) (original magnification ×400).

reach the statistical significance when compared only with secondary colorectal adenocarcinoma ($P = 0.0854$), it was significant when primary colorectal adenocarcinomas were combined ($P = 0.0019$). Again, urothelial carcinomas were negative for villin expression.

The frequency of AMACR positivity was similar between bladder and colorectal adenocarcinomas, observed in 11 (65%) and 28 (70%) cases, respectively ($P = 0.694$). The staining patterns were also similar between these two types of tumors, with diffuse positivity (Figure 3a) seen in 12 and 38% of the cases, respectively ($P = 0.052$), and focal (Figure 3b) in the remaining cases. Only two urothelial carcinomas (14%) showed positive staining for AMACR.

Table 2 shows that primary bladder adenocarcinomas less frequently coexpressed CDX2/villin, CDX2/AMACR and CDX2/villin/AMACR when compared with colorectal counterparts ($P < 0.0001$, $P = 0.008$ and $P = 0.0023$, respectively). Bladder adenocarcinomas also appeared to less frequently coexpress villin/AMACR, but the difference from colorectal adenocarcinomas was not statistically significant ($P = 0.1005$).

In total, 12 primary bladder adenocarcinomas and nine secondary colorectal adenocarcinomas included in this study have been previously examined for the expression of CK7, CK20, thrombomodulin and β-catenin.[2] A distinct expression pattern, characterized by CK7 and thrombomodulin negativity, CK20, CDX2 and villin positivity, and nuclear β-catenin localization, was observed in six secondary colorectal adenocarcinomas (67%). This was in marked contrast to primary bladder adenocarcinomas where none of the cases studied showed this expression profile ($P = 0.02$).

Discussion

Primary adenocarcinoma occurs only rarely in the urinary bladder, accounting for 0.5–2% of all primary bladder tumors.[3] Therefore, the possibility of secondary involvement should always be considered when an adenocarcinoma is encountered in the bladder. In this regard, colorectal adenocarcinoma is of great importance not only because it is the most common secondary tumor involving the bladder,[1] but also because of its morphological similarity to primary bladder adenocarcinoma. The distinction between these two types of tumors can

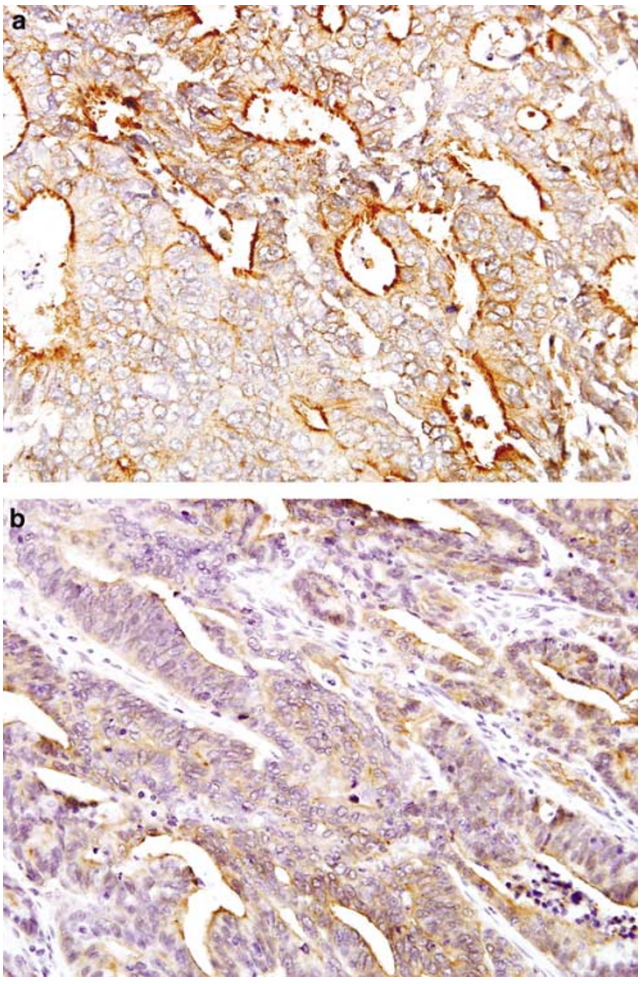

Figure 2 Diffuse cytoplasmic staining with linear apical accentuation for villin seen in a secondary colorectal adenocarcinoma (**a**). Similar staining pattern was also observed in some of the primary bladder adenocarcinomas (**b**) (original magnification $\times 400$).

