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Placental S100 (S100P) and GATA3: Markers for Transitional Epithelium and Urothelial Carcinoma Discovered by Complementary DNA Microarray

John P. T. Higgins, MD, Gulsah Kaygusuz, MD,† Lingli Wang, MD,‡ Kelli Montgomery, BA,* Veronica Mason, BS,* Shirley X. Zhu, MD,* Robert J. Marinelli, PhD,§ Joseph C. Presti Jr, MD,‡ Matt van de Rijn, MD, PhD,* and James D. Brooks, MD‡*

Abstract: The morphologic distinction between prostate and urothelial carcinoma can be difficult. To identify novel diagnostic markers that may aid in the differential diagnosis of prostate versus urothelial carcinoma, we analyzed expression patterns in prostate and bladder cancer tissues using complementary DNA microarrays. Together with our prior studies on renal neoplasms and normal kidney, these studies suggested that the gene for placental S100 (S100P) is specifically expressed in benign and malignant urothelial cells. Using tissue microarrays, a polyclonal antiserum against S100P protein stained 86% of 295 urothelial carcinomas while only 3% of 260 prostatic adenocarcinomas and 1% of 133 renal cell carcinomas stained. A commercially available monoclonal antibody against S100P stained 78% of 300 urothelial carcinomas while only 2% of 256 prostatic adenocarcinomas and none of 137 renal cell carcinomas stained. A second gene, GATA3, also showed high level expression in urothelial tumors by cDNA array. A commercially available monoclonal antibody against GATA3 stained 67% of 308 urothelial carcinomas, but none of the prostate or renal carcinomas. For comparison, staining was also performed for p63 and cytokeratin 5/6. p63 stained 87% of urothelial carcinomas whereas CK5/6 stained 54%. Importantly, when S100P and p63 were combined 95% of urothelial carcinomas were labeled by one or both markers. We conclude that the detection of S100P and GATA3 protein expression may help distinguish urothelial carcinomas from other genitourinary neoplasms that enter into the differential diagnosis.

Key Words: bladder, immunohistochemistry, cDNA

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From the Departments of *Pathology; §Biochemistry; ‡Urology, Stanford University School of Medicine, Stanford, CA 94305; and †Department of Pathology, Ankara University, School of Medicine, Ankara, Turkey.

Reprints: John P. T. Higgins, MD, Department of Pathology, Stanford University School of Medicine, Stanford, CA 94305 (e-mail: john.higgins@stanford.edu).

Both urothelial carcinoma and prostate carcinoma may produce a mass that protrudes into the lumen of the bladder. High grade carcinomas may present ambiguous morphologic features that do not permit definitive diagnosis and relatively few useful immunohistochemical markers are available. Prostate specific antigen and prostatic acid phosphatase, in particular, are often not expressed by high grade prostate carcinomas.[9] Furthermore, there are few markers that positively stain urothelial carcinomas. A clinical need for new markers clearly exists. To search for urothelial carcinoma markers, we analyzed several urothelial neoplasms using cDNA arrays and compared these with prostate and renal carcinoma profiles. S100P and GATA3 stood out as strongly associated with urothelial differentiation.

We undertook to study expression of S100P and GATA3 using tissue microarrays (TMAs). We studied over 1100 tissues including 321 urothelial carcinomas, 267 prostate carcinomas, and 150 renal cell carcinomas, including 125 clear cell, 9 papillary, 12 chromophobe, 2 collecting duct, and 2 that showed sarcomatoid dedifferentiation. Smaller numbers of carcinomas from other sites were also studied. These include colorectal, squamous, ovarian, lung, endometrial, gastric, thyroid, liver, pancreas, adrenal cortex, and breast.

MATERIALS AND METHODS

Complementary DNA Microarrays

Urothelial carcinomas were analyzed using 43,000 element cDNA arrays. Additional conventional renal cell carcinomas were also analyzed using these arrays in order to permit comparison with the urothelial carcinomas. The gene expression profiles of these renal tumors have not been previously published. Prostate carcinomas have been previously analyzed using this format and were therefore available for study using samples previously published by our group.[19]

Tissue Samples

Informed consent was obtained from all patients before tissue harvest. Urothelial carcinoma samples

were obtained from 3 fresh nephrectomy specimens, 1 cystectomy specimen, and 1 pelvic lymph node. Renal cell carcinomas were isolated from 5 fresh nephrectomy specimens. Tumor was harvested based on gross appearance and immediately frozen on dry ice and stored at −80°C. All samples were validated before RNA isolation using frozen sections stained with hematoxylin and eosin. Paraffin sections from each case were also reviewed by a single pathologist (J.P.T.H.) to confirm the diagnosis.

