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# Immunohistochemistry Biomarkers in Bladder Cancer: The Old and the New Immunohistochemistry Markers in Bladder Cancer

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## Abstract

**Introduction:** Bladder Cancer (BC) is split into subgroups and is primarily caused by environmental causes. Pathological classification can determine prognosis and clinical management. Immunohistochemistry (IHC) is an inexpensive technique that reveals specimens' important clinical information. Biomarkers are required to improve IHC, and combinations are currently in clinical use. We reviewed the main markers in use and emerging ones that may aid in BC management.

**Methodology:** A literature search was conducted on MEDLINE and PubMed databases from January 2017 to August 2022. Biomarkers chosen were cited three or more times.

**Results:** Out of 116 publications, 93 articles had their markers listed. Cytokeratin 5/6 (CK5/6), 14 (CK14), and 20 (CK20), GATA binding protein 3 (GATA3), tumor protein p53 (p53), marker of proliferation Ki-67 (Ki-67), and Uroplakin II (UPII) were discussed as markers in clinical use. Human Epidermal Growth Factor Receptor 2 (HER-2), Programmed Cell Death 1 receptor (PD-1) and Programmed Cell Death Ligand 1 (PDL-1), E-cadherin, vimentin, and Sex-Determining Region Y-box 2 (SOX2) were considered novel biomarkers.

**Conclusion:** Major markers in clinical use require additional research to better understand their applicability to BC subtyping. Novel markers show promising results since they can be used to evaluate the need for targeted therapy.

**Keywords: Clinical management; Immunostaining; Bladder neoplasia**

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## Introduction

BC accounts for 3% of all new cancer cases each year and it is estimated to have been the cause of death of approximately 200,000 patients in 2020 worldwide [1,2]. In Brazil, there were about 4,500 deaths of patients with BC diagnostics in 2019, 919 in the south area and 334 in Paraná [3,4]. It is also the seventh most common cancer among men in Brazil and is more prevalent in men than in women [4].

Advanced age, cigarette smoking, benzene chemical exposure, pelvic radiation, and genetic predisposition are all risk factors for BC [5]. BC is the major type of urothelial cancer and can be staged using TNM staging methods according to World Health Organization (WHO) standards from 2016, ranging from Ta (noninvasive carcinoma) to T4 (extravesical invasion) [5,6] (Figure 1A). Staging and providing an accurate diagnosis based on histopathological features is a challenge for pathologists and oncologists since the morphological traits resemble different stages or other types of malignancies of the adjacent organs with bladder invasion [7]. The distinction between Non-Muscle-Invasive BC (NMIBC) and Muscle-Invasive BC (MIBC) is significant for clinical management because they take different approaches to treatment and prognosis, as well as biomarkers [8,9].

Carcinoma of the bladder is the most frequent, accounting for 90% of all instances of urothelial origin (Figure 1B), and is classified as dysplasia, Carcinoma *in situ* (CIS), various kinds of invasive urothelial carcinoma, and metastatic carcinoma [7].

Diagnosis is based on clinical findings, as it can be painless, with microscopic hematuria, or symptomatic, with gross hematuria. Urinalysis is essential for diagnosis and urine cytology is often performed. Cystoscopy is used to get images of the urinary tract and to detect cancer. Additionally,

endoscopic resection and transurethral resection of the bladder tumor are important for pathological analysis and tumor staging [5,10].

In this context, various methodologies are available to aid in clinical prognosis and diagnosis. IHC is a tissue-based technique useful by pathologists as a diagnostic tool and prognostic predictor for patients with cancer that is often performed in cancer studies. IHC has been applied to histopathological studies since 1941, the year Coons et al. published a study that revealed the use of antibodies with a fluorescent group in mammalian tissues specific for antigens of interest [11]. Recently, there has been an improvement in the IHC technique with the use of monoclonal and polyclonal antibodies specific for tumor antigens, as well as the use of new technologies such as multiplex IHC or immunofluorescence [12,13]. The principle of IHC is based on the use of primary or secondary antibodies binding to specific biological molecules of interest in a tissue section in the cell membrane, nucleus, cytosol, or different organelles, and even in the extracellular matrix (Figure 2). The tissue is embedded in paraffin and stained with a specific antibody. 3,3′-Diaminobenzidine Tetrahydrochloride (DAB) is the most commonly used chromogen [14].

In BC, staging and establishing the tumor origin of metastasis evaluation is critical for differential diagnosis and classification in NMIBC and MIBC, and IHC is a non-expensive and widely available technology, but the molecular classification is not yet suitable for clinical routine in all patients [9]. Although several markers, such as CK20, CK5/6, CK14, and GATA3 are already in clinical usage, they are still unable to differentiate all the possible stages and types of BC [7,15,16]. The IHC staining combination of these markers is important for recognizing tissue organization, cancer staging, and clinical outcome prediction [7,17-19].

Classifying BC into luminal and basal subtypes is important for clinical management since they behave differently, altering the prognosis prediction [7]. Luminal BC is less invasive and metastatic than basal BC, but it is also less sensitive to conventional chemotherapy regimens [20-22]. The current review elaborates on the major markers already in clinical use and potential novel IHC biomarkers for BC.

## Methodology

Articles included in this review were selected from the PubMed and MEDLINE databases. The title should have contained the Medical Subject Reading Terms (MeSH Terms) "bladder carcinoma" or "bladder cancer" and, "biomarkers", "immunohistochemistry". Only journal articles conducted during the period from 2017 to 2022, in the English language and on humans were added. Here we show our PubMed advanced search builder term: ((("bladder carcinoma"[Title] OR "bladder cancer"[Title]) AND "immunohistochemistry"[MeSH Terms] AND "biomarkers"[MeSH Terms]) AND ((humans[Filter]) AND (English[Filter]) AND (2017:2022[pdat]))) AND ("journal article"[Publication Type])".

The eligibility criteria were assessed by analyzing all summaries of the 116 publications and by excluding reviews, meta-analysis, protocols, and guidelines; drug testing trials; evaluation of methodology and comparison of techniques; not using IHC as the analytical method and gallbladder research. Therefore, 93 publications were analyzed and the identification of all markers studied in each of these studies was listed. Biomarkers cited three or more times in different studies and the ones, which were the main objective of the articles, were included in this review. Supplementary Table 1 (ST 1) and 2 (ST 2) contain the list of markers and the papers excluded from this review, respectively.

