

Reassessment of Id1 Protein Expression in Human Mammary, Prostate, and Bladder Cancers Using a Monospecific Rabbit Monoclonal Anti-Id1 Antibody

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Abstract

Id proteins are a class of dominant-negative antagonists of helix-loop-helix transcription factors and have been shown to control differentiation of a variety of cell types in diverse organisms. Although the importance of Id1 in tumor endothelial cells is well established, the expression and role of the Id1 protein in human cancer cells is controversial. To explore this issue, we developed and characterized a highly specific rabbit monoclonal antibody against Id1 to assess its expression in human breast, prostate, and bladder malignancies. Our results show that in usual types of human mammary carcinomas, the Id1 protein is expressed exclusively in the endothelium. Interestingly, we detected nuclear expression of the Id1 protein in the tumor cells in 10 of 45 cases of poorly differentiated and highly aggressive carcinoma with metaplastic morphology. Similarly, only 1 of 30 prostate cancer samples showed Id1-positive tumor cells, whereas in almost all, endothelial cells showed high Id1 expression. Intriguingly, whereas normal prostate glands do not show any Id1 protein expression, basal layer cells of benign prostate glands in proximity to tumors expressed high levels of the Id1 protein. In contrast to the lack of Id1 expression in the usual types of mammary and prostate cancers, the majority of transitional cell bladder tumors showed Id1 protein expression in both tumor and endothelial cells. These results suggest that further refinement of Id1 expression patterns in a variety of tumor types will be necessary to identify and study the functional roles played by Id1 in human neoplastic processes. (Cancer Res 2006; 66(22): 10870-7)

Introduction

The Id proteins are a class of dominant-negative antagonists of helix-loop-helix transcription factors and have been shown to control differentiation of a variety of cell types in diverse organisms (for reviews, see refs. 1, 2). The expression and role of the Id1 protein in epithelial cancer cells, however, is controversial. For example, some investigators have reported that the neoplastic cells of breast, prostate, and bladder carcinomas express the Id1 protein,

and that this finding correlates in some cases with poor prognosis (3–6). Others, however, have reported Id1 protein expression in mammary tumors only in the endothelial cells of blood vessels associated with these lesions (7). Interestingly, mRNA expression is observed in a broad spectrum of tumors, suggesting posttranscriptional control mechanisms perhaps at the level of protein destabilization (8). Genetic elimination of the Id1 locus in mice predisposed to develop breast or prostate neoplasias shows a profound effect on vascularization and tumor integrity but little effect on tumor initiation: results are consistent with an endothelial cell-specific localization of the protein product (7, 9). Mobilization of endothelial cell progenitors from the bone marrow is also severely perturbed in Id knockout mice consistent with this hypothesis (10, 11). On the other hand, a role for Id1 in modulating tumor epithelial cell behavior is suggested by the fact that overexpression of Id1 in cell lines results in increased invasiveness and metastatic potential of these cells, whereas reduction of high levels of Id1 present endogenously in these lines leads to an inhibition of these properties (12–17).

It is likely that the discrepancy in the expression data is due to variability in the specificity of antibodies used in these studies that are obtained from commercial sources. To unequivocally determine the expression pattern of the Id1 protein in human breast, prostate, and bladder cancers, we developed a rabbit monoclonal antibody against purified murine Id1, which cross-reacts with the human orthologue. The rationale for isolating a monoclonal antibody with this cross-reactivity is that the demonstration of the specificity required in immunohistochemistry experiments is most easily achieved using mouse knockout strains as negative controls. Thus, to insure antibody specificity, we have imposed the following criteria: the antibody preparation must be monospecific in Western blots of tissue extracts that contain the Id1 protein but negative over a wide molecular weight range in extracts from which the gene product is known to be absent. In addition, we demanded that the signals detected by immunohistochemistry in a murine tissue known to express Id1 are absent in Id1 knockout controls.

By these criteria, we have identified a rabbit monoclonal antibody (195-14), which is suitable for human immunohistochemistry, and have used it to measure Id1 protein expression in human breast, prostate, and bladder tumor samples. Our results show that the Id1 protein is present in some breast tumor cells derived from a restricted subset of human mammary tumors known as metaplastic carcinomas, usually associated with a poor prognosis. Despite the purported role of Id1 in breast cancer metastasis, lymph node metastatic lesions of the common types of breast cancer also did not show Id1 staining outside of the endothelium. In human

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prostate cancers, only 1 of 30 samples showed *Id1* protein expression in the epithelial cells. However, basal layer cells from benign seeming glands in proximity to tumor areas showed high *Id1* protein expression. The most widespread and consistent expression of *Id1* among all tumors examined was seen in bladder cancer samples with a moderate to high expression rate of 28%. Importantly, all of the immunohistochemical data are internally controlled as positive endothelial cell staining is observed in essentially all tumors examined.

These studies show that a careful reexamination of *Id1* protein expression in human tumors is warranted to unequivocally identify specific lesions that may be influenced by *Id1* protein expression.

Materials and Methods

Development of Rabbit Monoclonal Anti-*Id1* Antibody

Antibody development. Rabbits were immunized with an *Id1* fusion protein. To generate rabbit hybridomas, splenocytes from the immunized rabbit were isolated and fused with the rabbit hybridoma fusion partner (Spieker-Polet et al., *Proceeding National Academy Science USA* 1995;92:9348-9352 and U.S. patent 5,675,063), which was done at Epitomics, Inc. (Burlingame, CA). Hybridoma clones secreting the *Id1* antibody were selected through ELISA screens of hybridoma supernatants (data not shown). Further screen of the ELISA-positive clones by Western blot and immunohistochemistry identified hybridoma clones suitable for these two applications.

