Immunohistochemistry for the Novel Markers Glypican 3, PAX8, and p40 (ΔNp63) in Squamous Cell and Urothelial Carcinoma

Michael P. Gailey, DO, and Andrew M. Bellizzi, MD

From the Department of Pathology, University of Iowa Hospitals and Clinics, University of Iowa Carver College of Medicine, Iowa City, IA.

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ABSTRACT

Objectives: To examine squamous cell carcinomas (SCCs) from diverse anatomic sites and invasive urothelial carcinomas (UCs) for expression of the oncofetal antigen glypican 3 (GPC3), the paired box transcription factor PAX8, and the ΔN isoform of p63 (p40).

Methods: Immunohistochemistry for GPC3, PAX8, and p40 was performed on whole sections of 107 SCCs from 11 anatomic sites and 49 UCs; evaluation included extent and intensity of staining.

Results: GPC3 was detected in 20% of SCCs and 12% of UCs and PAX8 in 3% of SCCs, limited to the uterine cervix, and 10% of UCs. p40 Was found in 99% of SCCs and 96% of UCs.

Conclusions: GPC3 expression is frequent in SCC/UC, awareness of which should guard against an incorrect diagnosis of hepatocellular carcinoma, while PAX8, limited in distribution, may have some use in suggesting a cervical or urothelial tract origin in a metastatic squamotransitional carcinoma of unknown primary. There is no drop-off in sensitivity for the diagnoses of squamous cell and urothelial carcinoma with ΔNp63-specific immunohistochemistry, and if this performance can be extended to other applications, p40 may supplant the dominant “pan-p63” antibody clone.

Diagnostic immunohistochemistry (IHC) is widely used to determine tumor type and to suggest the site of origin of a carcinoma of unknown primary (CUP). IHC for p63 and high-molecular-weight cytokeratins (CKs), including CK5/6, is commonly employed to support a diagnosis of squamous cell carcinoma (SCC). Compared with its application in adenocarcinoma, IHC is generally not considered very useful in determining the site of origin of an SCC. An exception to this teaching is p16, which has proven utility in supporting an origin from the anogenital tract1 or oropharynx2 (due to the protein’s upregulation in high-risk human papillomavirus–driven tumors).

With these two points in mind, we were intrigued by a few recent observations. First, glypican 3 (GPC3), a membrane-bound proteoglycan and oncofetal antigen whose
expression is most often used in diagnostic pathology to support an interpretation of hepatocellular carcinoma (HCC), was identified as a potential tumor suppressor in gene expression profiling of lung tumors, and was subsequently found to be overexpressed in pulmonary SCC at the protein and messenger RNA (mRNA) levels. Anecdotally, we have recently seen a metastatic pulmonary SCC to a cervical lymph node misdiagnosed as HCC based on diffuse, strong GPC3 expression. Second, a fraction of SCCs from a subset of anatomic sites was found to express the paired box transcription factor PAX8 in comprehensive tissue microarray (TMA) studies. Finally, p40, an antibody with specificity for the ∆N isoform of p63, is gaining traction in neoplastic pulmonary pathology based on superior specificity for the diagnosis of SCC (vs adenocarcinoma).

We studied expression of these three proteins in SCCs across diverse anatomic sites and in invasive urothelial carcinomas (UCs) from the urinary bladder, tumors that frequently show squamous differentiation and are virtually indistinguishable from SCCs at metastatic sites. Specifically, we sought to answer the following questions: (1) Does GPC3 expression in an SCC indicate a pulmonary origin, or is the marker more widely expressed? (2) Is PAX8 expression in SCC/UC limited in anatomic extent, as suggested by previous TMA-based studies? (3) Is there any drop-off in sensitivity for the diagnosis of SCC/UC across anatomic sites using an antibody specific for the ∆N isoform of p63 (relative to routinely employed "pan-p63" IHC)? We determined that GPC3 expression in SCC/UC is relatively common and is seen at most anatomic sites, PAX8 expression is very limited in anatomic distribution, and p40 IHC is incredibly sensitive for a diagnosis of SCC/UC.

### Materials and Methods

#### Study Design and Tumor Description

Study approval was obtained from the Institutional Review Board of the University of Iowa Hospitals and Clinics (UIHC), Iowa City, Iowa. Tissue blocks of SCCs (n = 107) and invasive UCs (n = 49) were retrieved from the surgical pathology archives of the UIHC Department of Pathology. The SCCs consisted of 17 well, 56 moderately, and 34 poorly differentiated tumors (grades as assigned in the conduct of clinical care). We performed IHC for GPC3, PAX8, and p40 on whole sections of all cases. Study slides were independently scored by two pathologists (M.P.G. and A.M.B.), blinded to diagnosis and anatomic site. Discrepancies were resolved over a two-headed microscope.