## Major Markers in Clinical Uses

### Cytokeratin's

Cytokeratin's are proteins found in the intermediate and superficial cells of the bladder urothelium. Abnormal expression of this molecule is linked to a poor prognosis and cancer stage classification [23]. CK20, expressed in the umbrella cells, is associated with relapses, increased proliferative activity, but correlation with progression is not as robust when co-expressed with Ki-67 [23,24]. Nonetheless, not all cytokeratin's, such as CK20, are luminal type markers. CK5/6 is expressed in basal progenitor cells and has been related to chemosensitivity and inflammatory and aggressive behavior [20,25,26]. This differential expression of cytokeratin's can also affect how the tumor will respond to chemotherapy, as seen by Lu et al. [27].

CK14, another basal marker, shows accurate discrimination of BC subtypes, being specific for basal cell differentiation [28]. In concordance with previous research, CK14 was evaluated as a differential marker for BC in a study by Al-Sharaky et al. [25]. By analyzing 90 BC samples, this group found that co-expression of CK5 with CK14 and negative CK20 (CK5+CK14+CK20-) was associated with a more aggressive and muscle invasiveness phenotype than those lacking CK14 (CK5+. CK14-, CK20-) [25]. Similar results are seen in other publications, where CK14 was correlated with worse overall survival, cancer-specific survival, and recurrence-free survival [29]. It is important to recognize that the same markers are expressed differently in each type of BC, MIBC or NMIBC [30]. In fact, regarding muscularis propria invasion in BC, CK5 is positive and linked to a higher tumor grade [31]. However, when IHC was performed on high-risk NMIBC, Garczyk et al. found CIS heterogeneity, with basal (CK5/6) and luminal (CK20) biomarkers mixed, highlighting the challenge in prognosis prediction by using these markers [32]. Similar results are seen by Wang et al., who found overlapping of basal (CK5/6) and luminal (GATA3) markers in 48.4% of MIBC cases after analyzing 109 patients' staining patterns of these markers [33].

### GATA3

GATA3 protein, discovered as a significant biomarker for BC, acts as a transcription factor driving hematopoiesis, T-cell development, proliferation, and differentiation of epithelial tissues [34]. Along with CK20, GATA3 is a marker already used for the luminal type of BC and was correlated with 80% accuracy when analyzed with the mRNA expression [26].

Therefore, GATA3 is often used in a pathological context and to distinguish it from the basal subtype, often marked as CK5/6 positive [26]. However, Wang et al. showed that samples with co-expression of both luminal and basal markers can be overlapped in MIBC cases [33]. This study covered 91 patients and co-expression of the markers was found in 48.35% of the MIBC samples. In most research, GATA3 has consistently been shown to be a luminal marker and with a significant correlation to clinical outcome among the markers studied, with higher expression of GATA3 indicating better recurrence-free survival, independent of stage and metastasis, and lower GATA3 staining associated with poor prognosis and overall survival, and may predict patients who will receive radical cystectomy [29,33].

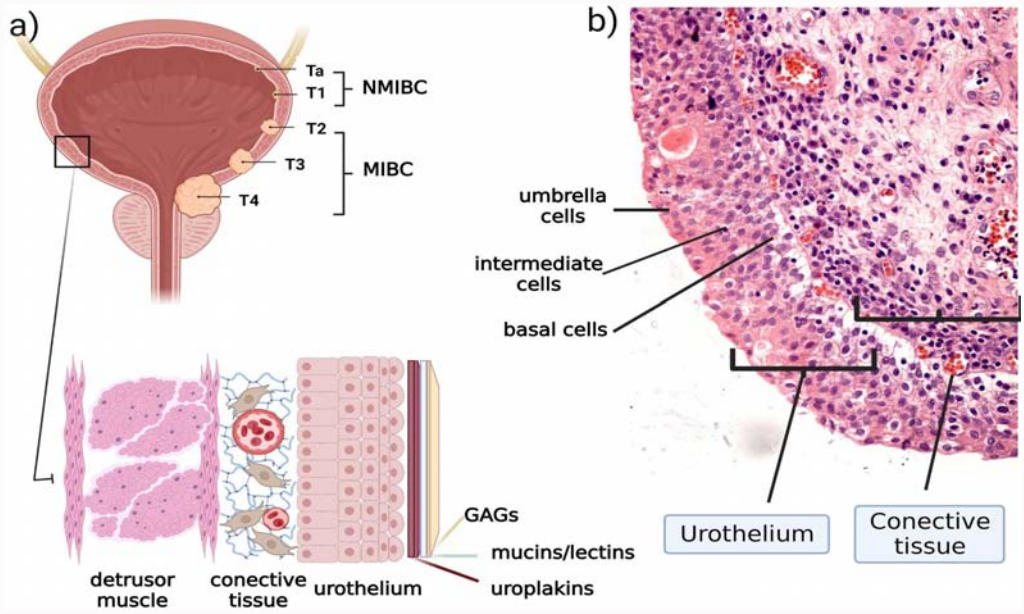


**Figure 1:** Anatomy of the bladder wall and urothelium. A) Shows the detrusor muscle layer, connective tissue, and urothelium from the innermost layer to the bladder lumen. Urothelium umbrella cells feature a unique apical cell membrane containing barrier components such as proteoglycans and GAGs, mucins and lectins, and uroplakins. The intermediate and basal layers are linked to the basement membrane beneath them. The connective tissue that contains the capillaries vessels is located beneath the urothelium. B) Normal (non-tumor) histology of bladder lumen stained with hematoxylin and eosin (400x magnification).

### *P53*

*TP53* is a tumor suppressor gene, and its altered expression leads to avoidance of growth suppression and genomic instability, important features for tumor progression [35,36]. Altered expression of *p53*, with nuclear accumulation and mutations, is present in BC [37]. *p53* staining patterns in the IHC method are heterogeneous in BC, being null staining and nuclear accumulation markers of alteration [32,33].