Microarray Analysis

The cDNA microarrays used in this study included about 28,000 unique characterized genes or expressed sequence tags (ESTs) represented by a total of 41,859 unique cDNAs printed on glass slides by the Stanford Functional Genomics Facility (http://www.microarray.org/sfgf/jsp/home.jsp). Approximately, 10,000 of these genes are characterized while the remainder represents ESTs. Methods for RNA extraction, hybridization to arrays, and interpretation of data have been described elsewhere[1,7,8,21,22] and detailed protocols are available at http://cmgm.Stanford.EDU/pbrown/.

Tissue was homogenized in Trizol reagent (Invitrogen) and total RNA was prepared. Preparation of Cy-3-dUTP (green fluorescent) labeled cDNA from reference RNA using Universal Human Reference RNA (Stratagene, La Jolla, CA) as an internal reference standard and Cy-5-dUTP (red fluorescent) labeled cDNA from tumor sample RNA, microarray hybridization, and subsequent analysis was performed as described.[7,22,25] After washing, the microarrays were scanned on a GenePix 4000 microarray scanner (Axon Instruments, Foster City, CA) and, after normalization of fluorescence intensities to control for experimental variation, fluorescence ratios (sample/reference) were calculated using GenePix software. The primary data tables and the image files are available from the Stanford Microarray Database (http://genome-www.stanford.edu/microarray)[10] and at the website that accompanies this publication: http://tma.stanford.edu/tma_portal/S100P/.

The 5 urothelial carcinomas and 5 renal cell carcinomas were compared with 5 prostate carcinomas randomly selected from the set of 62 prostate carcinomas previously published by our group.[19] We restricted our analysis to genes with a log(base2) R/G normalized ratio (mean) absolute value >1.5 for at least 2 of the samples. We also required the mean fluorescent hybridization signal intensity divided by the median background intensity was greater than or equal to 1.5 in either the sample or reference channel for at least 80% of the samples analyzed. In all, 8856 cDNAs (6210 unique Unigene clusters) met these criteria and their expression across the samples was organized by average linkage hierarchical clustering, where expression levels were centered relative to the mean for each gene and each array. It is this dataset that is described in this report.

Immunohistochemistry Using TMAs

TMAs

TMAs were prepared and evaluated as previously described.[14,17,20] A total of 8 different TMAs were used in this analysis. These contain a total 1211 tissue cores representing 321 bladder urothelial carcinomas (238 high grade, 83 low grade), 267 prostate adenocarcinomas from radical prostatectomies (46 Gleason score 3 + 3, 136 3 + 4, 48 4 + 3, 2 4 + 4, one 3 + 2, and one 4 + 5), 150 renal cell carcinomas (125 clear cell, 9 papillary, 12 chromophobe, 2 collecting duct, 2 sarcomatoid dedifferentiation), 86 colorectal carcinomas, 59 squamous CA (various sites), 43 ovarian surface epithelial neoplasms, 53 lung carcinomas, 31 endometrial adenocarcinomas, 26 stomach adenocarcinomas, 20 melanomas, 17 papillary thyroid carcinomas, 17 renal urothelial carcinomas, 14 hepatocellular carcinomas, 11 seminomas, 10 pancreatic adenocarcinomas, 7 bladder squamous cell carcinomas, 4 ductal breast carcinomas, 5 placentas, 3 adrenocortical carcinomas, and other tissues. Of the bladder carcinomas, 135 were obtained by partial or radical cystectomy and 186 by transurethral resection. Two hundred and eight were invasive at least into the lamina propria and 113 were noninvasive. Depending on the array, each tissue was represented either in duplicate or in quadruplicate. Cores that did not yield an interpretable result were excluded from the data analysis for that antibody. Staining was scored based on staining characteristics of the neoplastic cells in the cores taken from neoplastic tissues and based on the predominant cell type in normal tissues. Positive results were scored as either weak or strong based on both the extent and the intensity of staining. For a strong positive score, intense staining was required in more than 50% of the scored cells. Diffuse weak staining and strong staining in fewer than 50% of the total cell number were both scored as weak positive. When scores for replicate cores were discrepant, the highest positive score was taken to represent the overall score, because this most closely approximates clinical practice. Images of the bladder cores stained with hematoxylin and eosin, S100P, and GATA3 can be viewed at: http://tma.stanford.edu/tma_portal/S100P/.