### Immunohistochemistry

Immunohistochemistry was performed in the Histology Research Laboratory of the UIHC Department of Pathology using the following commercially available antibodies: GPC3 (Cell Marque, Rocklin, CA), PAX8 (Proteintech, Chicago, IL), and p40 (CalBiochem/EMD Biosciences, Billerica, MA). The antibody clone/catalogue number, type, dilution, and vendor are summarized in Table 1.

IHC was performed on 4-μm sections, which were first deparaffinized, rehydrated, and subjected to heat-induced epitope retrieval (HIER) in citrate buffer (pH 6). For GPC3, HIER was performed in a pressure cooker placed in a 1,000-W microwave, while for PAX8 and p40, a Decloaking Chamber (Biocare Medical, Concord, CA) was used. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide. After incubation with the primary antibody, the DAKO Envision Kit (DAKO, Carpinteria, CA) was used for detection. Sections of HCC (for GPC3), serous carcinoma (PAX8), and pulmonary SCC (p40) were used as positive controls, and for negative controls, citrate buffer was substituted for the primary antibody.

For each marker, both the extent (percentage of cells) and intensity (1+, 2+, and 3+) of staining were assessed. For GPC3, only granular, cytoplasmic staining in 5% or more of tumor cells was considered positive. For the transcription factors PAX8 and p40, any definite nuclear staining was considered positive.

### Results

The IHC results are summarized in Table 1 and Table 3, with Table 2 containing aggregate results by tumor type (SCC vs UC), as well as the range, mean, and median

**Table 1**

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Clone/Catalog No.</th>
<th>Type</th>
<th>Dilution</th>
<th>Vendor</th>
</tr>
</thead>
<tbody>
<tr>
<td>GPC3</td>
<td>1G12</td>
<td>Mouse monoclonal</td>
<td>1:100</td>
<td>Cell Marque, Rocklin, CA</td>
</tr>
<tr>
<td>PAX8</td>
<td>10336-1-AP^b</td>
<td>Rabbit polyclonal</td>
<td>1:800</td>
<td>Proteintech, Chicago, IL</td>
</tr>
<tr>
<td>p40</td>
<td>PC373^b</td>
<td>Rabbit polyclonal</td>
<td>1:1500</td>
<td>CalBiochem/EMD Biosciences, Billerica, MA</td>
</tr>
</tbody>
</table>

^a Clone.  
^b Catalog No.
percentage of cells staining (for positive cases), while in Table 3, protein expression is broken down by anatomic site. PAX8, p40, and GPC3 slides were scored with 100%, 99%, and 94% concordance, respectively.

**GPC3**

GPC3 staining was present in 27 (17.3%) of 156 tumors from nine (75.0%) of 12 anatomic sites, including anus (1/10; 10.0%), cervix (3/11; 27.3%), esophagus (2/7; 28.6%), larynx (3/10; 30.0%), lung (6/12; 50.0%), tongue base/tonsil (1/8; 12.5%), urinary bladder (6/49; 12.2%), ventral tongue/floor of mouth (1/8; 12.5%), and vagina (4/10; 40.0%). SCCs from the penis (0/10), skin (0/10), and vulva (0/11) did not express GPC3. Staining occurred in three predominant patterns: diffuse, advancing edge, and focal. None of the patterns was specific to anatomic site (data not shown). **Image 1A** and **Image 1B** depict a vaginal SCC showing diffuse GPC3 expression in the invasive tumor. **Image 1C** and **Image 1D** show positive GPC3 staining at the advancing edge of a pulmonary SCC.

**PAX8**

PAX8 expression was detected in three (2.8%) of 107 SCCs, all from the uterine cervix, and five (10.2%) of 49 UCs. In positive cases, expression was seen in 10% to 60% of tumor cells and was moderately or strongly intense (2+ or 3+). **Image 2A** and **Image 2B** show PAX8 expression in a uterine cervical and urinary bladder tumor, respectively. Lymphocytes in the background stained faintly positive (due to a known cross-reactivity of this antibody with PAX5).
p40

p40 proved to be a highly sensitive marker for SCCs at all anatomic sites as well as for UCs, expressed in 106 (99.1%) of 107 and 47 (95.9%) of 49 tumors, respectively. In moderately to poorly differentiated tumors, p40 protein expression was diffuse and intense Image 3A and Image 3B. However, in more differentiated tumors, protein expression was diminished to absent in areas of keratinization Image 3C and Image 3D, qualitatively similar to normal squamous epithelium and urothelium in which protein expression is concentrated in the basal/progenitor cell layer. The lone negative SCC was an esophageal tumor after neoadjuvant chemoradiotherapy, characterized by the presence of minimal residual, extremely well-differentiated carcinoma Image 3E and Image 3F. The two negative invasive UCs had no special features.

