Overexpression of Glutathione S-Transferase α in Clear Cell Renal Cell Carcinoma

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Key Words: Glutathione S-transferase α; GST-α; Renal cell carcinoma; Complementary DNA microarray; Immunohistochemistry

DOI: 10.1309/AQXR6B2QPUGD638C

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Abstract

To determine its diagnostic value, we evaluated glutathione S-transferase α (GST-α) expression in a large number of renal cell carcinomas (RCCs). GST-α messenger RNA (mRNA) levels from 70 renal neoplasms were analyzed with complementary DNA (cDNA) microarray chips containing 21,632 cDNA clones. Furthermore, 348 primary renal tumors and 24 metastatic RCCs were subjected to immunohistochemical analysis with a GST-α–specific antibody. GST-α mRNA was elevated significantly (11.4-fold) in a majority of clear cell RCCs (28/43 [65.1%]; 28/39 [71.8%] with adjustments for informative spots) compared with other kidney tumors (1/27 [3.7%]). Strong and diffuse GST-α immunoreactivity was demonstrated in a majority of clear cell (166/202 [82.2%]; mean intensity, 2.41) and metastatic clear cell RCCs (17/24 [70.8%]; mean intensity, 2.62). Other renal tumor types did not exhibit significant GST-α immunoreactivity, confirming mRNA results. Through cDNA microarrays and immunohistochemical analysis, we demonstrated GST-α as a biomarker for clear cell RCCs.

Renal cell carcinoma (RCC), the most common adult renal neoplasm, represents approximately 2% of all malignant neoplasms and 2% of cancer-related deaths. The most prevalent subtype of RCC, clear cell (conventional) type, accounts for the vast majority of deaths and morbidity associated with renal cancer.[1] The diagnosis of RCC can be problematic because of the lack of symptoms in the early stages. Prompt treatment is imperative for cure owing to the high metastatic potential of this neoplasm in advanced stages. The challenge, therefore, is to identify molecular markers for the precise diagnosis of RCC.

Application of advanced complementary DNA (cDNA) microarray technology in analyzing renal cell neoplasms has provided promising results for identifying novel markers for renal cancer.[2-4] Based on cDNA expression profiling, Young et al[5] reported β defensin-1, parvalbumin, and vimentin as diagnostic markers for different subtypes of RCC. The overexpression of glutathione S-transferase α (GST-α) and α-methylacyl coenzyme A racemase in RCC also has been reported by using cDNA microarrays[6] and immunohistochemical analysis.[7]

Soluble cytosolic GSTs are a supergene family of dimeric ubiquitous enzymes that conjugate glutathione with an electrophilic substrate.[8] The importance of GST lies in the biotransformation and detoxification of many xenobiotics. However, its function also might lead to the activation of potent carcinogens, induction of renal tumors, and development of resistance to chemotherapeutic drugs.[9-11] The human kidney contains 3 isoenzymatic cytosolic GSTs, which are referred to as α (GST-α), μ, and π.[12] The distribution of these isoenzymes is heterogeneous along the nephron, with GST-α expressed predominantly in the convoluted proximal tubule where clear cell RCCs generally are believed to derive their origin.[12] Some level of GST-α expression also is seen in the loop of Henle.[12]

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DOI: 10.1309/AQXR6B2QPUGD638C

There are limited reports on GST-α in renal cell tumors, with controversial results documented. Some reports demonstrated GST-α gene overexpression, while others suggested its downregulation in RCC through the use of different methods. The aims of the present study were to evaluate the expression of GST-α at the messenger RNA (mRNA) and protein levels in a large number of renal neoplasms with defined histologic classifications.

Materials and Methods

Case Selection for RNA Extraction

Freshly frozen tissues of 70 renal neoplasms,[6] including clear cell RCC (n = 43), papillary RCC (n = 10), chromophobe RCC or oncocytoma (n = 8), Wilms tumor (n = 6), and urothelial carcinoma of the renal pelvis (n = 3) were obtained and subjected to RNA extraction. Briefly, total RNA was isolated from renal tumor tissues using Trizol (Invitrogen, Carlsbad, CA) reagent. RNA from pooled normal kidney tissues was extracted and used as a comparison.

cDNA Microarray Analysis

The levels of GST-α mRNA in these tumors were measured by comparison with pooled normal renal tissue, including renal cortex and medulla. Microarrays were performed using gene chips, custom-made at the Van Andel Research Institute Core Facility (Grand Rapids, MI), containing 21,632 cDNA clones. Polymerase chain reaction–amplified bacterial colonies were transferred into 384-well plates and printed onto aminosilane-coated glass slides by means of a custom-made robotic microarrayer.

