



# Glutathione S-transferases as molecular markers of tumor sub-type and prognosis in clinically confined renal cell carcinoma

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**Glutathione S-transferases as molecular markers of tumor sub-type and prognosis in clinically confined renal cell carcinoma**

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**Glutathione S-transferases as molecular markers of tumor progression and prognosis in clinically confined renal cell carcinoma**

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*Running title: GST-alpha in human RCC*

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## Abstract

### Aims.

Renal cell carcinoma (RCC) often recurs as distant metastasis; there is thus a need for new indicators to identify high-risk patients. Glutathione S-transferases (GST)  $\alpha$  and  $\pi$  are involved in the renal bioactivation of toxic metabolites. We investigated whether their expression could hold diagnostic and prognostic value.

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### Methods and Results.

Western blotting of microdissected normal kidney and immunostaining of histological RCC microarrays shows expression of GST- $\alpha$  in proximal tubular cells while GST- $\pi$  was found in the distal nephron. Of the primary 174 RCC cases examined, GST- $\alpha$  immunoreactivity was restricted to conventional RCC (n = 76, 68% positive) and was not seen in any other RCC sub-types. The cross-tabulation of the GST- $\alpha$  scores with other prognostic indices demonstrated that GST- $\alpha$  immunostaining was significantly more frequent in low grade tumours ( $\chi^2$ :  $p < 0.004$ ), and that conventional GST- $\alpha$  positive RCC patients had a mean disease-free survival of 6.0 years (95% CI 5.33 – 6.63), compared with 4.7 years (3.54 – 5.90) in GST- $\alpha$  negative tumours (Kaplan-Meier survival analysis,  $p = 0.011$ , log rank test).

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### Conclusions.

GST- $\alpha$  is a highly specific diagnostic marker for primary conventional RCC, where it is a prognostic marker if grade is omitted from the multivariate analysis.

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### Keywords:

Glutathione S-transferases; renal cell carcinoma; tissue microarray; prognostic indicators; western blotting; immunohistochemistry

## Introduction

Renal cell carcinoma (RCC), the most common adult renal neoplasm accounts for approximately 3% of cancers and 2% of all cancer-related deaths (1). It has been reported that 50-60% of RCC patients will either present with, or develop metastasis, because RCC is difficult to diagnose in the early stages due to the patients being asymptomatic (2). Furthermore, stage, grade, presence of vascular invasion and histological subtype are the accepted prognostic indicators, but these parameters are notoriously inaccurate to provide confident prognostication and therapy selection (3). Therefore, there is the need to identify more accurate molecular markers for the precise diagnosis of RCC and for the assessment of the risk of disease re-occurrence (4) to form the basis for future treatments.

Oxidative and electrophilic stress play a key role in RCC growth and progression (5, 6) and humans have an array of anti-oxidants and enzymes to limit the damage caused by certain xenobiotics and carcinogens. Glutathione S-transferases (GSTs) play an essential role in this detoxification process (7). They belong to a supergene family of ubiquitous dimeric enzymes that conjugate glutathione with electrophilic substrates, including potential alkylating agents where they are eliminated. The resulting S-conjugates are water-soluble, and are transported via the circulation to the kidney (8). The resulting glutathione conjugates can be metabolically activated in the kidney by gamma-glutamyltransferase and beta-lyase to create cytotoxic/carcinogenic metabolites, acting on the proximal tubule, the main site of toxicity (9). In these circumstances, increased GST expression levels have been associated with a protective effect against the toxicity evoked by certain alkylating agents. However, because the kidney metabolises many of these S-conjugates, higher activity of GSTs have been reported in RCC and is believed to account for the formation of even more reactive glutathione S-conjugates. These conjugates can ultimately cause renal damage directly (10). Indeed, it has been previously shown that GSTs are involved in the renal bio activation of toxic metabolites (5, 6, 11) and that selective toxicity and carcinogenicity by the proximal tubular cells can be accounted for the active accumulation of toxic glutathione S-conjugates (9). Moreover, GSTs also inactivate several anticancer drugs (*i.e.*, adriamycin, mitoxantrone and melphalan) (12 – 14), and their overexpression is believed to exacerbate disease severity and progression. Whether due to direct target of xenobiotics or to their ability to bioactivate

glutathione conjugates, these observations suggest that differential GST expression in the kidney could contribute to disease outcome by either affecting the cancer risk and/or the response to chemotherapeutic agents.