Immunohistochemistry

A polyclonal antiserum to S100P (S0084) was raised by injecting peptides predicted from the S100P gene sequence (AGI, Sunnyvale, CA). The peptides were conjugated to keyhole limpet haemocyanin, and injected into 2 outbred rabbits. The serum was harvested after the rabbits demonstrated significant antipeptide titer. Affinity-purified antiserum was obtained by binding the antiserum to an affinity column conjugated with the 3 peptides; the bound antibodies were eluted with a pH gradient. A commercially available monoclonal antibody against S100P (BD Transduction Laboratories, San Jose, CA; 1:50) was also used. Immunohistochemistry was performed according to previously published protocols.[13] To avoid interference from endogenous biotin, a biotin

free method, EnVision, was used for amplification of the signal (Dako, Carpinteria, CA). For both the antiserum and the monoclonal antibody, placental cores served as a positive control with strong positive staining of trophoblasts. Nuclear staining was interpreted, and was frequently but not always associated with cytoplasmic staining.

Mouse monoclonal anti-GATA3 was obtained from Santa Cruz biotechnology (Santa Cruz Biotechnology, Inc, Santa Cruz, CA; 1:50) and was stained using the EnVision method.

Additional immunohistochemical markers used were monoclonal anticytokeratin 5/6 (Zymed, South San Francisco, CA; 1:200) and monoclonal anti-p63 (Dako, Carpinteria, CA; 1:200). The cytokeratin 5/6 staining was performed on a Ventana Benchmark automated immunostainer (Ventana Medical Systems, Tucson, AZ) with standard antigen retrieval, whereas the p63 staining was perfomed on a Dako automated immunostainer with citrate retrieval and the EnVision biotin free method.

RESULTS

Gene Expression Profiling

Samples analyzed consisted of 5 urothelial carcinomas, 5 prostate carcinomas, and 5 renal cell carcinomas. When analyzed by unsupervised hierarchical clustering, each of the 3 tumor classes shows distinct patterns of gene expression. The different classes of tumor were clustered together by diagnosis with only 1 urothelial carcinoma grouping with the renal cell carcinomas (Fig. 1).

Urothelial Carcinoma Cluster

Four of the 5 urothelial carcinomas tightly grouped together in a distinct branch of the dendrogram. One joined the cluster of clear cell carcinomas of renal origin. This urothelial carcinoma was a renal primary. Although this suggests the possibility that the similarity between it and the renal cell carcinomas could have been related to contaminating normal kidney in both samples, this is unlikely because frozen section examination was performed on each array tissue and did not show benign contaminating renal tissue in any of the samples. It is more likely that the grouping results from the array print run that was common to these 2 tumors, but not shared with any of the other samples.

By visual inspection of the heatmap, a group of 220 genes was highly expressed in urothelial carcinoma relative to the other 2 tumor types. Within this cluster of genes, S100P stands out as 1 gene that is uniformly expressed in the urothelial tumors but not in the other samples. Other genes that show this pattern include: GATA3, several members of the cytochrome P450 family, keratin 7, Lipocalin 2, Envoplakin, and Follistatin and also less well-characterized genes and ESTs such as transcribed locus Hs.537345 and hypothetical protein LOC348938. Two genes in this cluster were of particular interest, keratin 5 and tumor protein p73-like, commonly known as p63. These genes are known to be expressed in urothelial carcinomas and expression of these 2 proteins identified by immunohistochemistry is used in clinical practice to support a diagnosis is urothelial carcinoma. However, the differential expression pattern for these genes is less striking than it is for S100P in that the separation between the urothelial tumors and the others is not as sharp.