Fifty micrograms of total RNA from each tumor and corresponding reference was reverse transcribed with oligo (dT) primer and Superscript II (Invitrogen) in the presence of Cy5–deoxycytidine triphosphate and Cy3–deoxycytidine triphosphate (Amersham Pharmacia Biotech, Peapack, NJ). The Cy5- and Cy3-labeled cDNA probes were mixed with probe hybridization solution containing formamide and hybridized to prewarmed (50°C) slides for 20 hours at 50°C. After hybridization and washing, slides were scanned with the use of a Scan Array Lite, operating at 532- and 635-nm wavelengths (GSI Lumonics, Billerica, CA). Images were analyzed further with GENEPIX PRO 3.0 software (Axon Instruments, Foster City, CA). The gene ratios were median centered before hierarchical clustering via CLUSTER and visualized with TREEVIEW (Eisen Lab, http://rana.lbl.gov). Cluster identification tool software was used to locate genes that were expressed differentially (using a Student *t* test) between one histologic subtype and the others. To identify significant discriminating genes, 10,000 *t* statistics were calculated by randomly placing patients into 2 groups.

Tissue Microarrays

Tissue microarray (TMA) blocks were constructed by the use of 2 methods: "sausage" (containing 10 × 10 × 2-mm tissue fragments) and tissue cores (1.5- or 1.0-mm diameters, Beecher Instrument, Sun Prairie, WI). Among the 348 renal tumors, there were 13 cases displaying sarcomatoid transformation (8 associated with clear cell RCC, 4 with papillary RCC, 1 with chromophobe RCC). An additional 24 cases of metastatic clear cell RCC were constructed in 1 TMA block.

Immunohistochemical Analysis

Immunohistochemical studies were performed on 5-µm sections of formalin-fixed, paraffin-embedded tissue as previously described.[7] Antigen retrieval was carried out with a 0.1-mol/L concentration of citrate buffer, pH 6.0, in an 800-W microwave oven for 15 minutes before immunostaining. The slides then were stained on an automated immunostainer (DAKO, Carpinteria, CA) using an avidin-biotin complex staining procedure. The sections were incubated with a rabbit polyclonal antibody specific for GST-α (LabVision, Fremont, CA) at a dilution of 1:100 for 45 minutes followed by brief buffer washings. Sections were incubated with a cocktail of biotinylated antirabbit IgG for 30 minutes. The sections then were washed, incubated in avidin-peroxidase complex for 30 minutes, washed again, and further reacted with diaminobenzidine and hydrogen peroxide to visualize the end product. The sections finally were counterstained with hematoxylin. The nonspecific rabbit IgG was used as a negative control sample.

Evaluation of Immunohistochemical Study Results

The GST-α immunoreactivity intensity of each case was graded semiquantitatively as follows: negative, 0; weakly positive, 1+; moderately positive, 2+; or strongly positive, 3+. The distribution of positive cells was divided into focal (<30%) and diffuse (≥30%).

Results

GST-α mRNA Overexpression in Clear Cell RCC

Isoenzymes GST-α2 and GST-α3, which share 91% homology in protein sequences, were evaluated at the mRNA level. The GST-α2 mRNA (accession ID T73468 of the corresponding cDNA clone) was elevated substantially in the majority of clear cell RCCs (28/43 [65.1%]) compared with other kidney tumors (1/27 [3.7%]) measured by cDNA microarrays. If 4 noninformative gray spots (missing data) were eliminated in the clear cell group **Image 1**, 71.8% (28/39) clear cell RCCs showed GST-α2 overexpression. The average increase of GST-α2 mRNA in clear cell RCCs was 11.4-fold, placing it as one of the top 3 most overexpressed genes in clear cell RCC.