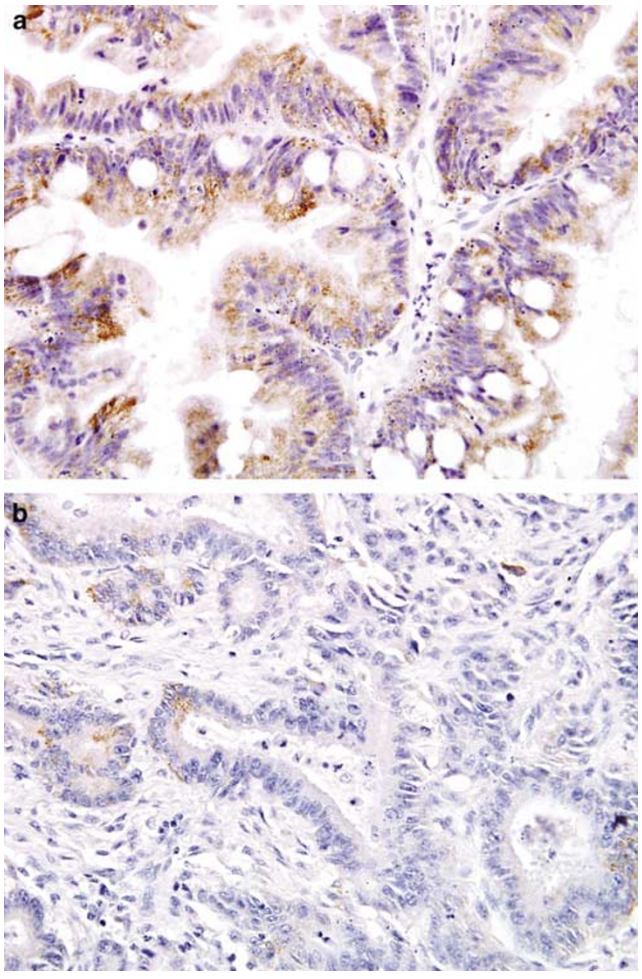

Figure 3 Diffuse cytoplasmic staining for AMACR noted in a primary bladder adenocarcinoma (**a**). Focal positivity was also noted in both primary bladder adenocarcinomas and colorectal adenocarcinomas. The example shown here was a colorectal adenocarcinoma (**b**) (original magnification $\times 400$).

be extremely challenging, particularly when the diagnostic material is a small biopsy and when the clinical information is incomplete. Currently, there are only a few immunomarkers available for the distinction but their use is limited because a significant proportion of bladder adenocarcinomas share the same immunophenotype with colorectal adenocarcinomas. It is thus desirable to evaluate additional markers with established tissue specificity, to exploit their potential in aiding in the differential diagnosis.

CDX2 is a mammalian homeobox gene encoding a nuclear transcription factor whose expression appears to be strictly limited to the intestinal epithelium.[4,5] In addition to serve a key role in regulating normal intestinal development and homeostasis, CDX2 may also function as a tumor suppressor.[4,6–8] In mouse model, reduced expression of CDX2 results in colonic polyposis via the activation of the mTOR pathway, the mammalian target of rapamycin,[9] that leads to chromosomal instability, accelerated G_1–S transition, suppression of apoptosis, decreased expression of p27 and increased cyclin E-associated kinase activity.[10] CDX2 also

Table 2 Coexpression of CDX2, villin and AMACR in primary bladder adenocarcinoma (PBA), secondary colorectal adenocarcinoma (SCA), primary colorectal adenocarcinoma (PCA), and urothelial carcinoma (UC)

Marker	*No. (%) positive*			
	PBA (n = 17)	*SCA (n = 17)*	*PCA (n = 23)*	*UC (n = 14)*
CDX2/villin	7 (41)	16 (94)	23 (100)	0
CDX2/AMACR	5 (29)	12 (71)	15 (65)	0
Villin/AMACR	8 (47)	13 (77)	15 (65)	0
CDX2/villin/AMACR	4 (24)	12 (71)	15 (65)	0

regulates the expression of p21,[11] a cyclin-dependent kinase inhibitor, and COX-2,[12] an essential enzyme in prostaglandin synthesis that is often upregulated in colorectal adenocarcinomas.