To obtain anti-*Id1* IgG cDNA, total RNA was isolated from the desired hybridoma using RNeasy kit (Qiagen, Valencia, CA). cDNA was made from total RNA by oligo-dT-directed reverse transcription (reverse transcriptase, Promega Improm II, from Promega, Madison, WI). Rabbit IgG cDNAs were amplified with heavy- or light-chain specific primers (designed by Epitomics and synthesized by Elim Biopharmaceuticals, Hayward, CA) by PCR (DNA polymerase, TaqPlus Precision, from Stratagene, La Jolla, CA). The IgG heavy- and light-chain sequences were obtained by sequencing the PCR products (sequencing services provided by Elim Biopharmaceuticals). For recombinant expression of anti-*Id1* antibody, the PCR product was cloned into pCEP4 or pTT5 vectors. Recombinant *Id1* antibody was transiently expressed in HEK 293 cells by plasmid DNA transfection using standard protocols.

Western blotting. Whole-cell extracts from HeLa cells, wild-type murine embryonic fibroblasts (MEF), and *Id1*^{-/-} MEFs were quantified, and 40 µg of each sample was used for Western blot analysis. Working concentrations of specific antibody lots from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) and BioCheck, Inc. (Foster City, CA) were determined by the manufacturers' estimates. The primary antibodies used were mouse-specific *Id1* rabbit monoclonal 37-2 (BioCheck; concentration 0.05 mg/mL, diluted 1:1,000), anti-mouse-human *Id1* cross-specific rabbit monoclonal 195-14 (BioCheck; concentration 0.51 mg/mL, diluted 1:1,000), and anti-*Id1* polyclonal SC-488 (Santa Cruz Biotechnology; diluted 1:1,000).

Immunohistochemistry. Immunohistochemical detection was done with a Discovery XT system (Ventana Medical Systems, Tucson, AZ). Slides were blocked with 10% normal goat serum and 2% bovine serum albumin for 30 minutes. Primary antibody incubation was done for 2 hours with anti-mouse-specific *Id1* rabbit monoclonal 37-2 (diluted 1:100) and anti-mouse-human *Id1* cross-specific rabbit monoclonal 195-14 (diluted 1:500) followed by incubation with biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA; 1:200 dilution) for 8 minutes. Vascular endothelial cells were identified using anti-CD31 (SC-1506 antibody, Santa Cruz Biotechnology). Endogenous biotin blocking kit, blocker D, streptavidin-horseradish peroxidase, and 3,3'-diaminobenzidine detection kit were used according to the manufacturer's instructions (Ventana Medical Systems).

Immunohistochemistry Staining for the *Id1* Protein: Human Material

All human tissue samples were formalin fixed and paraffin embedded. Immunohistochemical staining for *Id1* was done using anti-*Id1* clone 195-14.

Breast samples. Immunohistochemical staining for *Id1* was done on 5-µm-thick sections of the following breast tissue samples: normal tissue obtained by reduction mammoplasty from six women with no personal history of breast carcinoma or atypia; usual subtypes of breast carcinoma, both primary and metastatic to lymph nodes, from 28 patients and one case of metaplastic breast carcinoma. In 16 patients, two to four sections were available for evaluation, whereas one section was analyzed for the rest. Overall, 56 slides were analyzed in this group.

Tissue microarrays (TMA) containing triplicate 0.6-mm tissue cores of each sample were constructed using an automated microarrayer (Beecher Instruments, Sun Prairie, WI). Five-micrometer-thick sections of the TMAs were used for immunohistochemical studies. The cases in the TMAs consisted of so-called "triple negative" [meaning estrogen receptor (ER), progesterone receptor (PR), and HER-2/*neu* negative] breast carcinoma samples from 122 patients, including 44 cases of metaplastic carcinomas, the latter being of proven epithelial origin but with pseudosarcomatous morphology.

Two slides of commercially available (Folio Biosciences, Columbus, OH) TMAs were also analyzed. The two slides included a total of 143 cores, of which 62 samples were common types of breast cancer, and the rest of the samples were benign (slides #ARY-HH0058, "Breast Carcinoma and Normal TMA" and #ARY-HH0088, "Breast Carcinoma and Matching Normal TMA").

Prostate samples. In total, prostate cancer paraffin sections from 30 patients were studied. Three of them were commercially available (Folio Biosciences; CA-PR-0001). These tumors were of moderately to poorly differentiated pathology (Gleason score of 6-9/10). Additionally, three different normal prostate sections (Folio Biosciences) were evaluated as well (NOR-PR-0001).

One slide of a commercially available (Folio Biosciences) TMA was analyzed. In total, thirty 1.5-mm cores were analyzed, which were derived from 17 normal prostates, 6 prostate hyperplasias, 1 smooth muscle, and 6 prostatic carcinoma cases (slide #ARY-HH0068, "Prostatic Carcinoma, Hyperplasia, and Normal TMA").

Bladder samples. Ten full-section samples of bladder cancer were studied. Of these, four were carcinoma *in situ*, four were high-grade invasive urothelial carcinoma, and two were high-grade urothelial carcinoma invasive into normal prostate glands.

One TMA of 57 cases of high-grade invasive urothelial carcinoma was constructed and tested.