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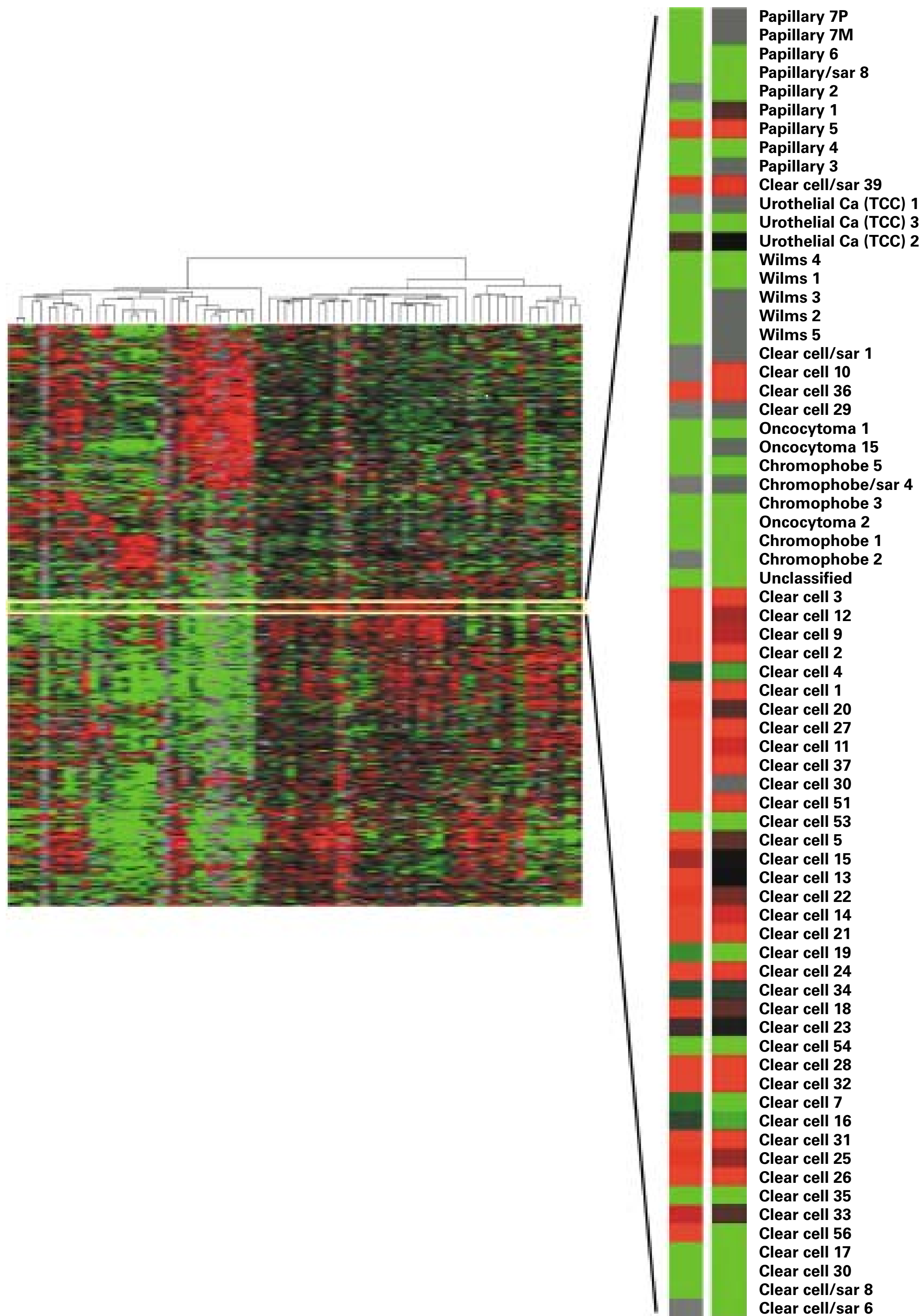

Image 1 Glutathione S-transferase α (GST-α) messenger RNA (mRNA) levels in 70 renal tumors by complementary DNA microarray with 2 probes: accession number T73468 (GST-α2) and accession number N30096 (GST-α3). Red bar, increase in mRNA level (above that of normal kidney); green bar, decrease in mRNA level (below that of normal kidney); black bar, no change in mRNA level (equal to that of normal kidney); gray bar, noninformative case (inadequate RNA or missing data). Brackets indicate molecular clustering (classification) of kidney tumors based on their gene expression profiles. Ca, carcinoma; sar, sarcoma; TCC, transitional cell carcinoma.

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An additional cDNA clone encoding GST-α3 (accession ID N30096) also was elevated in most clear cell RCCs (25/43 [58.1%]; 25/41 [61%] when adjusted for noninformative spots), with an average increase in the level of 6.6-fold, ranking it the 14th most overexpressed gene in clear cell RCC. Only 1 (3.7%) of the 27 remaining renal tumors (papillary, case 5) showed significant GST-α2 and GST-α3 mRNA levels. One tumor (clear cell, case 10) demonstrated a high level of GST-α3 mRNA but was noninformative for GST-α2. Therefore, in combination, a total of 29 clear cell RCCs showed elevated levels of GST-α2 and/or GST-α3 mRNA (29/43 [67.4%]; adjusted rate, 29/39 [74.4%]), as summarized in **Table 1**. Isoenzymes GST-α2 and GST-α3 share 91% homology in protein sequences; therefore, the antibody used in this study reacted with both proteins. Among 6 sarcomatoid RCCs analyzed (4 associated with clear cell RCC, 2 associated with papillary RCC) only one (clear cell 39) displayed increased GST-α mRNA.