Despite the earlier findings that the expression of CDX2 was downregulated during colorectal carcinogenesis,[13,14] recent studies have clearly shown CDX2 to be a highly sensitive marker for colorectal adenocarcinoma.[15–18] The reported frequency of positive CDX2 immunostaining ranged from 98 to 100% in three series,[15,17,18] and was 86% in one study where tissue microarrays containing only one core per tumor were used.[16] The vast majority of the cases exhibited a strong and diffuse staining pattern. Although not entirely specific, the documented high sensitivity makes CDX2 a relatively reliable marker for adenocarcinoma of colorectal origin, which may be helpful in distinguishing metastatic colorectal adenocarcinoma from primary adenocarcinomas of the lung,[15,17,19] ovary,[17,20] and uterine cervix.[21]

CDX2 expression in primary bladder adenocarcinoma, a morphological mimic of colorectal adenocarcinoma, has not well been investigated to date. In fact, only three cases of bladder adenocarcinoma (including one urachal carcinoma) have been examined by Werling *et al*,[18] which all showed positive CDX2 nuclear staining. In the current study, we examined 17 cases and demonstrate that although nearly 50% of primary bladder adenocarcinomas express CDX2 in a similar fashion to colorectal adenocarcinomas, a significant proportion of the cases exhibit negative CDX2 immunoreactivity under the identical immunostaining conditions.

Villin is an actin-binding protein that serves a critical role in the maintenance of the brush border organization.[22] It is expressed with a relatively high specificity in epithelial cells of the gastrointestinal tract that possess brush border microvilli and in adenocarcinomas derived from these cells.[23–25] Using a multistep protocol including genomic, proteomic and tissue array profiling, villin was recently identified as the best diagnostic marker that distinguishes colorectal adenocarcinoma from ovarian adenocarcinoma.[26]

The potential use of villin in distinguishing primary bladder adenocarcinoma from colorectal adenocarcinoma has been investigated in a limited number of cases. In the study by Tamboli *et al*,[27] positive villin immunostaining was observed in all four primary bladder adenocarcinomas and all 13 colorectal adenocarcinomas they examined. The three bladder adenocarcinomas examined by Werling *et al*[18] were also positive for villin expression. By examining a larger number of cases in this study, we show that while the majority of bladder adenocarcinomas stain positive for villin, one-third of the cases exhibit negative immunoreactivity in contrast to only one of 40 colorectal adenocarcinomas included in the study.

AMACR, also known as P504S, is a well-characterized enzyme that serves an essential role in β-oxidation of dietary branched-chain fatty acids and bile acid intermediates.[28] It is a novel immunomarker for prostatic adenocarcinoma and its precursor lesion high-grade prostatic intraepithelial neoplasia.[29] Recently, AMACR has been shown to be expressed in 69–83% of colorectal adenocarcinoma,[30,31] although the underlying mechanism(s) remains to be established. Its expression has also been detected in approximately 30% of urothelial carcinomas,[31,32] but has not yet previously been examined in bladder adenocarcinomas. The frequency of positive AMACR immunoreactivity in colorectal adenocarcinomas in our study (70%) is comparable to those reported previously.[30,31] However, a similar frequency (65%) is also observed in adenocarcinomas of the bladder origin.

In summary, the data presented in this report extend our previous observations[2] that primary bladder adenocarcinoma is not entirely identical immunophenotypically to colorectal adenocarcinoma. Since essentially all colorectal adenocarcinomas express CDX2 and nearly all express villin, negative staining of one or both of these two markers strongly suggests a bladder primary in the appropriate clinical setting. This may be particularly true of CDX2 since it appears to be less frequently expressed than villin in bladder adenocarcinomas. Immunostains for CDX2 and villin are thus useful additions to the limited list of diagnostic markers (including CK7, CK20, thrombomodulin and β-catenin) for the distinction between primary adenocarcinoma of the bladder and secondary colorectal adenocarcinoma. On the contrary, AMACR does not appear to contribute to the differential diagnosis. In the future, it would be of interest to perform gene expression profiling to identify additional markers that are positive in bladder adenocarcinomas but negative in colorectal adenocarcinomas to complement CDX2 and villin.

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