Additionally, one slide of a commercially available (Folio Biosciences) TMA was analyzed. This TMA comprised of eighty 1.5-mm cores, including 40 urothelial carcinoma (low and high grade) cases, 29 normal bladder tissues from bladder cancer specimens, and 11 normal controls (slide #ARY-HH0087, "Bladder Carcinoma and Normal TMA").

Results

Anti-*Id1* Antibody Development

To develop a reliable *Id1*-specific antibody, we screened 17 anti-mouse/human *Id1* rabbit monoclonal hybridomas from Biocheck, by Western blotting and immunohistochemical staining. Mouse-specific monoclonals 37-2 and 195-14 reacted with a single protein species of ~17 kDa in wild-type MEFs (consistent with the molecular weight of murine *Id1*) but failed to detect a similar sized band in *Id1*^{-/-} MEFs, thus establishing their specificity for mouse *Id1* (Fig. 1A and B). Of note is that a faint nonspecific 34-kDa band appears on Western blots when high concentrations of the 37-2 antibody are used on the different extracts, including that from *Id1* null MEFs (Fig. 1A). However, nonspecific bands are completely absent when comparable titers of 195-14 antibody are used (Fig. 1B). To examine cross-reactivity with human *Id1*, whole-cell extracts from HeLa cells, which express endogenous human *Id1*, were used in a Western blot analysis. Clone 37-2 failed to detect the human *Id1* protein, whereas 195-14 showed a prominent band at the appropriate molecular weight (compare Fig. 1A and B). Thus, clone

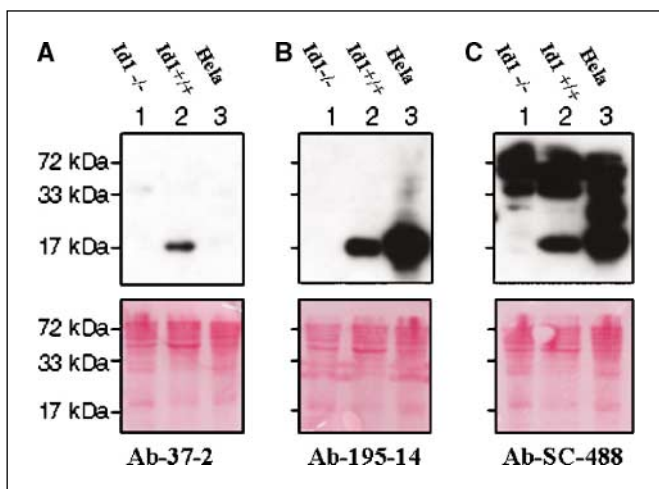


Figure 1. *Top*, Id1 Western blot; *bottom*, corresponding Coomassie stain. *Lane 1*, whole-cell extract from Id1^{-/-} MEFs; *lanes 2*, whole-cell extract from Id1^{+/+} MEFs; *lane 3*, whole-cell extract from HeLa cells. *A*, rabbit monoclonal anti-Id1 antibody 37-2. *B*, rabbit monoclonal anti-Id1 antibody 195-14. *C*, rabbit polyclonal anti-Id1 antibody SC-488.

37-2 is specific for mouse Id1, whereas clone 195-14 is cross specific and detects both mouse and human Id1.

We also tested a commercially available anti-Id1 antibody from Santa Cruz Biotechnology (SC-488) in our Western blot analysis. This antibody has been used in the majority of Id1 immunohistochemical studies. The Santa Cruz antibody detects the 17-kDa Id1 band in HeLa extracts as well as in a wild-type MEF extract. However, a number of additional higher molecular weight bands of equal or greater intensity are detected in addition to the 17-kDa Id1

band (Fig. 1C), making this preparation unsuitable for unequivocal Id1 detection in tissue samples.

Based on the Western blot analysis, clones 37-2 and 195-14 were selected for further immunohistochemical analysis. Immunohistochemical staining was done on paraffin-embedded HER-2/*neu*-dependent mouse mammary tumor sections from both wild-type and Id1^{-/-} animals. As Id1 is a known marker of endothelial cells in tumor blood vessels, this staining served as an internal positive control. Both clones 37-2 and 195-14 positively stained endothelial cells in wild-type tumors, whereas the tumor epithelial cells were negative (Fig. 2A and C). Additionally, Id1^{-/-} tissue was completely negative, thus establishing the Id1 specificity of these antibodies (Fig. 2B and D). Because two independent antibody clones that recognize different epitopes (as evidenced by differences in human Id1 cross-reactivity) give identical staining patterns, it is extremely unlikely that cell type-specific epitope masking accounts for the lack of Id1 staining in the tumor cells. Based on these analyses, clone 195-14 was chosen for immunohistochemistry of a variety of human tumor samples.