Most publications cannot show a correlation between p53 expression and clinical outcomes, such as grade, recurrence, or progression [33,38,39]. Usually, *p53* is investigated in conjunction with other markers, such as DDX31, Ki-67, pRb, or p21, and its clinical evaluation is relevant when associated with other IHC markers [33,39-41]. Simultaneously, Daizumoto et al. reported no correlation between *p53* IHC expression and grading and the TNM classification system, but only with cancer-specific survival among 77 samples of BC [40]. In concordance, Semeniuk-Wojtas et al. reported an independent association of *p53* with cancer recurrence survival [42]. Lloreta et al. analyzed 162 BC samples and found an association between *p53* expression and high-grade, staging, tumor progression, and disease recurrence, but all outcomes were better evaluated when expression with FOXO1 was evaluated [43].

By testing a different *p53* IHC expression scoring method with a cut-off the classification of 0% or more than 50% as abnormal, and 1% to 49% as wild type for 344 samples of BC, Hodgson et al. provided 100% sensitivity and 100% negative predictive value [44]. Therefore, this research highlights the importance of updating the IHC technique when analyzing markers' expression and the difficulty of providing a consensus on the *p53* prediction value for oncologic outcomes.

### Ki-67

Ki-67 is a proliferation marker linked to cancer cell aggressiveness and risk-recurrence, and is negatively correlated with basal NMIBC (CK5+) and positively associated with luminal NMIBC (CK20+) (24,42). Da Silva et al. showed in 93 BC samples an association between Ki-67 and high-grade BC, but not with muscle invasion and cancer recurrence ($p>0.05$) [38].

In addition to its correlation with staging and grading, other publications state that Ki-67 could indeed predict clinical outcomes, as seen by Fossum et al. who demonstrated Ki-67 correlation with aggressive disease and local-only recurrence in 42 MIBC samples [45]. Han et al. classified 48 BC samples into luminal and basal subtypes, in addition to a specific basal type called Claudin-Low Subtype (CLS), enriched with Epithelial-to-Mesenchymal Transition (EMT) and immune infiltration markers [46]. As a result, CLS had poorer oncologic outcomes and was marked with higher Ki-67 than other BC subtypes [46]. At the same time, in pT1 stages, Ki-67 has previously been associated with poorer outcome with tumor recurrence prediction, and Ziaran et al. proposed the implementation of Ki-67 expression evaluation on NMIBC risk stratification [39]. Still analyzing pT1 BC stage, Culpan et al. also found positive expression of Ki-67 association with high-grade tumor, and worse recurrence-free, progression-free, and cancer-specific survival when analyzing 101 patients' samples with BC. Therefore, Ki-67 shows great potential for clinical prediction as an IHC marker for BC.

### UPII

Uroplakins (UPs) are a group of 4 proteins associated with the urothelium apical surface, which helps maintain the homeostasis of the bladder mucosa. Recently, UPII has shown great sensitivity and specificity for urothelial BC [47-49]. It has been consistently associated with luminal BC, being a great marker of differentiation from basal BC [18].

However, Guo et al. showed that UPII and CK20 had overlapping expression patterns using IHC in 74 tissue microarrays and this can infer staining for these two markers could not be effective [26]. This is inferred from Lu et al. research, in which CK20 had a

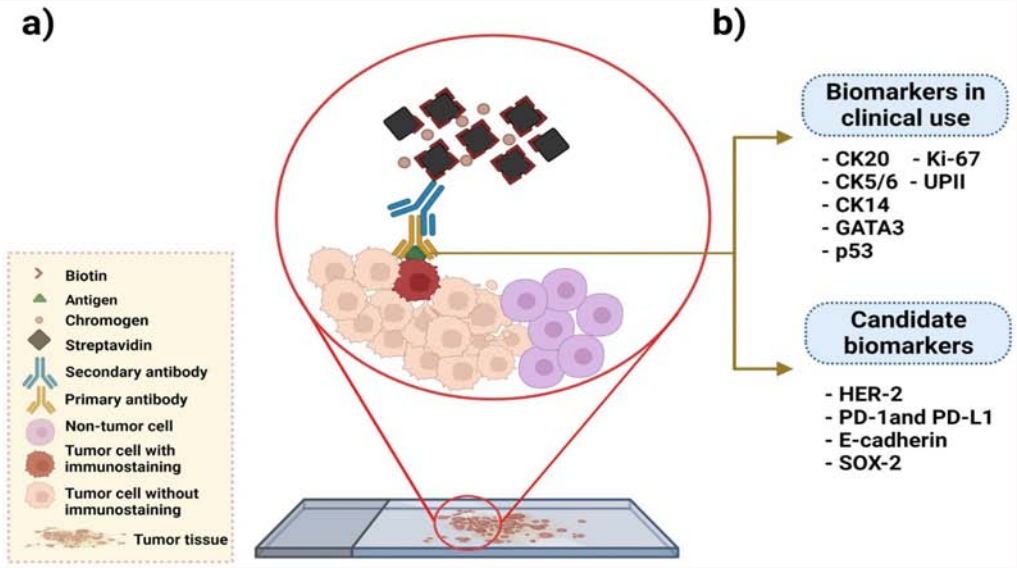


**Figure 2:** a) Technique for immunohistochemistry using antibodies specific for tumor antigens and streptavidin [biotin complex. B) Proteins currently in clinical use as predictive biomarkers in bladder cancer, as well as putative biomarkers examined in this work. Keratin 20 (CK20), cytokeratin 5/6 (CK5/6), keratin 14 (CK14), GATA binding protein 3 (GATA3), tumor protein 53 (p53), phosphatase 1(interacting protein (Ki-67), and recombinant human uroplakin II (UPII) are clinically used biomarkers, while candidate biomarkers include human epidermal growth factor receptor 2 (HER-2), programmed cell death protein 1 (PD-1), programmed death-ligand 1 (PD-L1), epithelial cadherin (E-cadherin) and SRY-box transcription factor 2 (SOX-2).

better performance in discriminating different types of luminal profiles, with distinct clinical outcomes, and whether UPII was positive for both subtypes [27]. UPII performance was compared to GATA3 in distinguishing urothelial carcinoma from other BC mimickers, studied by Leivo et al. such as nephrogenic adenoma, papillary nephrogenic adenoma, endometriosis/endosalpingiosis, inflammatory myofibroblastic tumor, and ectopic prostate tissue, and malakoplakia [50]. This study, although the number of samples from each mimicker was small, provided that UPII had high specificity for urothelial lesions and GATA3 stained for most of the mimickers (59%) [50]. In conclusion, even though the studies on UPII staining for different BC subtypes could overlap with CK20, it could be a powerful IHC tool for differentiating BC from other organ cancers.