While the involvement of GSTs in the pathogenesis or progression of RCC is undisputed, there is discrepancy in the reported levels of expression of the different GST isoforms in renal cell tumours. For instance, some studies have reported over-expression of GSTs in RCC (1, 15), while others a decrease in their expression (16, 17). Thus, it is currently difficult to establish conclusively the possibility of using GST levels as tools for diagnosis, sub-classification and disease progression.

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The normal human kidney expresses high levels of cytosolic GSTs (18). GST- $\alpha$  is predominantly expressed in the proximal convoluted tubule while GST- $\pi$  is expressed in the distal convoluted tubule and collecting duct (19, 20). The proximal tubule is the origin of conventional RCC (21). It is in this nephron segment that GST- $\alpha$  is specifically and selectively localised, suggesting a potential (although not exclusive) association with this type of renal cancer. In contrast, GST- $\pi$  is expressed in the distal nephron (19, 20) and it is well established that its expression is increased in the majority of human renal tumours (20, 22). Furthermore, human-derived cell lines selected for studies of resistance to anticancer drugs over express GST- $\pi$  (23, 24). All of these observations point to the possibility that GST- $\alpha$  and GST- $\pi$  may be useful to discriminate the different types of RCC and that they could potentially be used to predict disease outcome.

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The aim of this study was, therefore, to evaluate the immunohistochemical distribution profile of GST- $\alpha$  and GST- $\pi$  in normal human kidney and in different types of RCC, with the view of assessing whether GSTs hold any value as molecular markers for RCC, and to test their ability to predict long-term prognosis.

## Materials and Methods

### Tissue

Human tissue samples were taken from kidneys surgically resected for RCC. Nephrectomy specimens were delivered to the laboratory within 1 hour of removal and dissected fresh. Samples of cortex, medulla and papilla were dissected from non-neoplastic kidney tissue. Half of the samples were fixed in buffered formalin and routinely processed into paraffin wax and the other half were frozen in liquid nitrogen and stored at -70 °C until processed for western blotting.

To study the intra-renal distribution of GSTs, RCC tumour tissue arrays from a series of 174 surgically resected kidney samples from two hospitals were used. The series has been described in detail previously (25). Briefly, the array is derived from sequential localised (non-metastatic) tumours surgically resected between 1989 and 1998 from patients followed up for up to 10 years. Pathological data on each case included: histological type according to the Heidelberg classification; size, Furrman grade, level of vascular invasion (IVC, renal vein or microvascular) stage (IUCC 1997) and cellular invasion of fat (26, 27). The median age of the patients was 65 (34–88) years; 119 of the patients were men and 55 women. Conventional (clear cell) carcinomas accounted for 119 of the tumours, 23 were papillary, 5 chromophobe and 27 of the tumours were unclassified by conventional histology. **There were 12 conventional, 2 papillary and 5 unclassified tumours with sarcomatoid features.** Complete clinical follow-up was carried out as previously described (28), and the following information being extracted from the patients records: date of birth, sex, date of surgery, date last seen, date of death, cause of death and the date on which recurrent or metastatic disease was first identified.

### Western Blot Analysis

The frozen kidney cortex, medulla and papilla samples were dissected by a pathologist immediately after surgery and individually homogenised in RIPA buffer for 30 seconds. The homogenates were placed on ice for 1 minute and homogenised for further 30 seconds. The samples were then centrifuged at 10,000 x g for 5 minutes to remove insoluble debris. The supernatant was aliquoted and kept at -70 °C until used.

Before the electrophoretic separation, the samples were heated at 70 °C for 10 minutes and reduced using 1X NuPage Reducing Agent (Invitrogen Ltd., Paisley, UK). Proteins were resolved by SDS-polyacrylamide gel electrophoresis using NuPage Novex 10% Bis-Tris gels