GST-α Protein Overexpression in Clear Cell RCC by Immunohistochemical Analysis

The vast majority of clear cell RCCs (166/202 [82.2%]) exhibited strong GST-α immunoreactivity, with most cases (152/166 [91.6%]) demonstrating diffuse staining patterns **Image 2**. The other renal tumors did not illustrate significant GST-α immunoreactivity, confirming the mRNA results. The mean GST-α staining intensity in all clear cell RCCs (2.41) was significantly higher than that of papillary RCCs (0.30), chromophobe RCCs (0.02), and oncocytomas (0.20) **Image 3**. Metastatic clear cell RCCs displayed GST-α immunoreactivity in percentage and intensity similar to that of primary clear cell RCCs (17/24 [70.8%] and 2.62, respectively). All 13 RCCs with sarcomatoid transformation lacked detectable GST-α immunoreactivity. The majority of these tumors were associated with lower grade clear cell RCC components exhibiting GST-α immunoreactivity in 5 of 8 cases studied. The results of GST-α immunoreactivity in renal tumors are summarized in **Table 2**.

Discussion

Although renal cancer is less common than other malignant neoplasms of the genitourinary tract such as prostate and bladder cancers, it has a high risk of metastasis and a relatively higher mortality. Clear cell RCC, the most common subtype of RCC, accounting for 50% to 70% of renal cell neoplasms, is the major cause of mortality and morbidity related to kidney cancer. Compounding its grim nature is the challenge of early diagnosis of RCC and its notoriously high metastatic nature. Metastatic RCC can occur anywhere in the body and histologically mimic many primary tumors, posing additional challenges in the differential pathologic diagnosis. The identification of a new molecular marker for RCC such as GST-α might facilitate early diagnosis and effective treatment.

The discovery and study of GST dates back to 1970. This protein belongs to a family of enzymes capable of cellular protection through the detoxification of xenobiotics and carcinogens by the conjugation of glutathione with these toxins.[13] Nevertheless, GST also can lead to the generation of potent mutagens and carcinogens with possible induction of renal tumors.[14,15] The evaluation of the GST isoenzymes in renal tumors has been performed by various methods, including enzyme assays, immunoblots, Northern blot (mRNA), and immunohistochemical analysis.[16-19] In most of the previous studies, the subtypes of RCCs were not classified further. Based on our recent cDNA microarray analysis, different subtypes of RCCs display significantly distinct gene expression patterns, with GST-α one of the significantly up-regulated genes in the clear cell subtype of RCC.[6]

In 1991, Di Ilio et al[17] reported a reduction in total GST enzymatic activity in 25 of 26 RCCs analyzed. Although the authors attributed the decreased activity predominantly to a decline in GST-α activity, they could not exclude the possibility of the change in level of specific GST isoenzymes, GST-α1, GST-α 2, and GST-α 3. Klone et al[18] found reductions of GST-α mRNA in 27 of 30 cases and of protein levels in 8 of 9 cases. Again, the specificity of the assays used in the study did not distinguish different GST isoenzymes.[18] Similarly, Clairmont et al[20] reported decreased expression of GST-α in

Table 1
GST-α Messenger RNA Levels in 70 Renal Tumors*

	CCRCC (Informative)	Overexpression	Positive Rate	Adjusted Positive	Other Tumors (Informative)	Overexpression	Positive Rate	Adjusted Positive
GST-α2 (T73468)	43 (39)	28	65 (28/43)	72 (28/39)†	27 (23)	1	4 (1/27)	4 (1/23)†
GST-α3 (N30096)	43 (41)	25	58 (25/43)	61 (25/41)‡	27 (18)	1	4 (1/27)	6 (1/18)‡

CCRCC, clear cell renal cell carcinoma; GST-α, glutathione S-transferase α.
* Data are given as number of cases or percentage (number of cases/total cases).
† For GST-α2, 4 of 43 CCRCC spots did not provide sufficient information for analysis (gray spots), thus only 39 cases of CCRCC were informative. Within other tumors, 23 of 27 were informative. Therefore, the positive rates were adjusted using informative spots.
‡ For GST-α3, 41 of 43 CCRCCs were informative, and 18 of 27 other types of renal tumors were informative. The positive rates were adjusted accordingly.