## New BC Biomarkers for IHC

### *HER-2*

The *HER-2* or *ERBB2* gene is found on the long arm of human chromosome 17 [51]. In some types of cancer, it is overexpressed by mechanisms of constitutive activation, leading to cellular proliferation and tumorigenicity, seen in breast, lung, bladder, and gastric cancers, for example [52,53].

In breast cancer, monoclonal antibody targeting *HER-2* is the preferred treatment when overexpression and/or amplification of this gene are present [53]. Unsurprisingly, this immunotherapy is a historical success in science when considering targeting a molecular marker in cancer. Nowadays, different types of monoclonal antibody therapies are being used and combined in the clinical context of breast cancer, with IHC being a powerful tool for screening these patients [54]. *HER-2* overexpression in BC was first published in 1990 when they showed it as a possible diagnostic or prognostic marker, and it is considered a luminal marker for BC [32,55]. Still, the treatment of BC targeting *HER-2* is not well established.

Cimpean et al. analyzed *HER-2* expression in 45 samples using the IHC technique and found the role of this protein in the invasion of urothelial tumors (+2 and +3 IHC score) and its overexpression correlating with lymphovascular involvement [56]. For NMIBC, *HER-2* was studied in 67 patients with stage T1 (T1G3) BC in a paper published in 2017 [57]. *HER-2* overexpression was defined as a predictor of disease-free survival better than prognostic factors (sex, tumor size/number of recurrences) and the use of BCG treatment [57]. Li et al. demonstrated in 56 samples of bladder transitional cell carcinoma that 37.5% were positive for HER-2 and this expression was related to the staging of BC compared to non-cancer samples [58].

However, Franceschini et al. found a distinct expression in urothelial carcinoma of the bladder by different techniques. Using chromogenic and fluorescent in situ hybridization methodologies could detect the amplification of *HER-2* in more samples than IHC. Moreover, they also stated that this overexpression was not only related to the micropapillary histotype of BC but it was found in usual type BC [59].

By analyzing different subtypes of BC and its response to novel therapies targeting tyrosine kinase receptors, Wucherpfennig et al. reported that 95% of the squamous differentiated BC was positive for EGFR and potentially suitable for anti-*HER2* therapy [60]. Hence, although there is clear evidence of overexpression of *HER-2* in BC, more investigations toward this molecule and its association with subtyping BC are needed for possible prognostic and treatment choice predictor use.

### PD-1 and PD-L1

Inhibition of immune checkpoints has been an ally to neoadjuvant and adjuvant therapy in cancer, as stromal cells are important for tumor establishment and metastasis [61]. PD-1 acts as an effector of apoptosis and negatively regulates T cell function when bound to PD-L1, also known as B7-H1 [62,63]. Tumors expressing PD-1 have been associated with tumor evasion from the immune system and have been studied as a monoclonal antibody target for novel cancer

therapies [64].

Atezolizumab was the first inhibitor of PD-L1 to be approved by the FDA for treating urothelial cancer in 2016 [65]. In this clinical trial, 310 patients with locally advanced (T4b) or metastatic urothelial carcinoma received one dose of the drug every three weeks. IHC was used as a tool for identifying the patients with the expression of the marker and samples were from primary lesions, metastatic sites, transurethral resection of bladder tumors, and biopsy for unknown lesions. Atezolizumab demonstrated durable activity and good tolerability in pre-treated local and advanced or metastatic BC patients, correlating with higher levels of PD-L1 expression in tumor-infiltrating lymphocytes [65].

Following the approval of Atezolizumab for the BC treatment, various studies have attempted to define and group patients who may benefit from immunotherapy. One study, conducted in 2018, analyzed immunotypes as a method to predict survival in 258 patients with MIBC who underwent radical cystectomy and were selected for adjuvant chemotherapy [66]. They divided patients' samples after chemotherapy for IHC into two phenotypes: Stromal immunotype A with cytotoxic T lymphocytes high, NK cells high, regulatory T cell low, macrophage low, and mast cells low (CTL high NK high Treglow Macrophage low MClow) and immunotype B (CTL low NK low Treghigh Macrophage high MChigh). Immunotype B patients showed worse 5-year overall survival and 5-year disease-free survival, but a better response to platinum-based chemotherapy compared with immunotype A. Simultaneously, immunotype A expressed higher immune checkpoint molecules (PD-L1, PD-1, and CTLA-4), characterizing tissue inflammation with more T cell population than in immunotype B [66]. In conclusion, immunotype A patients could benefit from immunotherapy targeting immune checkpoints such as PD-L1 or PD-1 and immunotyping patients could predict the use of this therapy [66].

Furthermore, in another study of MIBC, they investigated the ability to classify samples from MIBC with local recurrence to normal using PD-L1 and Ki-67 markers for IHC [45]. Therefore, they compared 42 samples from patients who underwent radical cystectomy compared with normal bladder tissue from those same patients. PD-L1 score was higher in tumor cells in IHC, and these patients would benefit from immunotherapy [45]. Nevertheless, when analyzing recurrence, PD-1 upregulated in tumors was associated with fast relapse in 40 samples of high-grade MIBC in association with low inflammation markers, such as CD3, CD4, CD88, and CD20 [67].

Still, Li et al. demonstrated in 98 patients who underwent urothelial cancer surgery with no neoadjuvant therapy that overexpression of PD-L1 was associated with higher grade and lower survival [68]. Thus, PD-1 and its ligand are well-established targets for immunotherapy and the assessment of their expression by IHC is extremely important for prognosis and deciding therapeutic procedures.

### E-cadherin

EMT is essential for invasion and metastasis establishment. Modulation of cell-cell adhesion molecules is key for EMT and phenotype switching in cancer and E-cadherin is involved by being downregulated in different types of cancer [69,70].

In 2017, a group from Zagazig University in Egypt analyzed 54 patients with BC and histological normal samples from these same patients. Among those samples, 46.3% were of low malignancy and 53.7% were of high malignancy. Normal epithelial expressed E-cadherin and 55.6% of tumor samples showed an aberrant (negative) expression of this marker. Increasing the tumor stage was significantly associated with decreased E-cadherin immuno-expression, where it showed positivity in 20% of pTis cases but negative in all pT3 and pT4 cases ($p<0.001$) [71].