Immunohistochemistry

S100P

On the basis of the gene expression patterns, protein expression was pursued for S100P. We studied S100P protein expression using an antiserum (S0084; AGI, Sunnyvale, CA) and a monoclonal antibody (BD Transduction Laboratories). On a total of 8 different TMAs, there were a total of 321 urothelial carcinomas, of which S0084 staining could be interpreted for 295. The remaining 26 were excluded for technical reasons such as none of the cores remained on the glass slides, or none contained diagnosable neoplasm. S0084 showed nuclear expression in 86.1% of the interpretable urothelial carcinomas. This was usually accompanied by cytoplasmic staining (Fig. 2). In 231 cases, the staining was scored as strong positive (90.9% of positive cases), whereas in the remaining 23 it was weak (Table 1). Also importantly, the quality of the staining was such that the result was easily scored as positive or negative in the majority of cases. S0084 staining was also subclassified according to grade. There were 215 valuable cores from high grade urothelial neoplasms. In all, 80.9% of these showed staining for S0084. Hundred percent of 80 low grade carcinomas stained.

In contrast, S0084 showed staining in only 2.7% of 260 prostate carcinomas and 0.75% of 133 renal cell carcinomas (1 clear cell carcinoma showed weak staining). Other neoplasms in this survey that showed a high rate of positivity included 61% of 72 colon carcinomas, 58% of 19 stomach carcinomas, and 67% of 6 pancreatic carcinomas. Esophageal adenocarcinomas showed a 100% positive rate, but only 4 cases were examined. Interestingly, none of 13 interpretable melanomas on the arrays stained. Complete results are depicted in Table 2.

The monoclonal S100P antibody showed slightly reduced sensitivity but increased specificity relative to the S0084 antiserum. There were 300 interpretable urothelial carcinomas scored for this antibody. Positive staining was seen for 78% of these and 185 of these were scored as strong positive (79.1%) (Table 1). The distribution of staining was essentially identical to that for the antiserum with nuclear staining required for a positive score and usually accompanied by cytoplasmic staining (Fig. 2). Staining decreased with increasing tumor grade with 71.2% of 219 high grade and 96.3% of 81 low grade carcinomas staining. Only 2% of 256 prostate carcinomas stained and none of 137 renal carcinomas did. Other neoplasms that stained included 62% of 21 gastric carcinomas, 60% of 5 esophageal adenocarcinomas,