Id1 Protein Expression in Human Breast, Prostate, and Bladder Tumors

Epithelial staining in normal human breast tissue and neoplasms. The presence of the Id1 protein was assessed in normal human mammary tissue and in a wide range of primary mammary neoplasms. Tissue obtained by reduction mammoplasty from six patients with no personal history of breast carcinoma or atypia was used to assess the expression of Id1 in normal breast. No immunoreactivity for Id1 was identified in the normal mammary epithelium and myoepithelium lining ducts and lobules, as well as in the mammary stroma from any of the six samples (representative section shown in Fig. 3B). Vascular endothelium was identified by CD31 staining on consecutive tissue sections

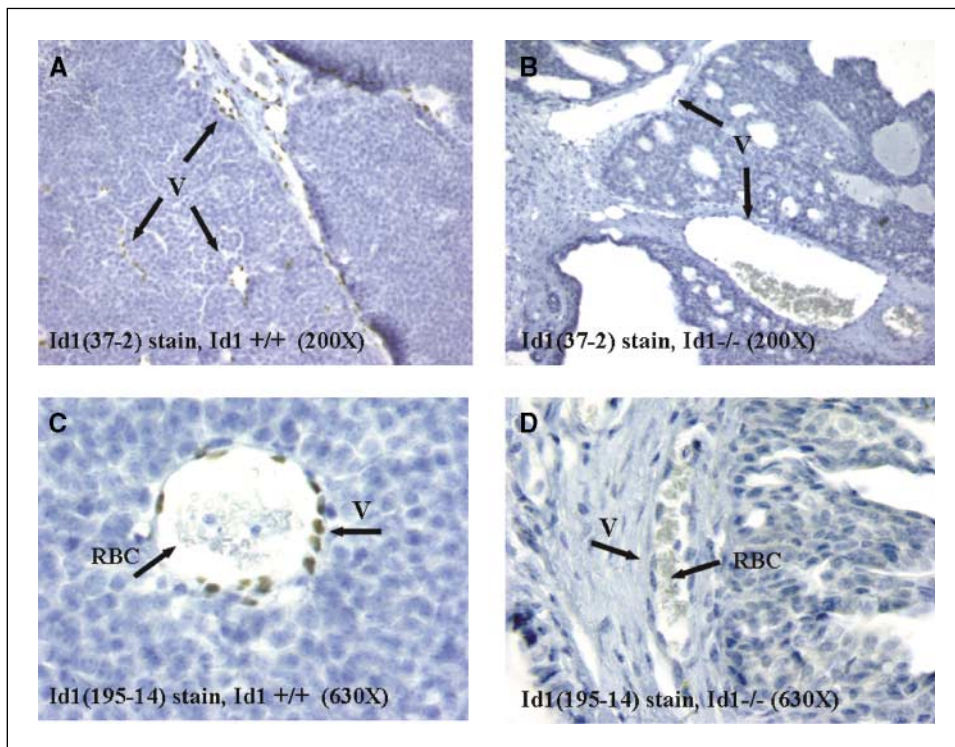
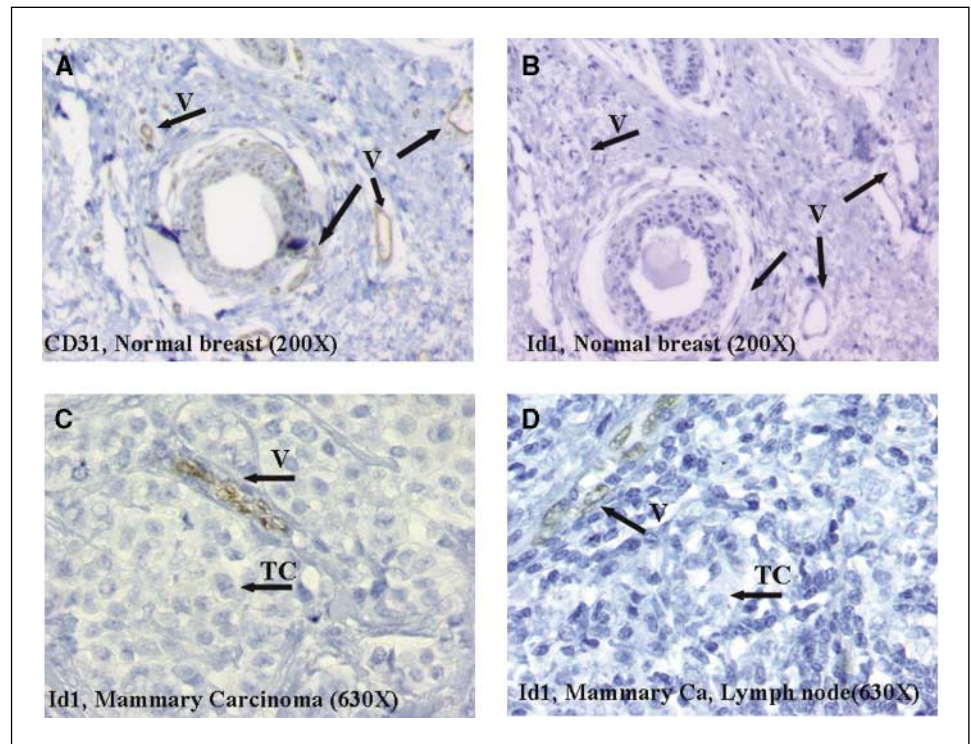


Figure 2. *A*, HER-2/*neu* mammary tumor in Id1^{+/+} mouse stained for Id1 using anti-Id1 antibody 37-2. Arrow pointing to Id1-expressing vascular (V) endothelial cells. Magnification, $\times 200$. *B*, HER-2/*neu* mammary tumor in Id1^{-/-} mouse stained for Id1 using anti-Id1 antibody 37-2. Arrows pointing to Id1-deficient vascular endothelial cells. Magnification, $\times 200$. *C*, HER-2/*neu* mammary tumor in Id1^{+/+} mouse stained for Id1 using anti-Id1 antibody 195-14. Arrow pointing to Id1-expressing vascular endothelial cells. Blood RBC. Magnification, $\times 630$. *D*, HER-2/*neu* mammary tumor in Id1^{-/-} mouse stained for Id1 using anti-Id1 antibody 195-14. Arrows pointing to Id1-deficient vascular endothelial cells. Blood RBC. Magnification, $\times 630$.