Ziaran et al. reported, in a study of 224 patients with different stages of BC, an association between low expression of E-cadherin with CIS, and worse progression free-survival with pT1 and pTa stages. Simultaneously, normal expression of E-cadherin was correlated with improved cancer-specific survival [39]. In contrast, Ottley et al. showed in 26 IHC high-grade pT1 BC that E-cadherin expression was lower in invasive areas compared to papillary, CIS, and normal regions and it was inversely correlated with N-cadherin expression, which marks mesenchymal transition and invasion [72]. However, the authors could not find an association between this EMT marker and disease-specific survival [72].

Therefore, the E-cadherin marker could be an important prognosis predictor in BC studies, as its lower expression is correlated with poor survival rates and invasiveness. Nonetheless, further research is needed to validate its use in clinical practice and to create a consensus as a survival predictor.

### SOX-2

Stem cell markers are also studied in cancer for their characteristics similar to some tumor cells when considering self-renew and poorly differentiated stages, known as Cancer Stem Cells (CSC) [73,74]. SOX-2 is located on chromosome 3 and is important for stem cell phenotype, embryonic development, and tumor progression [75,76]. The high expression of this CSC marker was previously associated with BC poor prognosis, low recurrence-free survival, muscle invasion, tumor size, and grade [77,78]. Zhu et al. showed higher SOX2 expression in 22 BC samples compared to 7 para-tumor tissue samples and the correlation of this marker with tumor progression [74].

SOX2 expression in neuroendocrine-like tumors indicates they are differentiating into the neuroendocrine phenotype, a rare aggressive type of bladder carcinoma [79]. This publication is an important result, as it addresses the possibility of a diagnosis of neuroendocrine tumors earlier and provides adequate treatment for these patients [79]. However, when analyzing low-grade NMIBC, high-grade NMIBC, and MIBC using the IHC technique [80]. The authors explained the difficulty of determining the CSC population in BC, as it represents only a small fraction of the tissue [80]. Indeed, biomarkers for CSC, such as SOX2, are an intriguing and prospective topic, but still with difficult applicability in a clinical context using the IHC technique.

### Vimentin

Vimentin is an intermediate filament expressed in mesenchymal tissue, related to metastatic carcinomas, and rarely expressed in the muscularis propria, and has moderate to strong expression in the muscularis mucosa [81,82]. Usually, vimentin IHC evaluation is done along with smoothelin or desmin markers since they have differential expression patterns in MP and MM, aiding invasion classification using the IHC technique [82]. Although difficult, the pathological distinction between these two muscular layers in Transurethral Resection (TUR) samples is essential, as MP invasion indicates an advanced stage with a worse prognosis and higher chances of more

radical treatment [82,83].

Poletajew et al. analyzed the expression of vimentin in 47 TUR remains and found that vimentin alone could not reliably distinguish the two muscular layers, but showed differential expression between them; suggesting vimentin could be used in a panel together with other muscular markers [83]. Elkady et al. demonstrated vimentin expression in 87.5% of MM, mostly (67.5%) of mild expression, and no expression within the smooth muscle bundles of MP, showing 80% sensitivity and 100% specificity [84]. Still, when comparing vimentin expression with smoothelin and, when both combined, sensitivity turned 100% [84].

Vimentin can also be used as an EMT marker for BC, as seen in Ottley et al. [72]. Vimentin expression was assessed, being negatively correlated to CK20, and positively correlated to basal markers [72]. Tumor progression was associated with vimentin expression, but no clinical outcome association could be inferred [72]. Thus, vimentin is an important marker for pathological classification of BC muscular layers and can provide clinical management aid when used with other muscular markers.

## Conclusion

Defining biomarkers for IHC staining in BC can be challenging. The IHC technique, cut-off points used for each marker, and establishing marker combinations add to the difficulty of the pathologist's knowledge in providing the best diagnosis and prognosis in BC. As seen in this review, even the markers already in clinical use for BC could not show a convergent recommendation among publications, as BC subtyping is not always straightforwardly classified into luminal and basal subtypes, mostly because of BC heterogeneity. We also highlight the use of novel biomarkers for the choice of treatment in patients with BC and the importance of their availability for use in clinical routines. In this sense, further research on BC biomarkers is needed to provide solid marker recommendations and novel marker investigation, as it has seen promising results in *HER-2* and PD-L1 staining.