(Invitrogen Ltd., Paisley, UK). The gels were transferred onto a PVDF membrane (Invitrogen Ltd., Paisley, UK) using an X-Cell sure lock mini-cell and blot module (Invitrogen Ltd., Paisley, UK) set at 40V for 70 minutes. Transfer efficiency was checked by incubating the blots with 0.1% ponceau S (Sigma-Aldrich, Poole, UK). Subsequently, the blots were rinsed in 1% acetic acid for 10 minutes to remove the stain. Membranes were blocked in Tris-buffered saline-Tween 0.05% with 5% non-fat dried milk (NFDM) overnight at 4 °C. The PVDF blots were incubated for 1 hour at room temperature with a 1:1000 dilution of GST- $\alpha$  mouse monoclonal antibody (Argutus Medical, Dublin, Ireland) and a 1:500 dilution of the GST- $\pi$  mouse monoclonal antibody (Abcam Inc., Cambridge, MA, USA) diluted in 5% NFDM on a rocking platform. After thorough rinsing, the immunoblots were incubated for 1 hour at room temperature on a rocking platform with horseradish peroxidase-conjugated guinea pig anti mouse IgG antibody (Abcam Inc., Cambridge, MA, USA) diluted in 5% NFDM. The protein bands were detected by chemiluminescence using an ECL Plus western blotting system (Amersham International Plc, Buckinghamshire, UK) and developed on Hyperfilm<sup>TM</sup> ECL (Amersham International Plc, Buckinghamshire, UK).

### **Tissue Microarray Construction**

A tissue microarray (TMA) was constructed using archived paraffin-embedded tumour samples. Representative peripheral regions of each tumour were identified in haematoxylin eosin-stained sections and marked for sampling with the TMAs. Each individual core was 0.6 mm in diameter and was punched from the identified region for each tumour. The cores were precisely arrayed into recipient paraffin blocks using a Manual Arrayer, Type I, Beecher Instruments (Sun Prairie, WI, USA) and the orientation was recorded. Normal renal parenchyma and cores from human placenta were also included as controls and orientation markers respectively. Full sections of normal kidney (from nephrectomy specimens) were used to assess the normal distribution of the antigens.

### **Immunohistochemistry**

4 $\mu$ m-thick sections were cut from the resulting TMA and from the normal kidney tissue paraffin blocks onto strongly adhesive slides (Superfrost Plus, Thermo Scientific, Waltham, MA, USA). The sections were deparaffinised in xylene (Sigma-Aldrich Company Ltd. Dorset, England) and rehydrated in ethanol. Immunohistochemistry was performed using the



Envision+ system (DAKO, Ely, Cambridgeshire, UK). Endogenous peroxidase activity within the rehydrated tissue was quenched using the supplied peroxidase block for 5 minutes. The sections were incubated with the primary antibodies [GST- $\alpha$  (mouse IgG; 1:350 – Argutus Medical, Co. Dublin, Ireland) and GST- $\pi$  (mouse IgG; 1:3000 – Abcam Inc., Cambridge, MA, USA)] for 1 hour and then washed off using 1X phosphate-buffered saline (PBS) added with 0.05% Tween 20 (3 washes for 5 minutes each). Mouse Horse Radish Peroxidase (HRP) labelled polymer (DAKO Cambridgeshire, UK) was applied to the sections for 30 minutes and the immunoreactivity was visualized using 3,3'-diaminobenzidine (DAB) (DAKO, Ely, Cambridgeshire, UK) as the chromogenic peroxidase substrate. The slides were counterstained with haematoxylin and mounted using DPX mounting medium (BioChemika, Sigma-Aldrich Company Ltd. Dorset, England). A mouse isotype control (DAKO, Ely, Cambridgeshire, UK) was used to replace the primary antibody as a negative control. All immunoreactions were carried out at room temperature.

### Scoring of Stained RCC Tissue Microarrays

Evaluation of sections was carried out blinded by the research assistant and the histopathologist over a conference microscope; observers were masked to outcome (29). Scoring was agreed by consensus; cases were graded semi-quantitatively as follows: negative, 0: [no detectable reaction product (deposit) in tumour cells]; weakly positive, 1+: very light diffuse or focal deposit in tumour cell cytoplasm, moderately positive, 2+: light diffuse or moderate focal deposit (may include very small areas of heavy deposit); strongly positive, 3+: tumour containing areas of heavy deposit in most or all of the tumour cells.

### Data and Statistical Analyses

The association of the positive markers with recognised tumour prognostic variables (grade, stage, size, vascular invasion, capsular invasion and tumour type) was examined by cross-tabulation and the chi-squared test or Fisher's exact test, as appropriate. Correction for multiple comparisons was made by the Holm-Bonferroni method. Analysis of disease-free survival of patients with tumours showing different scores of staining for each marker was carried out by Kaplan-Meier method using log-rank test, where the first appearance of a metastasis was considered an event. Patients last seen alive without metastasis or who died due to causes other than RCC were considered censored at the date last seen or date of death,

respectively. Scores were converted to a binary simple covariate (positive or negative) by thresholding according to the most informative split on the Kaplan-Meier using the log-rank statistical test. A score of 0 was recorded as negative, scores of 1+, 2+ or 3+ were considered as positive.