Discussion

GPC3

GPC3 is a member of a family of heparan sulfate proteoglycans anchored to the cell membrane by glycosyl-phosphatidylinositol. To date, six glypicans (GPC1-GPC6) have been described in vertebrates.11,12 The glypicans are...
predominately expressed during fetal development and are considered critical to organogenesis through their interaction with various growth factors, chemokines, and structural proteins within the extracellular matrix, influencing cell differentiation and growth.\textsuperscript{3,5,12} Germline GPC3 mutations cause Simpson-Golabi-Behmel syndrome, an X-linked disorder (the gene is located at Xp26) characterized by pre- and postnatal overgrowth, “coarse” facial features, and increased incidence of certain embryonal tumors, including Wilms tumor, hepatoblastoma, and neuroblastoma.\textsuperscript{3,5,12} The carcinogenic role of GPC3 appears dichotomous, acting as a tumor suppressor in some tissues, such as ovary, breast, and lung (adenocarcinoma), while in other tissues it functions as an oncoprotein, especially liver, lung (SCC), and embryonal tumors.\textsuperscript{11,13}

Most studies have focused on the utility of GPC3 to differentiate HCC from benign hepatocellular lesions.\textsuperscript{14-18} Other reports highlight a broader neoplastic role, with expression in embryonal tumors,\textsuperscript{19-21} germ cell tumors,\textsuperscript{22-24} and melanoma.\textsuperscript{25} Baumhoer et al\textsuperscript{11} reported TMA data from more than 4,000 samples, including nearly 140 tumor categories, and three dozen nonneoplastic/preneoplastic tissue types. One novel finding from their study was that GPC3 was expressed by SCCs from several anatomical sites, including lung, where 54% (27/50) of tumors were positive. Concurrently, Aviel-Ronen et al\textsuperscript{3} reported GPC3 expression in 55% (17/31) of pulmonary SCCs and 8% (5/59) of adenocarcinomas, and not in normal lung controls. Most recently, Tsuta et al,\textsuperscript{26} studying the utility of a variety of markers to distinguish pulmonary SCC from adenocarcinoma, found that 46% (70/150) of pulmonary SCCs expressed GPC3, again compared with 8% (12/158) of adenocarcinomas.

These studies led us to question whether and how frequently GPC3 was expressed by SCC (and UC). Although the literature had drifted to suggesting that GPC3 expression was characteristic of pulmonary SCC, we wanted to confirm the finding of Baumhoer et al\textsuperscript{11} that expression occurred at a variety of sites. We found most frequent expression in lung tumors (50.0%; 6/12), similar to rates described previously,\textsuperscript{5,11,26} but we also found somewhat less frequent expression at most of the anatomical sites examined and detected expression in six (12.2%) of 49 UCs. Table 4A compares our site-by-site findings with those of Baumhoer et al.

PAX8

PAX8 is a member of the paired box gene family, which encodes transcription factors vital to embryogenesis.\textsuperscript{27} To date, nine members (PAX1-PAX9) have been described, which participate in the development of critical target tissues, including central nervous system, skeleton, skeletal muscle, and neural crest, among others.\textsuperscript{27} Specifically, PAX8 is crucial in the development of the thyroid gland (especially) and the kidney.\textsuperscript{7,27} Cell lineage–specific transcription factors have emerged as incredibly useful diagnostic markers in oncology, since tumors tend to maintain or recapitulate the transcriptional programs of their tissues of origin.

In this study, PAX8 expression was restricted to SCC of the uterine cervix (27%) and UC of the bladder (10%). While PAX8 has been extensively studied in ovarian tumors, very limited data are available regarding its expression in the cervix. Laury et al\textsuperscript{7} reported positivity in two (100%) of two cervical SCCs and two (100%) of two high-grade squamous intraepithelial lesions. However, a more extensive investigation by Tacha et al\textsuperscript{8} found expression in only one (2%) of 60 cervical SCCs, one (33%) of three adenosquamous carcinomas, and five (83%) of six adenocarcinomas, while PAX8 was not expressed in normal cervix. Finally, Ozcan et al\textsuperscript{9} failed to detect PAX8 expression in nine cervical SCCs.