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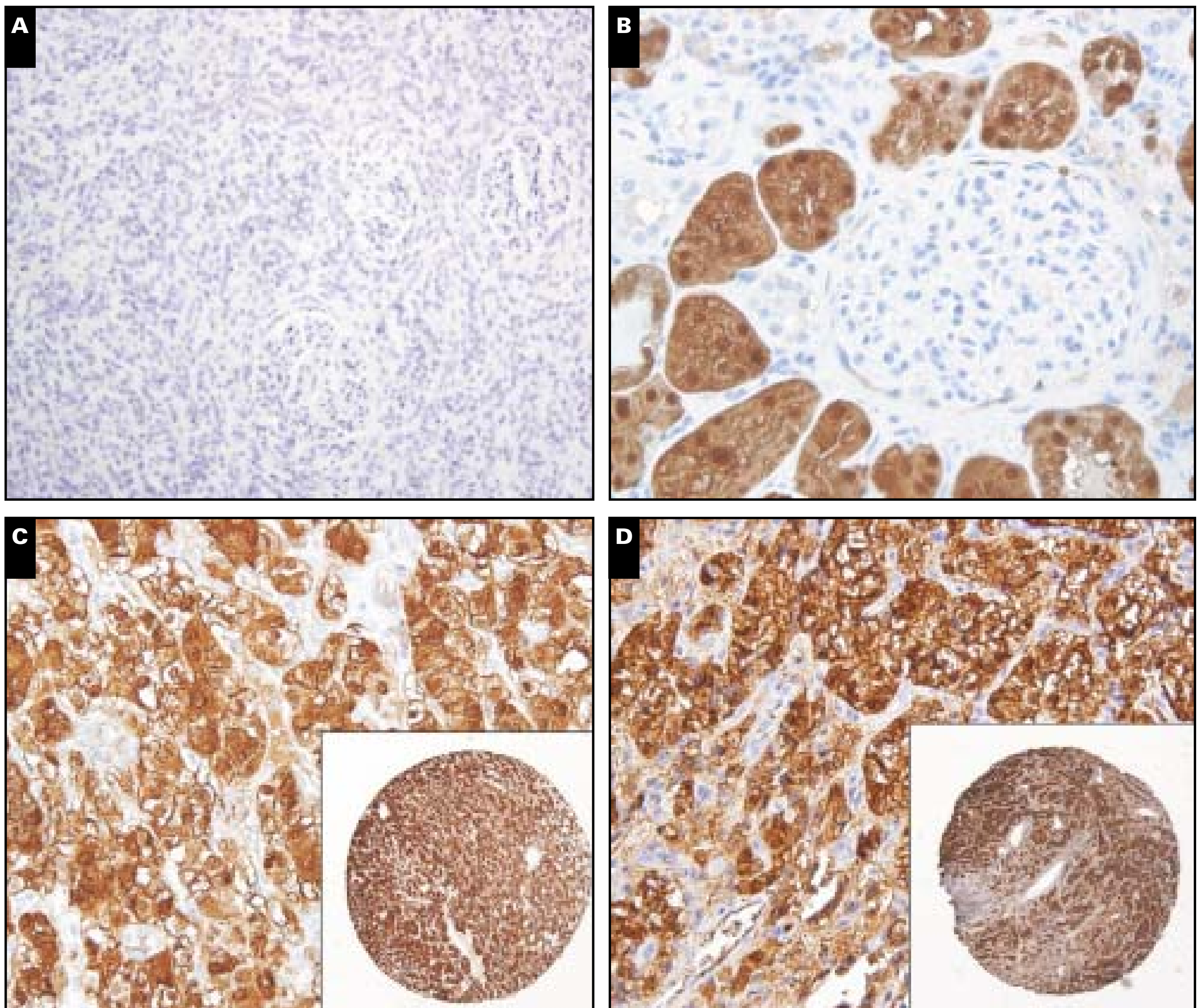

❚Image 2❚ Glutathione S-transferase α immunostaining. Comparison of a negative control sample, nonspecific IgG (**A**, ×400), normal renal cortex (**B**, ×400), primary clear cell renal cell carcinoma (**C**, ×400), and metastatic clear cell renal cell carcinoma (**D**, ×400).

11 of 14 RCCs by Western blot analysis. Emphasis needs to be made on the limitation of such a study, which included only 14 cases of RCC without specific classification based on the current knowledge of the genetic diversity of different subtypes of RCC.