Figure 3. Human mammary tissues and tumors stained for Id1 using anti-Id1 antibody 195-14. V, vessels; TC, tumor cells. A, CD31 stain for endothelial cells in a normal human mammary gland. Magnification, $\times 200$. B, consecutive section of (A) stained for Id1 using anti-Id1 antibody 195-14. Notice lack of vascular staining. Magnification, $\times 200$. C, invasive ductal carcinoma ("usual type"). Notice only vascular endothelial cells express the Id1 protein. Magnification, $\times 630$. D, invasive ductal carcinoma ("usual type") metastatic to a lymph node. Notice only vascular endothelial cells express the Id1 protein. Magnification, $\times 630$.



(Fig. 3A). Vessels that expressed CD31 did not express the Id1 protein in this normal tissue (Fig. 3B).

Nineteen invasive ductal carcinomas, three *in situ* ductal carcinomas, and six invasive lobular carcinomas (five also containing foci of carcinoma *in situ*) from a total of 28 patients were evaluated as representative of the most frequent types of mammary epithelial neoplasia. In addition, two slides of commercially available TMAs with 62 samples of common types of breast cancer were analyzed. The neoplastic cells from all these common variants of primary breast carcinomas were negative for Id1 protein expression (representative sample shown in Fig. 3C). Carcinomas metastatic to lymph nodes from seven of the patients above were studied as well, and the neoplastic cells of all were found negative for the Id1 protein (Fig. 3D). Id1 staining in the endothelium of these samples served as an internal positive control (see also below).

We assessed Id1 expression in 122 unusual primary breast carcinomas, all of them proven to be negative for PR, ER, and HER-2/*neu*. Of these 122 triple negative cases, 78 were poorly differentiated breast carcinomas, retaining epithelial morphology. Only 2 of these 78 highly aggressive mammary carcinomas showed weak nuclear immunoreactivity for the Id1 protein (Fig. 4B), whereas the rest only showed staining limited to the endothelium of blood vessels (Fig. 4A).

Forty-four of the 122 triple negative cases as well as one case represented by a standard section were metaplastic mammary carcinomas. This rare subgroup of malignant mammary tumors shows at least focal immunoreactivity for cytokeratins but has sarcomatoid morphology, with spindle cells and/or myxoid and chondroid areas (18). The neoplastic cells of 10 of these 45 metaplastic carcinomas (22%) showed strong positive nuclear immunoreactivity for the Id1 protein (Fig. 4C and D).

In summary, the Id1 protein was only observed in tumor cells from a subset of unusual and aggressive types of mammary cancers.

Epithelial staining in normal human prostate tissue and neoplasms. No immunoreactivity for Id1 was identified in either the epithelial or basal cells in any of the 20 normal prostate tissue samples or the six benign prostatic hyperplasia samples that were examined (Fig. 5A). Of the 30 cases of moderately to poorly differentiated prostatic adenocarcinoma (Gleason score 6-9), only one case expressed the Id1 protein (Fig. 5B and C).

Surprisingly, in all the prostate cancer sections examined, on average, 40% (range = 6-93%) of benign prostate glands showed moderate to high nuclear Id1 expression in the basal layer cells (Fig. 5D). This suggests that Id1 expression may be elevated in response to adjacent tumor influence and may perhaps serve as an early marker of disease.

Epithelial staining in normal human bladder tissue and neoplasms. Normal bladder tissue from 11 patients with no cancer history was evaluated for Id1 expression on TMA. Only 2 of the 11 cancer free samples showed weak nuclear Id1 protein expression (18%), whereas the rest were all negative (Fig. 6A1 and A2).

Absent or weak Id1 staining was also noted in most normal urothelium areas in four slides containing bladder carcinoma *in situ* (Fig. 6C). Interestingly, however, in 29 cases assembled in a TMA and 10 whole sections, benign urothelium adjacent to neoplastic invasive urothelium showed weak to moderate Id1 protein expression in 74% (29 of 39) of the samples (Fig. 6B1).

Tumor samples of a total of 107 bladder tumors represented in full sections as well as on TMAs were evaluated for Id1 protein expression. In the four sections that contained urothelial carcinoma *in situ*, the Id1 protein was overexpressed in the neoplastic cells. This expression was stronger than that of adjacent nonneoplastic urothelium (Fig. 6C).

The remaining 103 samples included invasive bladder urothelial carcinoma (6 full sections and 97 assembled in TMAs). The six full sections, two of which were of bladder cancer-invading prostate glands, were all high-grade tumors and showed significant nuclear