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## References

1. Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, et al. Global Cancer Statistics 2020: GLOBOCAN Estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin. 2021;71(3):209-49.
2. Richters A, Aben KKH, Kiemeney LALM. The global burden of urinary bladder cancer: An update. World J Urol. 2020;38(8):1895-904.
3. Instituto Nacional de Câncer José Alencar Gomes da Silva. Atlas Online de Mort alidade. 2019.
4. Instituto Nacional de Câncer José Alencar Gomes da Silva. Estimate/2020 - Cancer Incidence in Brazil. INCA, editor. Rio de Janeiro: INCA; 2019.
5. Lenis AT, Lec PM, Chamie K. Bladder cancer: A review. J Am Med Assoc. 2020;324(19):1980-91.
6. Kirkali Z, Chan T, Manoharan M, Algaba F, Busch C, Cheng L, et al. Bladder cancer: Epidemiology, staging and grading, and diagnosis. Urology. 2005;66(6 Suppl 1):4-34.
7. Akgul M, MacLennan GT, Cheng L. The applicability and utility of immunohistochemical biomarkers in bladder pathology. Hum Pathol. 2020;98:32-55.
8. Witjes JA, Bruins HM, Carrión A, Cathomas R, Compérat EM, Efstathiou JA, et al. EAU Guidelines on Muscle-invasive and Metastatic Bladder Cancer. In: EAU Annual Congress Amsterdam. Amsterdam: EAU Guidelines; 2022.
9. Babjuk M, Burger M, Gontero P, Liedberg F, Palou J, van Rhijn BWG. EAU Guidelines on Non-muscle-invasive Bladder Cancer (TaT1 and CIS). In: EAU Annual Congress Amsterdam. Amsterdam: EAU Guidelines; 2022.
10. DeGeorge KC, Holt HR, Hodges SC. Bladder cancer: Diagnosis and treatment. Am Fam Physician. 2017;96(8):507-14.
11. Coons AH, Creech HJ, Jones RN. Immunological properties of an antibody containing a fluorescent group. Proc Soc Exp Biol Med. 1941;47(2):200-2.
12. Tan WCC, Nerurkar SN, Cai HY, Ng HHM, Wu D, Wee YTF, et al. Overview of multiplex immunohistochemistry/immunofluorescence techniques in the era of cancer immunotherapy. Cancer Commun (Lond). 2020;40(4):135-53.
13. Tuffaha MSA, Guski H, Kristiansen G. Immunohistochemistry in tumor diagnostics. 1st Ed. Switzerland: Springer, Cham; 2017.
14. Sukswai N, Khoury JD. Immunohistochemistry innovations for diagnosis and tissue-based biomarker detection. Curr Hematol Malig Rep. 2019;14(5):368-75.
15. Razzaghdoust A, Ghajari M, Basiri A, Torbati PM, Jafari A, Fattahi M-R, et al. Association of immunohistochemical markers of tumor subtype with response to neoadjuvant chemotherapy and survival in patients with muscle-invasive bladder cancer markers of chemotherapy response in bladder cancer. Investig Clin Urol. 2021;62(3):274-81.
16. Cox RM, Magi-Galluzzi C, McKenney JK. Immunohistochemical pitfalls in genitourinary pathology: 2018 Update. Adv Anat Pathol. 2018;25(6):387-99.
17. Akhtar M, Rashid S, Ben GM, Taha M, Al Bozom I. CK20 and CK5/6 immunohistochemical staining of urothelial neoplasms: A perspective. Adv Urol. 2020;2020;4920236.
18. Ikeda J, Ohe C, Yoshida T, Kuroda N, Salto R, Kinoshita H, et al. Comprehensive pathological assessment of histological subtypes, molecular subtypes based on immunohistochemistry, and tumor-associated immune cell status in muscle-invasive bladder cancer. Pathol Int. 2021;71(3):173-82.
19. Labban M, Najdi J, Mukherji D, Abou-Kheir W, Tabbarah A, El-Hajj A. Triple-marker immunohistochemical assessment of muscle-invasive bladder cancer: Is there prognostic significance? Cancer Reports (Hoboken). 2021;4(2):e1313.
20. Choi W, Czerniak B, Ochoa A, Su X, Siefker-Radtke A, Dinney C, et al. Intrinsic basal and luminal subtypes of muscle-invasive bladder cancer. Nat Rev Urol. 2014;11(7):400-10.
21. Choi W, Porten S, Kim S, Willis D, Plimack ER, Hoffman-Censits J, et al. Identification of distinct basal and luminal subtypes of muscle-invasive bladder cancer with different sensitivities to frontline chemotherapy. Cancer Cell. 2014;25(2):152-65.
22. Dadhania V, Zhang M, Zhang L, Bondaruk J, Majewski T, Siefker-Radtke A, et al. Meta-analysis of the luminal and basal subtypes of bladder cancer and the identification of signature immunohistochemical markers for clinical use. eBioMedicine. 2016;12:105-17.
23. Ranzi AD, da Silva JNL, Graziottin TM, Annels N, Bica CG. Immunohistochemistry biomarkers in nonmuscle invasive bladder cancer.

Appl Immunohistochem Mol Morphol. 2017;25(3):178-83.

24. Breyer J, Wirtz RM, Otto W, Erber P, Kriegmair MC, Stoehr R, et al. In stage pT1 Non-Muscle-Invasive Bladder Cancer (NMIBC), high KRT20 and low KRT5 mRNA expression identify the luminal subtype and predict recurrence and survival. Virchows Arch. 2017;470(3):267-74.

25. Al-Sharaky DR, Abdelwahed M, Asaad N, Foda A, Abdou AG. Stratification of urinary bladder carcinoma based on immunohistochemical expression of CK5, CK14 and CK20. J Immunoassay Immunochem. 2021;42(3):236-51.

26. Guo CC, Bondaruk J, Yao H, Wang Z, Zhang L, Lee S, et al. Assessment of luminal and basal phenotypes in bladder cancer. Sci Rep. 2020;10(1):9743.

27. Lu J, Zhang Y, Wu C, Chu C, Liu Z, Cao Y. Impact of immunohistochemistry-based molecular subtype on predicting chemotherapy response and survival in patients with T1 stage bladder cancer after bladder-preserving treatment. Jpn J Clin Oncol. 2021;51(3):424-33.

28. Hardy CSC, Ghaedi H, Slotman A, Sjödahl G, Gooding RJ, Berman DM, et al. Immunohistochemical assays for bladder cancer molecular subtyping: Optimizing parsimony and performance of lund taxonomy classifiers. J Histochem Cytochem. 2022;70(5):357-75.

29. Yuk HD, Jeong CW, Kwak C, Kim HH, Moon KC, Ku JH. Clinical outcomes of muscle invasive bladder Cancer according to the BASQ classification. BMC Cancer. 2019;19(1):897.

30. Muilwijk T, Akand M, Van der Aa F, De Coninck V, Claessens M, Hente R, et al. Cytokeratin 5 and cytokeratin 20 inversely correlate with tumour grading in Ta non-muscle-invasive bladder cancer. J Cell Mol Med. 2021;25(16):7890-900.

31. Hashmi AA, Hussain ZF, Irfan M, Edhi MM, Kanwal S, Faridi N, et al. Cytokeratin 5/6 expression in bladder cancer: Association with clinicopathologic parameters and prognosis. BMC Res Notes. 2018;11(1):207.

32. Garczyk S, Bischoff F, Schneider U, Golz R, Rundstedt F-C von, Knüchel R, et al. Intratumoral heterogeneity of surrogate molecular subtypes in urothelial carcinoma in situ of the urinary bladder: Implications for prognostic stratification of high-risk non-muscle-invasive bladder cancer. Virchows Arch. 2021;479(2):325-35.

33. Wang C-C, Tsai Y-C, Jeng Y-M. Biological significance of GATA3, cytokeratin 20, cytokeratin 5/6 and p53 expression in muscle-invasive bladder cancer. PLoS One. 2019;14(8):e0221785.