There are even less existing data regarding PAX8 expression in SCCs outside of the uterine cervix. Laury et al\textsuperscript{7} studied only 26 other SCCs, detecting PAX8 expression in four (33%) of 12 pulmonary SCCs and none of two anal, seven esophageal, and five head and neck tumors. Tacha et al\textsuperscript{8} failed to detect expression in six esophageal, seven urinary bladder, and one endometrial SCC but did report positivity in a rare lung tumor (2%; 1/49). Ozcan et al\textsuperscript{9} failed to detect PAX8 expression in 20 SCCs from the uterine cervix (0/9), lung (0/4), skin (0/4), and larynx (0/3). Our results suggest that although PAX8 expression is fairly uncommon in SCC, its presence would seem to indicate an origin from the uterine cervix (or lung).

Table 4A

<table>
<thead>
<tr>
<th>Anatomical Site</th>
<th>Current Study, No. Positive/ No. Tested (%)</th>
<th>Baumhoer et al\textsuperscript{11}, No. Positive/ No. Tested (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anus</td>
<td>1/10 (10.0)</td>
<td>1/5 (20.0)</td>
</tr>
<tr>
<td>Cervix</td>
<td>2/11 (17.3)</td>
<td>6/41 (14.6)</td>
</tr>
<tr>
<td>Esophagus</td>
<td>2/7 (28.6)</td>
<td>3/37 (8.1)</td>
</tr>
<tr>
<td>Larynx</td>
<td>3/10 (30.0)</td>
<td>8/49 (16.3)</td>
</tr>
<tr>
<td>Lung</td>
<td>0/12 (0.0)</td>
<td>27/50 (54.0)</td>
</tr>
<tr>
<td>Penis</td>
<td>0/10 (0.0)</td>
<td>1/46 (2.2)</td>
</tr>
<tr>
<td>Skin</td>
<td>0/10 (0.0)</td>
<td>1/50 (2.0)</td>
</tr>
<tr>
<td>Tongue base/tonsil</td>
<td>1/8 (12.5)</td>
<td>NA</td>
</tr>
<tr>
<td>Urinary bladder (urothelial carcinoma)</td>
<td>6/49 (12.2)</td>
<td>7/43 (16.3)</td>
</tr>
<tr>
<td>Vagina</td>
<td>4/10 (40.0)</td>
<td>0/5 (0)</td>
</tr>
<tr>
<td>Ventral tongue/ floor of mouth</td>
<td>1/8 (12.5)</td>
<td>5/50 (10.0)</td>
</tr>
<tr>
<td>Vulva</td>
<td>0/11 (0.0)</td>
<td>5/42 (11.9)</td>
</tr>
</tbody>
</table>

NA, not tested.
papillary UC (16/16; 100%), as well as lack of expression in nine normal urothelial controls. Tong et al\(^7\) reported PAX8 positivity in 7% of 59 UCs, with expression confined to tumors from the renal pelvis (4/17; 23%) and not seen in two ureteral and 40 bladder tumors. They hypothesized that differential protein expression was related to the distinct embryologic origins of the upper urinary tract, derived from the ureteric bud, and the lower tract, derived from the urogenital sinus. In normal controls, they reported strong staining in the full thickness of the urothelium in the renal papilla and renal pelvis, with a gradual diminution in expression intensity and distribution (becoming limited to the basal layers) proceeding distally, as well as complete absence of expression in the urinary bladder. In their comprehensive IHC study, Ozcan et al\(^9\) reported no PAX8 expression in 13 renal-based UCs, 20 urinary bladder-based “urothelial neoplasm/dysplasia[s],” and 10 UCs. In contrast, similar to our findings, Laury et al\(^7\) and Tacha et al\(^8\) detected PAX8 expression in three (18%) of 17 and five (10%) of 50 UCs from the urinary bladder. Of note, we used the same rabbit polyclonal PAX8 antibody reported by the Tong, Laury, and Tacha groups, which, in our experience, has been the most widely clinically used PAX8 antibody to date.

p40 (ΔNp63)

*TP63* is structurally related to *TP53*, encoding transactivating, DNA-binding, and oligomerization domains, but p63 (protein) exists as multiple isotypes.\(^{30}\) The gene contains two separate promoters, resulting in the formation of protein products containing (TAp63) or lacking (ΔNp63) the transactivation domain. This critical distinction results in differing and, at times, opposing effects on target genes.\(^{31}\) In general, TAp63 functions as a tumor suppressor, like p53, while the ΔNp63 isoform (also known as p40) functions as an oncoprotein.\(^{10,26,30,31}\)