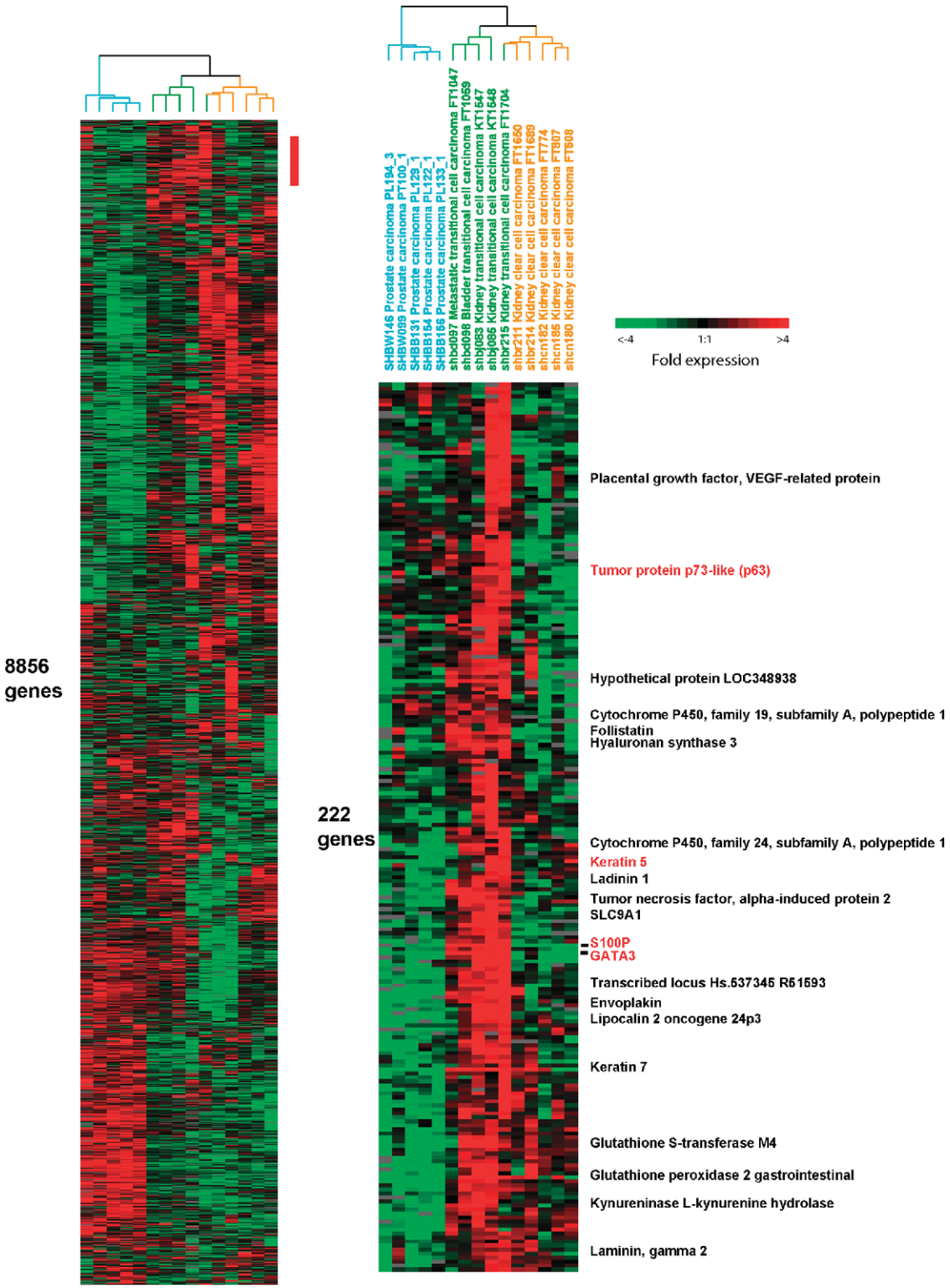

FIGURE 1. Genes highly expressed in urothelial carcinoma. The sample dendrogram at the top of the figure reflects the relative degree to which the samples show similar expression of 8856 genes. Each tumor type shows a reproducible pattern of gene expression that causes them to be grouped together in this dendrogram. One urothelial carcinoma is exceptional in that it is grouped with the renal carcinomas. The heatmap below the dendrogram represents 220 genes that are highly expressed in urothelial carcinomas. Selected gene names are provided to the right of the heatmap and their expression profiles across the samples can be read from the heatmap. Genes for which protein expression was investigated by immunohistochemistry include tumor protein p73-like (p63), keratin 5, S100P, and GATA3. These genes are highlighted in red.