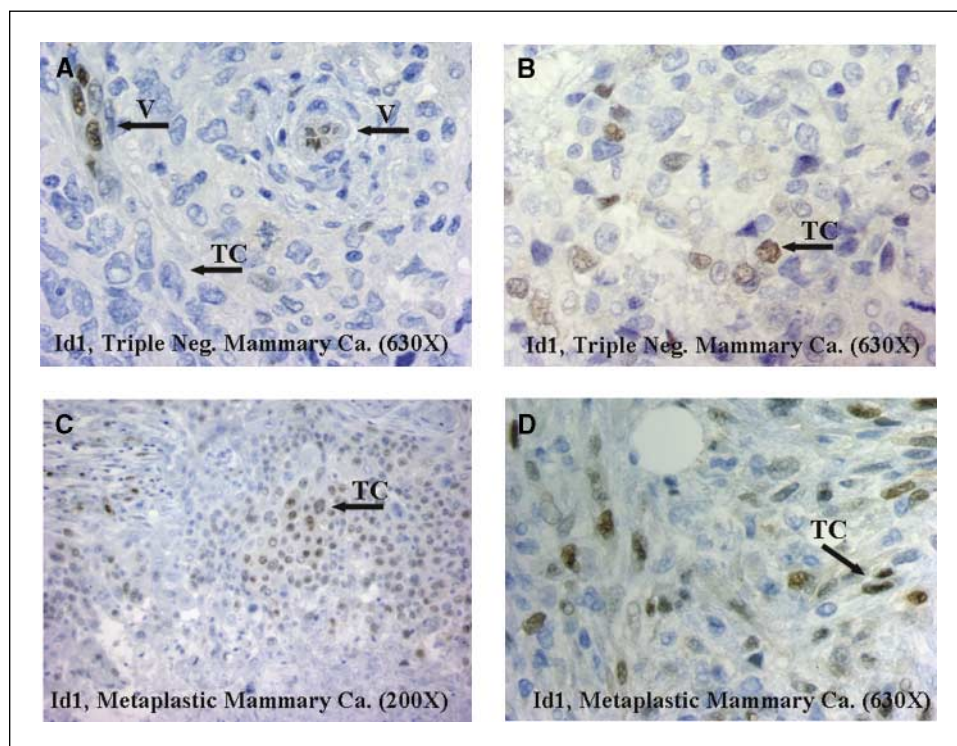


Figure 4. Human mammary tumors stained for Id1 using anti-Id1 antibody 195-14. V, vessels; TC, tumor cells. A, invasive ductal carcinoma: "triple negative" tumor where only vascular cells express the Id1 protein. Magnification, $\times 630$. B, invasive ductal carcinoma: triple negative. Notice several tumor cells express the Id1 protein. C, human mammary metaplastic carcinoma stained for Id1 using anti-Id1 antibody 195-14. Magnification, $\times 200$. D, metaplastic mammary carcinoma. Magnification, $\times 630$.

Id1 expression in the malignant urothelial cells (Fig. 6B2). Twenty of the remaining 97 tumor TMA samples showed moderate to high nuclear Id1 expression in the neoplastic tumor cells. In summary, a total of 30 of 107 (28%) of neoplastic bladder samples showed moderate to high expression of the Id1 protein as opposed to 0% moderate to high and 18% weak staining of noncancerous bladders.

Endothelial Id1 Protein Expression in Human Breast, Prostate, and Bladder Tumors

Endothelial staining in normal human mammary tissue and neoplasms. For each case of normal mammary tissue and of breast carcinoma of the usual types, blood vessels were identified based on morphology. Several consecutive sections were used to confirm

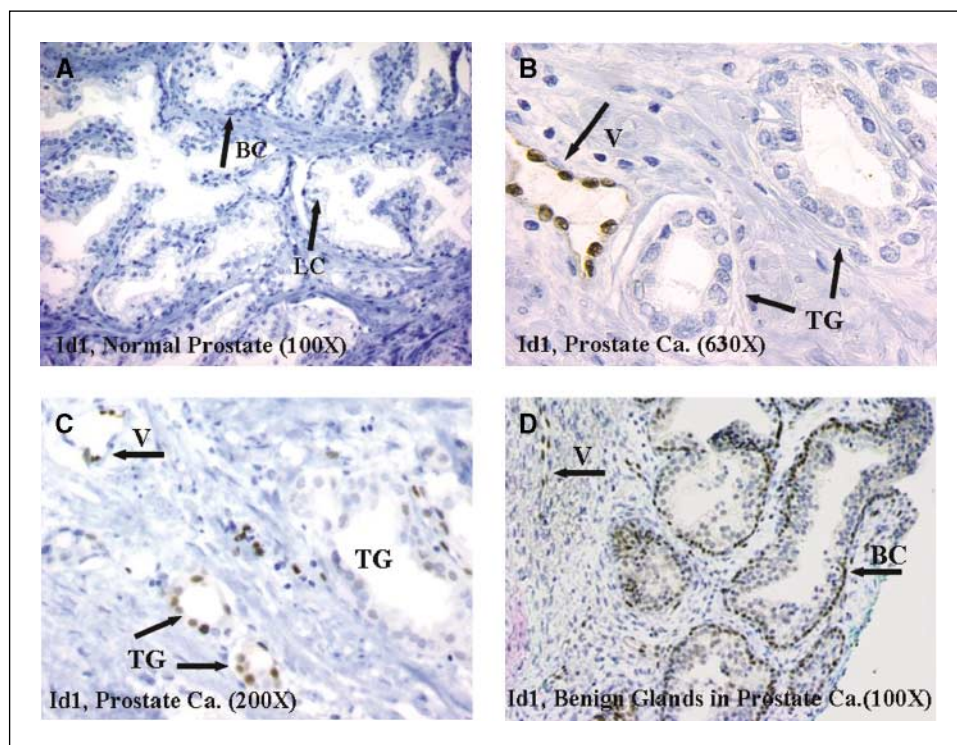
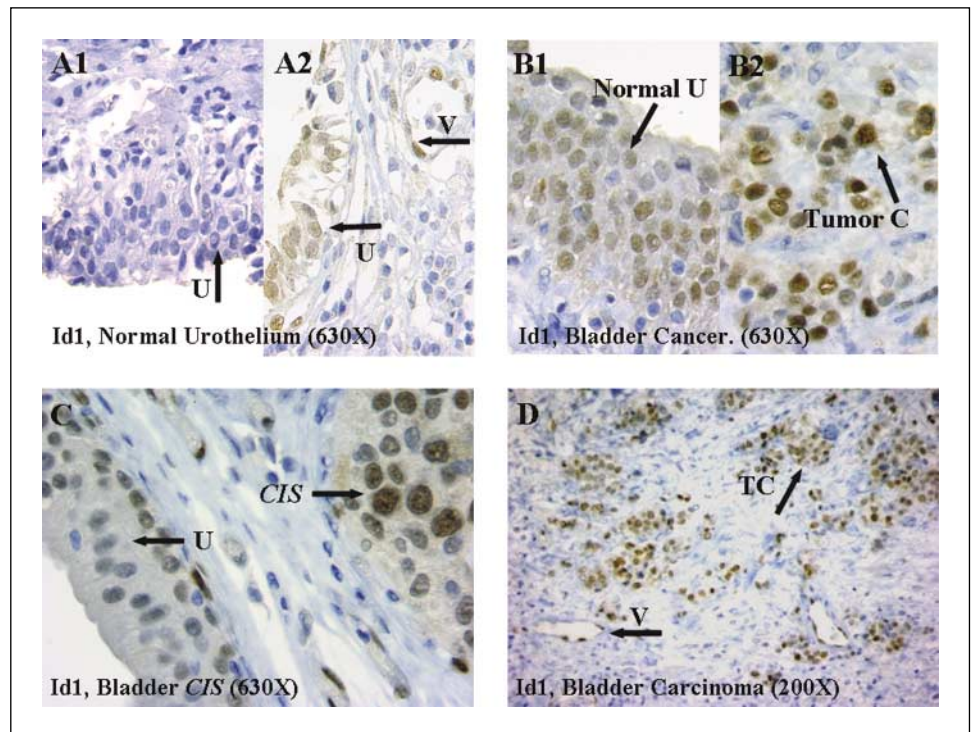