34. Wang C, Yang S, Jin L, Dai G, Yao Q, Xiang H, et al. Biological and clinical significance of GATA3 detected from TCGA database and FFPE sample in bladder cancer patients. Onco Targets Ther. 2020;13:945-58.

35. Livingstone LR, White A, Sprouse J, Livanos E, Jacks T, Tlsty TD. Altered cell cycle arrest and gene amplification potential accompany loss of wild-type p53. Cell. 1992;70(6):923-35.

36. Hanahan D. Hallmarks of Cancer: New Dimensions. Cancer Discov. 2022;12(1):31-46.

37. Mitra AP, Datar RH, Cote RJ. Molecular pathways in invasive bladder cancer: new insights into mechanisms, progression, and target identification. J Clin Oncol. 2006;24(35):5552-64.

38. da Silva JNL, Ranzi AD, Carvalho CT, Scheide TV, Strey YTM, Graziottin TM, et al. Cell cycle markers in the evaluation of bladder cancer. Pathol Oncol Res. 2020;26(1):175-81.

39. Ziaran S, Harsanyi S, Bevizova K, Novakova ZV, Trebaticky B, Bujdak P, et al. Expression of E-cadherin, Ki-67, and p53 in urinary bladder cancer in relation to progression, survival, and recurrence. Eur J Histochem. 2020;64(2):3098.

40. Daizumoto K, Yoshimaru T, Matsushita Y, Fukawa T, Uehara H, Ono M, et al. A DDX31/Mutant-p53/EGFR axis promotes multistep progression of muscle-invasive bladder cancer. Cancer Res. 2018;78(9):2233-47.

41. Wang G, Black PC, Goebell PJ, Ji L, Cordon-Cardo C, Schmitz-Dräger B, et al. Prognostic markers in pT3 bladder cancer: A study from the international bladder cancer tissue microarray project. Urol Oncol. 2021;39(5):301.e17-301.e28.

42. Semeniuk-Wojtaś A, Lubas A, Cierniak S, Brzóskowska U, Syryło T, Zieliński H, et al. Selected protein expression in a new prognostic model for patients with non-muscle-invasive bladder cancer. J Cancer Res Clin Oncol. 2020;146(8):2099-108.

43. Lloreta J, Font-Tello A, Juanpere N, Frances A, Lorenzo M, Nonell L, et al. FOXO1 down-regulation is associated with worse outcome in bladder cancer and adds significant prognostic information to p53 overexpression. Hum Pathol. 2017;62:222-31.

44. Hodgson A, Rhijn BWG van, Kim SS, Ding C, Saleeb R, Vesprini D, et al. Reassessment of p53 immunohistochemistry thresholds in invasive high grade bladder cancer shows a better correlation with TP53 and FGFR3 mutations. Pathol Res Pract. 2020;216(11):153186.

45. Fossum CC, Xiong Y, Magliocco A, Daneshmand S, Aron M, Mouw KW, et al. Role of Ki-67, MRE11, and PD-L1 as predictive biomarkers for recurrence pattern in muscle-invasive bladder cancer. Anticancer Res. 2021;41(8):3851-7.

46. Han L, Gallan AJ, Steinberg GD, Sweis RF, Paner GP. Morphological correlation of urinary bladder cancer molecular subtypes in radical cystectomies. Hum Pathol. 2020;106:54-61.

47. Wu X-R, Lin J-H, Walz T, Hiiner M, Yu J, Aebi U, et al. Mammalian uroplakins. A group of highly conserved urothelial differentiation-related membrane proteins. J Biol Chem. 1994;269(18):13716-24.

48. Smith SC, Mohanty SK, Kunju LP, Chang E, Chung F, Carvalho JC, et al. Uroplakin II outperforms uroplakin III in diagnostically challenging settings. Histopathology. 2014;65(1):132-8.

49. Tian W, Guner G, Miyamoto H, Cimino-Mathews A, Gonzalez-Roibon N, Argani P, et al. Utility of uroplakin II expression as a marker of urothelial carcinoma. Hum Pathol. 2015;46(1):58-64.

50. Leivo MZ, Tacha DE, Hansel DE. Expression of uroplakin II and GATA-3 in bladder cancer mimickers: Caveats in the use of a limited panel to determine cell of origin in bladder lesions. Hum Pathol. 2021;113:28-33.

51. Akiyama T, Sudo C, Ogawara H, Toyoshima K, Yamamoto T. The product of the human c-erbB-2 Gene: A 185-kilodalton glycoprotem with tyrosine kinase activity. Science. 1986;232(4748):1644-6.

52. Yarden Y, Sliwkowski MX. Untangling the ErbB signaling network. Nat Rev Mol Cell Biol. 2001;2(2):127-37.

53. Oh D-Y, Bang Y-J. HER2-targeted therapies - A role beyond breast cancer. Nat Rev Clin Oncol. 2020;17(1):33-48.

54. Morales S, Gasol A, Sanchez DR. Her2-positive cancers and antibody-based treatment: State of the art and future developments. Cancers (Basel). 2021;13(22):57-71.

55. Zhau HE, Zhang X, Von Eschenbach AC, Scorsone K, Babain RJ, Ro JY, et al. Amplification and expression of the c-erb B-2/neu proto-oncogene in human bladder cancer. Mol Carcinog. 1990;3(5):254-7.

56. Cimpean AM, Tarlui V, Cumpănaş AA, Bolintineanu S, Cumpănaş A, Raica M. Critical overview of HER2 assessement in bladder cancer: What is missing for a better therapeutic approach? Anticancer Res. 2017;37(9):4935-42.

57. Cormio L, Sanguedolce F, Cormio A, Massenio P, Pedicillo MC, Cagiano S, et al. Human epidermal growth factor receptor 2 expression is more important than Bacillus Calmette Guerin treatment in predicting the outcome of T1G3 bladder cancer. Oncotarget. 2017;8(15):25433-41.

58. Li W, Wang Y, Tan S, Rao Q, Zhu T, Huang G, et al. Overexpression of Epidermal Growth Factor Receptor (EGFR) and HER-2 in bladder carcinoma and its association with patients' clinical features. Med Sci

Monit. 2018;24:7178-85.

59. Franceschini T, Capizzi E, Massari F, Schiavina R, Fiorentino M, Giunchi F. Immunohistochemical over-expression of HER2 does not always match with gene amplification in invasive bladder cancer. Pathol Res Pract. 2020;216(8):153012.