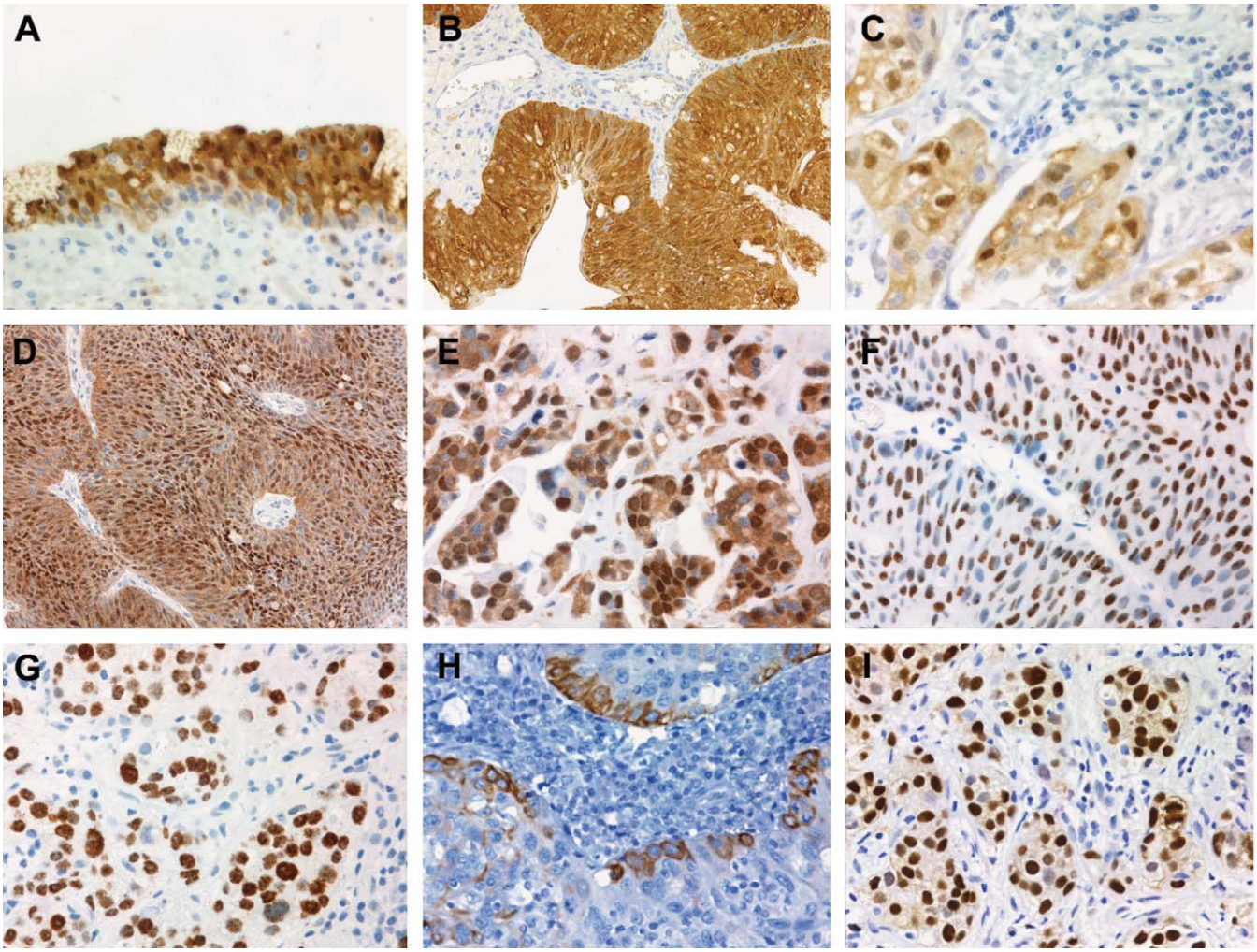

FIGURE 2. Immunohistochemistry. A, S0084 expression in normal urothelium. Both cytoplasmic and strong nuclear expression is seen. The basal layer is largely unstained. B, S0084 expression in low grade papillary urothelial carcinoma. Strong staining of the entire epithelium for S0084 is seen while the underlying stroma is negative. C, S0084 expression in high grade, invasive urothelial carcinoma. Pleomorphic, highly irregular nuclei are stained while cytoplasmic staining is weaker. D, Monoclonal S100P staining in low grade urothelial carcinoma. The pattern of staining is essentially identical to that seen for S0084 with both cytoplasmic and nuclear staining. E, Monoclonal S100P staining in high grade urothelial carcinoma. F, GATA3 staining in low grade urothelial carcinoma. There is no significant cytoplasmic staining. G, GATA3 staining in high grade, invasive urothelial carcinoma. H, Typical subset staining pattern of low grade urothelial carcinoma for cytokeratin 5/6. Strong staining is limited to those cells located next to the adjacent stroma. I, Typical, strong, specific nuclear staining of high grade, invasive urothelial carcinoma for p63.

30% of 10 pancreatic carcinomas, 20% of 80 colorectal carcinomas, 14% of 14 hepatocellular carcinomas, 10% of 48 lung carcinomas, and 7% of 41 ovarian carcinomas.

TABLE 1. Urothelial Carcinoma Immunohistochemistry

	S0084	S100Pm	GATA3	p63	CK5/6
Negative	41	66	102	38	136
Equivalent	26	21	13	29	28
Weak	23	49	38	20	94
Strong	231	185	168	234	63
Total cores	321	321	321	321	321
Total interpretable	295	300	308	292	293
% Positive rate	86.1	78.0	66.9	87.0	53.6
% Strong positive	90.9	79.1	81.6	92.1	40.1

None of the remaining tumors stained. Complete results are depicted in Table 2.

Because of the question of S100P regulation by androgen, we performed an analysis of S100P expression in urothelial carcinoma based on sex. Of our transitional cell carcinoma patients for whom sex status was available, 216 were male and 75 were female. Among the males, 88.9% expressed S100P whereas 80.0% of the females did. This difference was not statistically significant with a P value of 0.075 by Fisher exact test.