By using immunohistochemical analysis, Grignon et al[19] found that all 46 cases of RCC exhibited positive GST-α immunoreactivity. Similarly, our study, through the use of cDNA microarrays, immunohistochemical analysis, and tissue microarrays, demonstrated significant overexpression of GST-α in the majority of clear cell RCCs but not in other types of renal tumors. One of the factors that might contribute to the discrepant results between our study and previous studies is the lack of classification of RCCs in earlier studies. It is not possible to know what constituted the subtypes of the RCCs analyzed previously. Our study demonstrated that GST-α was overexpressed only in the majority of clear cell RCCs but decreased or not increased in the other 4 subtypes analyzed. It is interesting that we found lower GST-α immunoreactivity exhibited in RCCs with sarcomatoid transformation, consistent with the notion of "dedifferentiation" in RCC.[18] In fact, 50% (7/14) of the high-grade clear cell RCCs (14 tumors with poor clinical outcomes[6] on the far right side of clustering map, Image 1) also showed decreased GST-α expression. These findings of a possible dedifferentiation process of RCC could have diagnostic and therapeutic values. This observation was supported by our ongoing expression profiling studies of a larger number of cases using the Affymetrix (Santa Clara, CA) platform, confirming the presence of a subgroup of high-grade clear cell RCCs displaying lower GSTα mRNA levels

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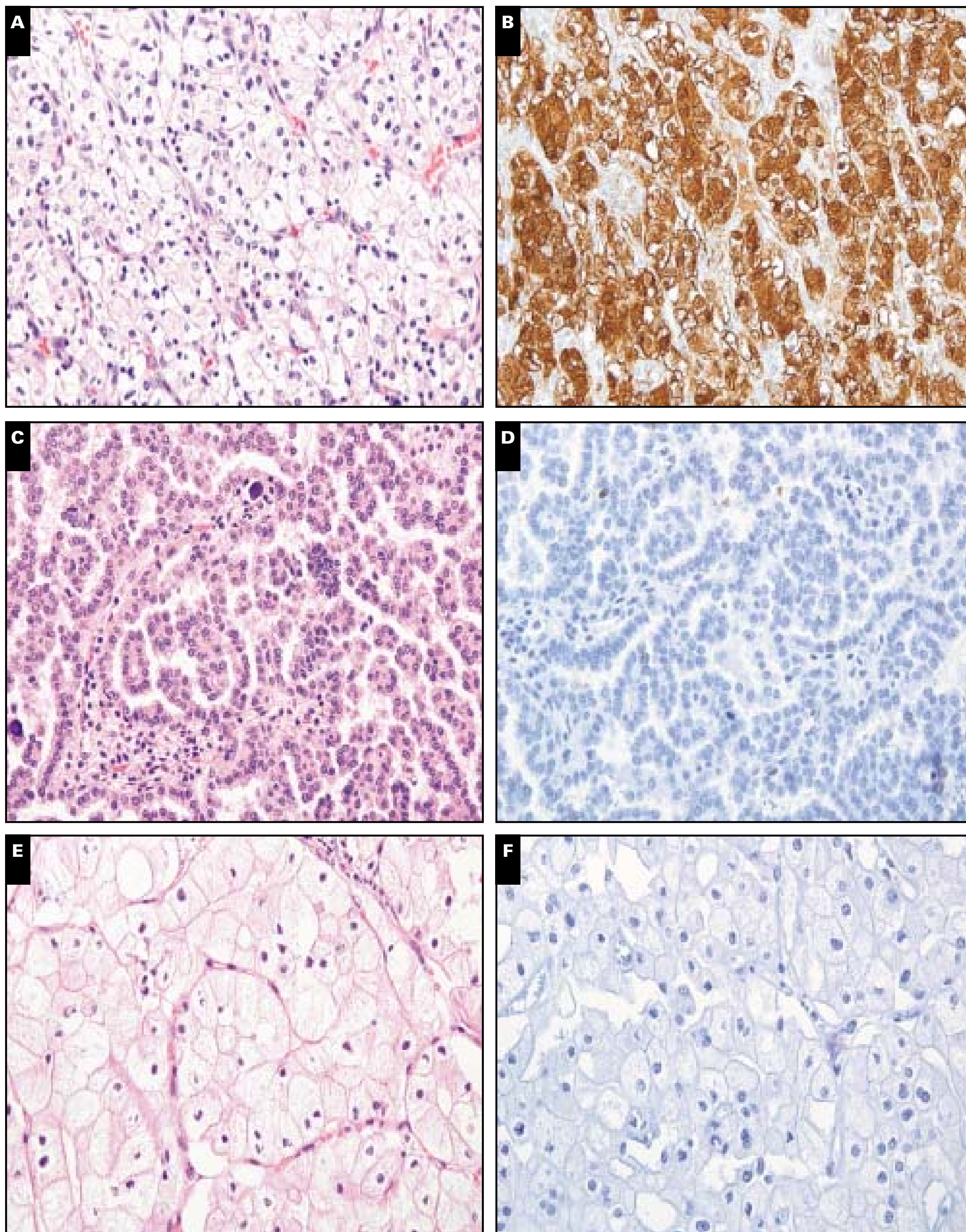