In 1998, Yang et al\(^10\) initially identified the various p63 isotypes, and in that landmark study, they also reported the production of multiple monoclonal antibodies recognizing an epitope common to TAp63 and ΔNp63, one of which (clone 4A4) they used in their experiments. Clone 4A4 has become synonymous with p63 IHC (supremacy through primacy) and is a workhorse in diagnostic IHC, where it is used to (1) support the presence of squamous, urothelial, or myoepithelial differentiation and (2) highlight the presence (or absence) of myoepithelial and basal cells in breast/salivary gland and prostate, respectively. That 4A4 positivity is seen in a broader group of tumor types, including some lymphomas, sarcomas, and, perhaps of most relevance, adenocarcinomas, is in our opinion not widely appreciated.\(^{10,26,32-37}\)

p63 (4A4) expression in a poorly differentiated carcinoma is generally assumed to represent unassailable proof of squamous differentiation, although in the literature, the rate of expression in pulmonary adenocarcinoma ranges from 15% to 65%.\(^{10,26}\) Although expression is typically weak and focal, we have occasionally encountered diffuse, strong expression. This “aberrant” staining risks the misclassification of adenocarcinomas as SCCs (or adenosquamous carcinomas), which could potentially deny a patient (1) testing for EGFR mutations or ALK rearrangement, the detection of which would be clinically actionable, or (2) use of bevacizumab (contraindicated in lung SCC).

ΔNp63 is the main form expressed in stratified epithelia, where it supports stem cell renewal.\(^{38}\) In 2000, Hibi and colleagues\(^39\) described the production of a polyclonal antibody specific for ΔNp63 (raised against amino acids 5-17 unique to ΔNp63). More than a decade later, interest in type-specific p63 antibodies is surging, since they have been shown to boost superior specificity in the diagnosis of pulmonary SCC, without sacrificing any sensitivity. For example, in the study by Bishop et al,\(^10\) while 4A4 IHC was positive in 31% of 237 lung adenocarcinomas and 100% of 81 lung SCCs (as well as 54% of 152 large cell lymphomas), the ΔNp63 antibody was positive in only 3% of 205 lung adenocarcinomas (all with staining in only 1%-5% of cells) and 100% of 81 lung SCCs (and none [0%] of 152 large cell lymphomas).

We detected ΔNp63 expression, using the same antibody (p40) as Bishop et al,\(^10\) in virtually all SCCs (106/107; 99.1%) and UCs (47/49; 95.9%). The one false-negative SCC was a small focus of residual tumor that showed therapy-related maturation. In normal squamous mucosa and urothelium, ΔNp63 demonstrates most intense expression in the basal layers, where it supports a basal-like phenotype. In our SCCs, moderate to poorly differentiated examples tended to show uniform expression throughout, while more well-differentiated tumors showed most intense expression at the advancing tumor front with diminution toward the more differentiated central portions of individual nests.

**Conclusion**

We have evaluated three commercially available immunohistochemical stains in a large cohort of SCCs from diverse anatomic sites and invasive UCs. We found that GPC3, which is commonly used to support a diagnosis of HCC, is also expressed by a significant minority of SCCs (~20%) and UCs (~10%-15%). Knowledge of this fact will make an incorrect diagnosis of HCC, as we have seen in our practice, less likely. Although GPC3 expression has been most widely investigated in lung SCC, and it appears to be most frequent at that anatomic site, it is seen with enough frequency at other sites to render it not especially useful in suggesting the site of origin of a metastatic SCC of unknown primary. PAX8 expression was distinctly uncommon in our

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series, seen only in SCC of the uterine cervix and UC. This is in keeping with previous results, and the only anatomic site at which PAX8 expression had been described by others and not seen by us was lung. As such, PAX8 IHC may have some application in assigning a uterine cervical origin or supporting the presence of urothelial differentiation in a metastatic squamotransitional CUP. Finally, lack of specificity of traditional p63 IHC, especially with regard to the distinction of pulmonary SCC from adenocarcinoma, represents a significant diagnostic pitfall. p63 Isoform-specific IHC has been rediscovered, and it has been shown to demonstrate superior specificity for the diagnosis of pulmonary SCC, without any diminution in sensitivity. We have shown that p40 (ΔNp63) IHC is quite sensitive for diagnoses of SCC throughout the body and for UC. If p40 can be proven equivalent to “pan-p63” in other diagnostic contexts (eg, as a marker of myoepithelial and basal cells), it may supplant the prevalent 4A4 antibody clone.

Address reprint requests to Dr Bellizzi: Dept of Pathology, University of Iowa Hospitals and Clinics, University of Iowa Carver College of Medicine, Iowa City, IA 52242; andrew-bellizzi@uiowa.edu.

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References


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