To permit a direct comparison with previously described markers of urothelial carcinoma, we stained the same 8 TMAs for p63 and for cytokeratin 5/6. For p63, 292 of the urothelial tumors yielded interpretable results.

TABLE 2. S100P Expression by Immunohistochemistry

Carcinoma	% S0084 Positive	% S100P Positive	No. Cases
Renal urothelial	94	80	17/15
Bladder urothelial, all	86	78	295/300
High grade	81	71	215/219
Low grade	100	96	80/81
Stomach	58	62	19/21
Esophageal adeno	100	60	4/5
Pancreas	67	30	6/10
Colorectal	61	20	72/80
Bladder squamous	0	14	7/7
Hepatocellular	36	14	11/14
Lung	27	10	41/48
Ovary	15	7	40/41
Prostate	3	2	260/256
Adrenal	0	0	3/3
Breast ductal	25	0	4/4
Cervix adeno	25	0	4/5
Cervix squamous	50	0	4/6
Endometrial	7	0	30/31
Esophageal squamous	0	0	7/8
Gallbladder	0	0	1/1
Melanoma	0	0	13/16
Clear cell renal CA	1	0	125/125
Papillary renal CA	0	0	9/9
Chromophobe renal CA	0	0	12/12
Renal collecting duct CA	0	0	2/2
Sarcomatoid renal CA	0	0	2/2
Seminoma	0	0	10/10
Squamous NOS	19	0	16/20
Thyroid	0	0	14/17

TABLE 3. GATA3 Expression by Immunohistochemistry

Carcinoma	% Positive	No. Cases
Breast ductal	100	4
Renal urothelial	67	15
Bladder urothelial, all	67	308
High grade	57	226
Low grade	95	82
Adrenal	0	3
Bladder squamous cell	0	7
Cervix adeno	0	4
Cervix squamous	0	6
Colorectal	0	71
Endometrial	0	27
Esophageal adeno	0	4
Esophageal squamous	0	8
Gallbladder	0	1
Hepatocellular	0	13
Lung	0	45
Melanoma	0	12
Ovary	0	39
Pancreas	0	6
Prostate	0	257
Clear cell renal CA	0	111
Papillary renal CA	0	9
Chromophobe renal CA	0	11
Renal collecting duct CA	0	2
Sarcomatoid renal CA	0	2
Seminoma	0	6
Squamous NOS	0	23
Stomach	0	15
Thyroid	0	18

Overall, 87.0% of the interpretable urothelial carcinomas expressed p63. Of these, 234 were scored as showing strong staining (92.1%). In general, staining for p63 was easily interpreted as positive or negative. None of 117 interpretable renal cell carcinomas expressed this marker and only 0.4% of 251 interpretable prostate carcinomas did. For CK5/6, 53.6% of 293 cases were scored as positive. However, only 40.1% were scored as strong positive and often only a few cells stained. Clearly invasive tumors would often show only staining of a layer of cells adjacent to the stroma. In all, 7.6% of 131 renal cell carcinomas (7 clear cell carcinomas) were positive and 4.3% of 254 prostate carcinomas were positive. This stain was more difficult to interpret than either the S100P stains or p63. When results for monoclonal S100P were considered together with those for p63, 94.9% of all urothelial carcinomas expressed one or both markers. This shows that S100P and p63 are in part complementary; each may identify urothelial carcinomas that were missed by the other. None of the prostate carcinomas expressed both p63 and S100P.

GATA3

GATA3 showed a similar profile on the gene expression dendrogram to S100P and for this reason, it was also pursued as a potential novel marker of urothelial differentiation. For the urothelial carcinomas, 66.9% of 308 interpretable cases were positive (Table 1). The strong positive rate was 81.6%. The pattern of staining was nuclear and usually not accompanied by cytoplasmic staining (Fig. 2).

Although the sensitivity of GATA3 for urothelial carcinoma was lower than that of S100P, the specificity was very high. None of 119 interpretable renal cell carcinomas or the 243 prostate carcinomas expressed GATA3. Each of 4 breast carcinomas stained (100%) but none of the remaining tissues expressed GATA3 (Table 3).