60. Wucherpfennig S, Rose M, Maurer A, Cassataro MA, Seillier L, Morsch R, et al. Evaluation of therapeutic targets in histological subtypes of bladder cancer. Int J Mol Sci. 2021;22(21):11547.

61. Roma-Rodrigues C, Mendes R, Baptista PV, Fernandes AR. Targeting tumor microenvironment for cancer therapy. Int J Mol Sci. 2019;20(4):840.

62. Ishida Y, Agata Y, Shibahara K, Honjo T. Induced expression of PD-1, a novel member of the immunoglobulin gene superfamily, upon programmed cell death. EMBO J. 1992;11(11):3887-95.

63. Agata Y, Kawasaki A, Nishimura H, Ishida Y, Tsubata T, Yagita H, et al. Expression of the PD-1 antigen on the surface of stimulated mouse T and B lymphocytes. Int Immunol. 1996;8(5):765-72.

64. Dong H, Strome SE, Salomao DR, Tamura H, Hirano F, Flies DB, et al. Tumor-associated B7-H1 promotes T-cell apoptosis: A potential mechanism of immune evasion. Nat Med. 2002;8(8):793-800.

65. Rosenberg JE, Hoffman-Censits J, Powles T, Van Der Heijden MS, Balar AV, Necchi A, et al. Atezolizumab in patients with locally advanced and metastatic urothelial carcinoma who have progressed following treatment with platinum-based chemotherapy: A single-arm, multicentre, phase 2 trial. Lancet. 2016;387(10031):1909-20.

66. Fu H, Zhu Y, Wang Y, Liu Z, Zhang J, Xie H, et al. Identification and validation of stromal immunotype predict survival and benefit from adjuvant chemotherapy in patients with muscle-invasive bladder cancer. Clin Cancer Res. 2018;24(13):3069-78.

67. Olkhov-Mitsel E, Hodgson A, Liu SK, Vesprini D, Bayani J, Bartlett JMS, et al. Can immune markers help identify fast relapse in patients with muscle invasive bladder cancer? Pathol Res Pract. 2020;216(11):153200.

68. Li Q, Li F, Che J, Zhao Y, Qiao C. Expression of B7 Homolog 1 (B7H1) is associated with clinicopathologic features in urothelial bladder cancer. Med Sci Monit. 2018;24:7303-8.

69. Sisto M, Ribatti D, Lisi S, Kurien T. Cadherin signaling in cancer and autoimmune diseases. Int J Mol Sci. 2021;22(24):13358.

70. Yu W, Yang L, Li T, Zhang Y. Cadherin signaling in cancer: Its functions and role as a therapeutic target. Front Oncol. 2019;9:989.

71. Hussein S, Mosaad H, Rashed HE, Ahmed S, Ragab A, Ismail EI. Molecular factors regulating E-cadherin expression in urothelial bladder cancer and their correlations with the clinicopathological features. Mol Biol Rep. 2017;44(4):365-77.

72. Ottley EC, Pell R, Brazier B, Hollidge J, Kartsonaki C, Browning L, et al. Greater utility of molecular subtype rather than Epithelial-to-Mesenchymal Transition (EMT) markers for prognosis in high-risk non-muscle-invasive (HGT1) bladder cancer. J Pathol Clin Res. 2020;6(4):238-51.

73. Hatina J, Schulz WA. Stem cells in the biology of normal urothelium and urothelial carcinoma. Neoplasma. 2012;59(6):728-36.

74. Zhu F, Qian W, Zhang H, Liang Y, Wu M, Zhang Y, et al. SOX2 is a marker for stem-like tumor cells in bladder cancer. Stem Cell Rep. 2017;9(2):429-37.

75. Zhang S, Xiong X, Sun Y. Functional characterization of SOX2 as an anticancer target. Signal Transduct Target Ther. 2020;5(1):135.

76. Leis O, Eguiara A, Lopez-Arribillaga E, Alberdi MJ, Hernandez-Garcia S, Elorriaga K, et al. Sox2 expression in breast tumours and activation in breast cancer stem cells. Oncogene. 2012;31(11):1354-65.

77. Kitamura H, Torigoe T, Hirohashi Y, Asanuma H, Inoue R, Nishida S, et al. Prognostic impact of the expression of ALDH1 and SOX2 in urothelial cancer of the upper urinary tract. Mod Pathol. 2013;26(1):117-24.

78. Ruan J, Wei B, Xu Z, Yang S, Zhou Y, Yu M, et al. Predictive value of Sox2 expression in transurethral resection specimens in patients with T1 bladder cancer. Med Oncol. 2013;30(1):445.

79. Costa JB da, Gibb EA, Bivalacqua TJ, Liu Y, Oo HZ, Miyamoto DT, et al. Molecular characterization of neuroendocrine-like bladder cancer. Clin Cancer Res. 2019;25(13):3908-20.

80. Belgorosky D, Girouard J, Langle YV, Hamelin-Morrissete J, Marino L, Agüero EI, et al. Relevance of iNOS expression in tumor growth and maintenance of cancer stem cells in a bladder cancer model. J Mol Med (Berl). 2020;98(11):1615-27.

81. Lane EB, Hogan BLM, Kurkinen M, Garrels JI. Co-expression of vimentin and cytokeratins in parietal endoderm cells of early mouse embryo. Nature. 1983;303(5919):701-4.

82. Council L, Hameed O. Differential expression of immunohistochemical markers in bladder smooth muscle and myofibroblasts, and the potential utility of desmin, smoothelin, and vimentin in staging of bladder carcinoma. Mod Pathol. 2009;22(5):639-50.

83. Poletajew S, Wolińska E, Wasiutyński A, Dybowski B, Radziszewski P, Górnicka B. Immunohistochemical differentiation between muscularis mucosae and muscularis propria for improving the staging of bladder cancer in patients undergoing transurethral resection of bladder tumours. Pol J Pathol 2017;68(3):218-24.

84. Elkady N, Abdou AG, Kandil M, Ghanem N. Diagnostic value of smoothelin and vimentin in differentiating muscularis propria from muscularis mucosa of bladder carcinoma. Int J Biol Markers. 2017;32(3):e305-12.