Multivariate survival analysis was carried out by Cox regression using the enter function with covariates considered as categorical. We had already determined that the most influential covariates predicting the disease-free survival of these patients are Fuhrman grade (grade 1 & 2 and grades 3 & 4 are pooled for analysis); any degree of vascular invasion (histological correlate of stage T<sub>3b</sub>), and histological invasion of perinephric tissue (the histological correlate of stage T<sub>3a</sub>). When these covariates are taken into account then tumour size and type have no influence on disease free survival (Peters and Roelofs, 1992). The statistical package SPSS 11.5 was used for analysis. All tests were two-tailed.

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## Results

GST- $\alpha$  and GST- $\pi$  have a molecular weight of approximately 45kD and are dissociable into subunits of approximately 25kD (8). Single bands of predicted molecular weight of ~ 25kD were detected for both GST- $\alpha$  (Figure 1A) and GST- $\pi$  (Figure 1D). Western analysis demonstrates that GST- $\alpha$  expression is confined to the kidney cortex and outer medulla but it is absent from the papilla (Figure 1A). In contrast, western analysis of equal amounts of kidney cortex extracts revealed that GST- $\pi$  expression was highest in the papilla and, to a lesser extent, in the cortex and medulla (Figure 1D), with immunoreactive species visible at ~25 and 45 kD. These observations are consistent with the immunohistochemical analysis of normal renal parenchyma that revealed GST- $\alpha$  localization in the cytoplasm of proximal tubule cells (Figure 1A-C). GST- $\pi$  immunoreactivity was found within the cytosol of the loop of Henle, distal tubule and collecting duct cells (Figure 1E-F). Both glomeruli and proximal tubules were devoid of GST- $\pi$  immunoreactivity. These findings indicate stronger GST- $\pi$  expression in the distal nephron, an observation which is consistent with previous reports (1, 19, 20).

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Comment [t1]: Insert Figure 1.

TMA's of clinically confined RCC showed that GST- $\alpha$  is exclusively and specifically expressed in the cytoplasm of clear cell RCC (Figure 2A-D). The figure also shows what



appears to be nuclear staining in all the different scores. *Vice versa*, GST- $\alpha$  is completely absent from papillary (Figure 2E) and chromophobe (Figure 2F) RCC specimens. We confirmed this in staining whole sections of three chromophobe carcinomas which showed no focal staining (not shown).

Comment [t2]: Insert Figure 2.

In contrast to that observed for GST- $\alpha$ , GST- $\pi$  immunoreactivity was more widespread and of different degree and intensity. For instance, GST- $\pi$  signal was specifically detected not only in the cytosol and the nuclei of clear cell RCC (Figure 3A-D), but also in those of papillary and chromophobe specimens (Figure 3E and F, respectively). Thus, while GST- $\alpha$  exhibits a high degree of specificity and is confined to proximal tubular cells of clear cell-positive specimens, GST- $\pi$  appears to be present all along the nephron and its expression pattern does not appear to have any distinctive feature between the different types of RCCs.

Comment [t3]: Insert Figure 3.

Of the 174 RCC samples on the array examined in the current study, 168 were assessable for GST- $\alpha$  and 155 for GST- $\pi$  immunostaining. The cross-tabulation of the grade of staining of GST- $\alpha$  and GST- $\pi$  with the conventional histological prognostic covariates are shown on Tables 1 and 2, respectively. Of the 112 conventional RCC cases examined, 76 were positive for GST- $\alpha$  (corresponding to 68% of the total); 22 cases of papillary RCC were examined, 100% of these were negative for GST- $\alpha$ ; 5 cases of chromophobe were examined, 100% of these were negative for GST- $\alpha$ ; 29 cases were unclassified and 13 of those were positive for GST- $\alpha$ , corresponding to 45%.



Comment [IS4]: Insert table 1

The striking result is the strong association of GST- $\alpha$  with low grade ( $p < 0.001$ ) and conventional type RCC ( $p < 0.001$ ). These associations retain their significance after correction for multiple comparisons ( $p = 0.006$ ). Indeed, in other specific types of RCC (papillary and chromophobe) GST- $\alpha$  immunoreactivity was completely absent. This finding prompted histological review of the 13 unclassified RCCs, which showed that in all 13 cases with any staining (grade 1+, 2+ and 3+) for GST- $\alpha$  the most likely histological diagnosis was considered conventional RCC.