DISCUSSION

DNA array technology has been used extensively to study a wide variety of neoplasms. With this technique, it is possible to rapidly survey the expression of a very large number of genes. In numerous cases, this has provided insight into the classification of tumors that had not been apparent based on light microscopic inspection.[1,19,21] It is frequently possible to subdivide morphologically identical neoplasms into 2 or more groups on the basis of expression of fewer than 100 genes and these different subclasses often have biologic or prognostic relevance. In this study, we have identified a large number of genes expressed in urothelial carcinoma using only a small number of samples. When searching for novel subclasses in a particular diagnostic group, usually a large number of cases need to be analyzed by gene arrays. However, when searching for differential diagnostic markers be-

tween different types of tumors, fewer arrays need to be run. Gene array analysis can thus be used as an initial screen with promising markers subsequently validated by TMA. Our search intended to find new markers specific for urothelial carcinoma that could be used in general practice. Our list of 220 urothelial carcinoma genes derived from these 5 samples contains the previously recognized markers, keratin 5 and p63. This attests to the validity of the results.

We have used cDNA array data to identify immunohistochemical markers that may be of clinical use. We first identified S100P as an urothelial marker based on gene expression patterns in the normal kidney.[15] We confirmed protein expression in the normal urothelium at that time. Here, we confirmed this finding by gene expression in urothelial carcinoma. We also found high level expression in urothelial carcinoma relative to other genitourinary neoplasms that may cause diagnostic confusion such as prostate carcinoma. Here too, S100P was a top gene for identifying the urothelial carcinomas. By immunohistochemistry, S100P shows a comparable sensitivity and specificity for urothelial neoplasms as the best markers in current clinical use and stains additional cases of urothelial carcinoma not marked by either of them. When used together with p63, a higher percentage of urothelial carcinomas is stained than with either marker alone. This indicates a definite role for S100P in diagnostic immunohistochemistry.

S100P is a member of the S100 family of proteins. It must be clarified that S100P is different from the polyclonal antiserum referred to as "S100" which is widely used in clinical immunohistochemistry as a marker for melanoma and nerve sheath neoplasms. S100P was first discovered in placenta and was thus designated S100P. S100 family proteins are characterized by 2 EF hand calcium binding motifs. The family is expressed in a wide range of cells and is thought to play a role in cell cycle progression and in differentiation. S100P binds 2 calcium ions and interacts with S100Z.[12] In addition to Ca^{++}, Zn^{++}, and Mg^{++} are bound by S100P.[11] There are at least 13 members of this family and many of these are encoded on the q21 region of chromosome 1. The S100P gene, however, is located at 4p16.[24] S100P was initially identified in the placenta,[4] a tissue in which we also found high level expression by immunohistochemistry. Expression of S100P has also been previously described in pancreatic carcinoma[5] and in esophageal squamous mucosa by immunohistochemistry,[23] which is also in line with our results. It has also been found to be expressed in breast carcinoma and to be associated with a poor prognosis.[26] It has been suggested that the expression of S100P is regulated by androgen[2] and that this gene may play a role in the etiology of prostate cancer.[3] It is therefore perhaps unexpected that we did not identify expression of S100P protein in a large sample of prostate carcinomas. However, patients with prostate carcinoma do not necessarily have elevated levels of circulating androgen and the level of S100P expression required for detection by our methods may be higher than that required for oncogenesis.

We have also confirmed urothelial expression of GATA3 at the RNA and protein levels. On the basis of cDNA array data, GATA3 showed comparable promise to S100P. By immunohistochemistry, GATA3 showed slightly lower sensitivity than S100P for urothelial carcinoma, but very high specificity. Although 66.9% of urothelial carcinomas expressed GATA3, the only other tumor that did so was ductal breast carcinoma of which each of 4 cases stained. Therefore, as a diagnostic marker, the greatest potential use of GATA3 would be to exclude most nonurothelial carcinomas from consideration.

Our results also serve to corroborate prior reports of p63 expression in urothelial carcinoma.[6,16,18] We have added a large number of cases to the reported literature and our rate of positive staining is comparable with that described previously. We would stress that, like S100P, p63 is a pleasant stain to evaluate and positive and negative cases are readily distinguished. Both can be useful markers in the differential diagnosis of urothelial carcinoma from poorly differentiated prostatic or renal carcinoma.

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The authors would like to dedicate this paper to Raymond Foley, a close friend who died of bladder cancer in 2002. His memory inspired this work.

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