Figure 5. Id1 staining in human prostate tissues. A, normal prostate tissue; no Id1 expression. BC, basal cells; LC, luminal epithelial cells. Magnification, $\times 100$. B, prostate carcinoma. Note exclusive endothelial cell expression of Id1. TG, tumor glands; V, vessel. Magnification, $\times 630$. C, prostate carcinoma. Note neoplastic cell and vascular endothelial cell expression of the Id1 protein. Magnification, $\times 200$. D, benign prostate glands in a prostate cancer section showing basal layer cells and vascular endothelial cells expressing Id1. BC, basal cells of benign gland. Magnification, $\times 100$.

Figure 6. Id1 stain; human bladder tissues. A, normal urothelium. A1, urothelium that does not express the Id1 protein (U). A2, urothelium and vascular endothelial cells (V) that do express Id1. Magnification, $\times 630$. B1, weak to moderate levels of Id1 expression in normal urothelium contained in a bladder cancer sample. Magnification, $\times 630$. B2, invasive urothelial carcinoma tumor cells (TC) expressing high Id1 levels. Magnification, $\times 630$. C, bladder urothelial carcinoma *in situ* (CIS) cells express strong levels of nuclear Id1. Very low levels of Id1 expression are detected in normal urothelium in the vicinity of carcinoma *in situ*. Magnification, $\times 630$. D, invasive urothelial carcinoma cells and vascular endothelial cells expressing Id1. Magnification, $\times 200$.



vessel identification by positive immunoreactivity for the endothelial cell marker CD31. The Id1 protein-positive vascular endothelial cells were manually counted. The endothelial cell density was determined by dividing the number of endothelial cells by the area of the section (endothelial cells per cm^2). The vascular endothelial cells of the normal breast displayed no to very focal and weak immunoreactivity for the Id1 protein, with an average density of 38.3 endothelial cells per cm^2 (range = 0-136; Fig. 3A and B).

In contrast, vascular endothelial cells from patients with the common forms of mammary cancer showed strong immunoreactivity to the Id1 protein (Fig. 3C). The average density of positive endothelial cells was 241.6 endothelial cells per cm^2 (range = 2-1,267). Nonetheless, we observed no correlation between density of Id1-positive endothelial cells and tumor histology. In the unusual types of breast carcinoma (i.e., poorly differentiated and metaplastic), which were studied by TMAs, vascular endothelial cells when present were positive for the Id1 protein (Fig. 4A). However, due to the small area of the sections, their density could not be scored reliably.

Endothelial staining in normal human prostate tissue and neoplasms. Endothelial cells of normal and hyperplastic prostates showed no detectable Id1 protein expression. In contrast, in all but one prostate tumor sample (29 of 30) the endothelial cells expressed the Id1 protein irrespective of the proximity of these vessels to the tumor area (Fig. 5B).

Endothelial staining in normal human bladder tissue and neoplasms. Endothelial cells of tumor-associated vessels in 10 slides of *in situ* and invasive carcinomas showed high Id1 expression. In TMA samples of invasive urothelial carcinoma, Id1 expression was observed in 58 of 97 (60%) cases (strong expression in 29 cases and weak in 29 cases; Fig. 6D; Supplementary Fig. S1). In contrast, strong Id1 expression was not observed in endothelial cells of any normal bladder samples, but weak expression was detected in 5 of 11 samples.