▌Image 3▌ Comparison of H&E staining and glutathione S-transferase α (GST-α) immunoreactivity in renal tumors. **A** and **B**, Clear cell renal cell carcinoma (RCC) (**A**, H&E, ×400; **B**, GST-α, ×400). **C** and **D**, Papillary RCC (**C**, H&E, ×400; **D**, GST-α, ×400). **E** and **F**, Chromophobe RCC (**E**, H&E, ×400; **F**, GST-α, ×400).

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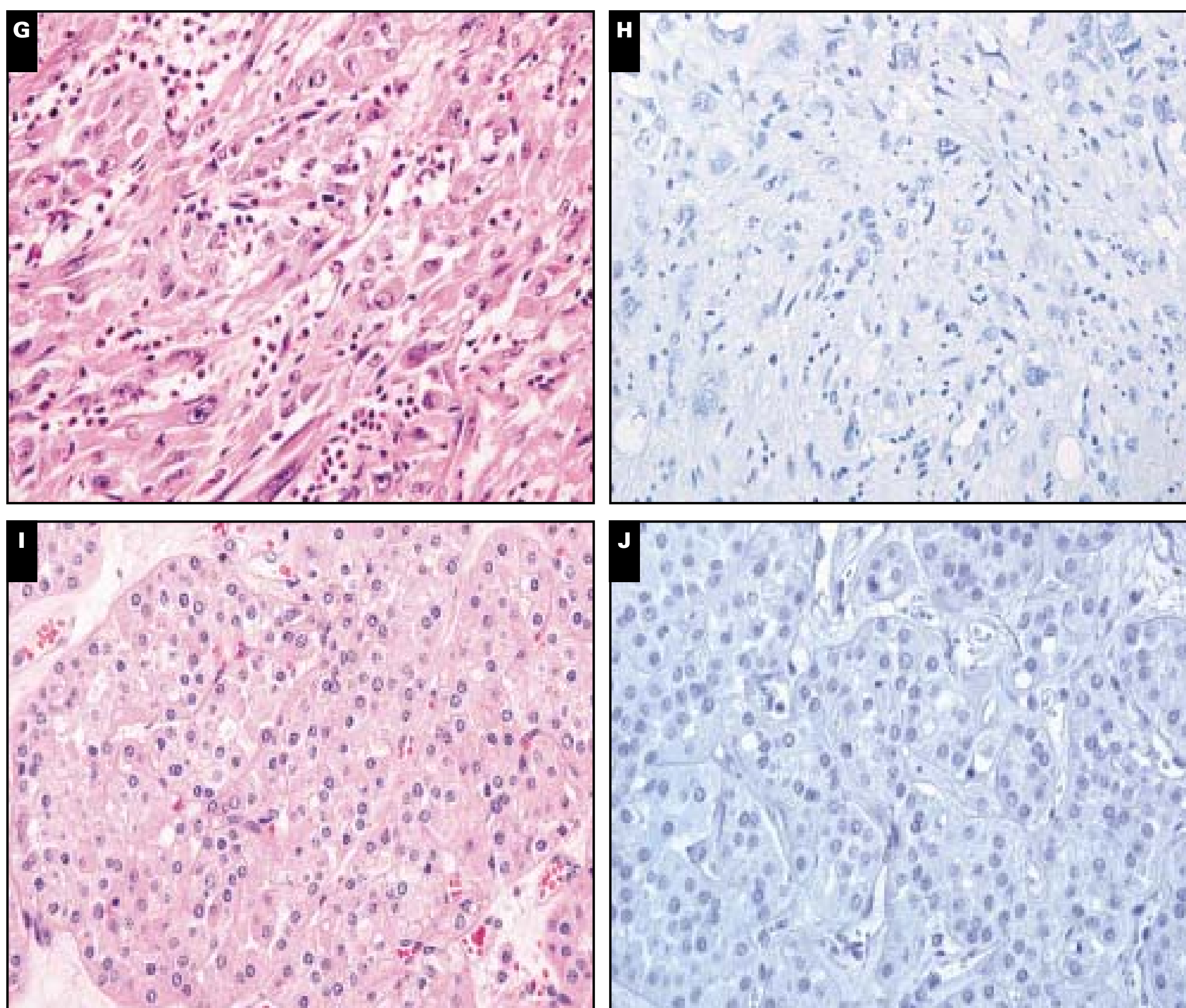

❚Image 3❚ G and **H**, Sarcomatoid RCC (**G**, H&E, ×400; **H**, GST-α, ×400). **I** and **J**, Oncocytoma (**I**, H&E, ×400; **J**, GST-α, ×400).