In summary, in most cases, endothelial cells in all three of the tumor types examined (breast, prostate, and urinary bladder) expressed high Id1 protein levels with weak or no staining of the corresponding endothelium in normal tissue.

Discussion

Id1 has been shown to be involved in fundamental regulatory cellular functions, such as inhibition of differentiation, cell cycle regulation, and proliferation. It also plays a role in non-cell-autonomous growth and differentiation through intercellular interactions (1, 19). Using a commercially available antibody, elevated Id1 protein expression levels have been reported in diverse human cancers, including epithelial malignancies of ovary, colon, pancreas, nasopharynx, stomach, and esophagus; in neural tumors; melanomas; Ewing's sarcoma; seminoma; leukemia; and, more recently, bladder transitional cell carcinoma (1, 5). Previous studies in prostate cancer found intense immunoreactivity in some tumor cells and related this to a poorer prognosis (4, 6). Similarly, studies in mammary cancers found that a majority of the tumors showed some level of Id1 protein expression (3). However, these results were controversial as other reports described Id1 expression only in the endothelium of human breast cancer (7).

In the current study, we showed the lack of specificity of the commercially available anti-Id1 antibody, confirming prior observations (20). Although there may have been lot-to-lot variability in the specificity of the commercially available antibody, a more reliable reagent was clearly needed. We have, therefore, developed and characterized a new, highly specific antibody to Id1, which is suitable for accurate immunohistochemical assessment of Id1 protein expression in mouse and human tissues. Using this antibody, we showed that Id1 protein expression is not detected in the neoplastic epithelium of the usual types of human mammary and prostate carcinomas. Importantly, despite the purported role

of Id1 in breast cancer metastasis, lymph node metastatic lesions examined did not show Id1 staining outside of the endothelium. We also found that most of poorly differentiated triple negative breast carcinomas were also negative for Id1 expression. Only metaplastic mammary carcinomas showed a considerable proportion (22%) of strong nuclear Id1 protein expression. This finding may be related to the altered differentiation pattern observed in these cells. In addition, the aggressive properties of these cells may be due to Id1 expression, a hypothesis that can now be tested experimentally. It will be of interest to determine if such behavior is influenced by the secretion of short-acting factors, such as matrix metalloproteinase 2 (MMP2; ref. 21) or MMP9 (22), known to be under Id1 control. Highly aggressive metastatic breast neoplasms, which are negative for ER and HER-2/*neu*, present a therapeutic challenge due to their limited treatment options. The expression of Id1 in a subset of these tumors supports its significance in determining the biological behavior of these tumors and therefore may prove to be a useful target in their treatment.

Human prostate cancer shares a variety of biological similarities with breast tumors, such as glandular origin (i.e., forming adenocarcinomas), hormonal involvement in the etiology, and high incidence of bone metastasis. Similar to the common types of breast cancer, in the vast majority of prostate cancer samples, tumor cells showed no Id1 protein expression. In our study, tumor cells of only one of the 30 cases examined were positive for Id1. However, the significance of this finding is unclear because this tumor did not have any unusual histologic features when compared with the other samples analyzed.

Curiously, in all prostate tumor samples, the basal cells of, on average, 40% of benign seeming glands were found to express Id1. Such expression was absent in the glands of normal and hyperplastic prostates. It has been proposed that these basal cells, which lay on the basement membrane and surround the luminal cell layer, contain the glandular stem cell population (23, 24). In accord with this hypothesis the basal cell layer compartment may have the capacity of regenerating benign prostate glands after androgen ablation therapy (25, 26). Moreover, some authors propose that prostate cancer progenitor cells may be part of the basal compartment and share some molecular features with normal basal cells as being androgen receptor (AR) negative,

prostate-specific antigen (PSA) negative, and CD133 positive (23, 24). As Id1 may have a role in the cellular self-renewal capacity (27), the expression of Id1 in basal cells may be an early, necessary, and transient event allowing them to differentiate into a frank malignancy. During this process, malignant cells would acquire AR and PSA expression while losing Id1 and CD133 protein expression.

Bladder cancer is a tumor type distinct from those of mammary and prostate tumors with an origin from the stratified urothelium. In this study, we found normal urothelium to be weak or negative for Id1 expression. Interestingly a high percentage of normal urothelium in cancer-containing bladders also show significant Id1 staining. This suggests the possibility that Id1 is expressed in the preneoplastic urothelium, and that it may be an early marker with a role in the transition from normal to malignant urothelium. In contrast to the observations in breast and prostate neoplasias, tumor cells of 28% of all *in situ* and invasive bladder cancers showed moderate to high expression of Id1. The importance of Id1 expression in bladder cancer can now be tested in animal models in which Id1 levels are manipulated.

In all the tumor types examined, the vascular endothelial cells showed a consistent pattern of high Id1 protein expression. This was in contrast to the pattern seen in normal breast, bladder, and prostate in which significantly fewer endothelial cells showed Id1 expression in rare focal areas. These findings provide further support to the distinctive nature of tumor vasculature.

In light of our current findings, a careful reexamination of Id1 protein expression in human tumors and metastatic lesions is warranted. Given its potential importance in determining the biological behavior of tumors, precise definition of Id1 expression and function in tumorigenesis will be necessary if it is ever to be successfully developed as a target for directed therapy against these highly aggressive neoplasms.

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