❚Table 2❚
GST-α Immunoreactivity in 348 Primary Renal Tumors and 24 Metastatic RCCs

	Clear Cell RCC (n = 202)	Papillary RCC (n = 54)	Chromophobe RCC (n = 52)	Oncocytoma (n = 40)	Metastatic RCC (n = 24)
No. (%) of positive cases	166 (82.2)	11 (20.4)	1 (1.9)	5 (12.5)	17 (70.8)
Mean intensity	2.41	0.30	0.02	0.20	2.62

GST-α, glutathione S-transferase α; RCC, renal cell carcinoma.

(M.H. Tan et al, unpublished material, 2004). Furthermore, the possibility that GST-α in clear cell RCC might lack enzymatic activity when analyzed by an enzymatic assay cannot be excluded, even though the protein levels might seem elevated. Further studies are necessary to determine this possibility.

Lately, gene expression microarrays have been shown to be powerful tools for identifying tumor-specific markers and aiding in problematic diagnoses. Recently, multiple specific genes were reported as overexpressed in different subtypes of renal cell neoplasms identified by cDNA microarrays.[4-6] Although increased mRNA expression of GST-α2 and GST-α3 were seen in clear cell RCC, it is of extreme importance that they be validated through the use of other methods such as immunohistochemical analysis. In addition, to confirm gene overexpression, distinction between genes overexpressed in tumor cells or in the tumor stroma must be made. Currently,

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the function of GST-α in the development of RCC is unclear. GST-α possibly might provide a growth advantage for the tumor cells, thereby promoting tumor development.

In the present study, the GST-α mRNA overexpression rate was lower than the immunoreactivity rate in clear cell RCC. Two factors might have contributed to this difference. First, the immunohistochemical antibody reacted with GST-α1 and GST-α2 proteins, whereas the cDNA levels demonstrated were GST-α2 and GST-α3. In other words, some RCCs with GST-α immunoreactivity might be due to the presence of the GST-α1 transcript. Furthermore, several noninformative cases (gray spots) were present in the clear cell group (Image 1), secondary to inadequate RNA for hybridization, which also might contribute to the discrepancy. If only informative spots (43 – 4 = 39) were counted, 72% of clear cell RCCs (28/39) showed GST-α2 overexpression and 74.4% of clear cell RCCs (29/39) showed overexpression of GST-α2 and/or GST-α3 mRNA.

GST-α immunostaining might be valuable in the differential diagnosis and subclassification of renal cell tumors. Markers for different subtypes of renal cell tumors have been reported[6,21]: α-methylacyl coenzyme A racemase for papillary RCC, ron for chromophobe RCC and oncocytomas, and cytokeratin 19 for transitional cell carcinoma. GST-α will be added to the panel of markers for differentiating renal cell tumors if difficulties should arise in classification. In a case of metastatic tumor of unknown primary origin, GST-α positivity would strongly suggest a renal origin.

Several studies have been performed to analyze the activity of GST in a wide range of tumors in the lung, colon, stomach, liver, bladder, ovary, and breast, with variable expression of GST-α. Tumors from the lung, colon, and stomach revealed an increase in GST-π but a decrease in GST-α.[22] A total increase in GST activity was seen in hepatocellular carcinomas, although the percentages of GST-π and GST-α were equivalent in these lesions.[23] McQuaid et al[24] noted an increase in the transcriptional activation of the GST-π gene in bladder and ureteric carcinomas. Intracytoplasmic staining of GST-α in mucinous ovarian carcinomas might represent up-regulation of some detoxification function as reported by Tiltman and Ali.[25] An increase in GST-π was seen in breast cancers.[22] As can be seen, a majority of the tumors from other organ sites usually exhibit an increase in GST-π but not GST-α, with the exception of mucinous ovarian carcinomas.

Through the use of cDNA microarrays, immunohistochemical analysis, and tissue microarrays, significant overexpression of GST-α in the majority of clear cell RCCs was identified but not seen in other types of renal tumors. These findings suggest the potential diagnostic value of GST-α as a molecular marker for clear cell RCC.

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Presented in abstract form at the 94th Annual Meeting of the United States and Canadian Academy of Pathology, Vancouver, Canada, March 2004.

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