Comment [t5]: Insert Table 2.

Table 2 shows that GST- $\pi$  did not show any significant association with any of the prognostic parameters. Although there was a trend suggesting it was more commonly expressed in



papillary RCC, this difference did not reach statistical significance after taking into consideration the correction for multiple comparisons ( $p = 0.49$ ).

Survival analysis for GST- $\alpha$  was restricted to the conventional RCCs as other specific tumours did not show any immunoreactivity for it. On Kaplan-Meier univariate survival analysis, patients with conventional RCCs that were positive for GST- $\alpha$  had a mean disease-free survival of 6.0 years (95% CI 5.33 – 6.63), compared with 4.7 years (95% CI 3.54 – 5.90) for those that were GST- $\alpha$  negative. On log-rank testing this difference is significant ( $p = 0.011$ ) and demonstrates a clear disease-free survival advantage for conventional RCCs expressing GST- $\alpha$  (Figure 4). The result is also significant if conventional and unclassified RCCs are pooled for analysis ( $p = 0.002$ ). In contrast, tumours expressing GST- $\pi$  showed no survival difference when compared with tumours without its expression (not shown).

Comment [t6]: Insert Figure 4.

Previously it has been shown that grade, as well as vascular and capsular invasions, are influential predictors of prognostic in this group of patients (28, 29). The current study shows that, by Cox regression analysis, neither GST- $\alpha$ , nor GST- $\pi$  constitute significantly influential covariates when including the covariates grade, vascular invasion and capsular invasion. “However, if grade is omitted from the analysis, GST- $\alpha$  adds prognostic information (Table 3)”

Comment [U7]: Inset Table 3.

## Discussion

This study of GST expression in normal human kidney tissue and in a large cohort of specimens from patients with RCC has two main results, both of which might have clinical impact: the strong, specific association of GST- $\alpha$  with conventional (or clear cell) RCC and the ability of GST- $\alpha$  to predict disease outcome in patients affected by this type of tumour.

Our study has clarified and added to knowledge of the distribution of GST- $\alpha$  and GST- $\pi$  in the normal kidney: in addition to using IHC, we have used tissue specifically taken from the cortex, outer medulla and papilla while others have used homogenates of whole kidney for protein or mRNA analysis. Our results add to previous findings in demonstrating that GST-

$\alpha$  is specific for the proximal tubule – in both the convoluted and straight sections (pars recta) and GST- $\pi$  to the loop of Henle and the distal nephron. As GST- $\alpha$  has a sparse distribution in other normal tissues, being found only in the liver in significant quantities, this may indicate GST- $\alpha$  is likely to be a good proximal tubular marker.

The most striking finding of our study is the strong association of GST- $\alpha$  expression with conventional (or clear cell) RCC; this association is to be expected as conventional RCC is postulated to be derived from the proximal tubular cell epithelium. However, the strength and specificity of this association is surprising. Our finding of 13 out of 29 unclassified RCCs staining for GST- $\alpha$  is explained by the fact that our original categorisation of the tumours was carried out on H&E sections alone with no support from IHC. We tried to ensure that no tumours were misclassified: if there was any doubt as the tumour type on morphological grounds, the tumour was designated unclassified; this was to ensure results were not contaminated by misclassified tumours. Subsequent review of the sections of the unclassified tumours that stained for GST- $\alpha$  revealed that in all these tumours the most likely morphological diagnosis was conventional RCC. Our results thus strongly support the findings of Lui *et al.*, 2007 (15) who, in a carefully categorised group of renal tumours, showed 41/45 (90%) conventional RCC staining for GST- $\alpha$  but no chromophobe carcinomas (22 cases) or oncocytomas (17 cases) (although we cannot comment on the expression of GST- $\alpha$  in oncocytomas as no cases were assessed). In contrast, although Chuang *et al.*, 2005 (1) and Takashi *et al.*, 2003 (30), confirmed GST- $\alpha$  immunoreactivity in a high proportion (90%+) of conventional RCCs, there was some reactivity with papillary (11 out of 54) chromophobe (2 out of 62) and oncocytoma (5 out of 40). Neither of these authors specifies how the original classification of these tumours was ascertained, and given the difficulty in classifying many renal tumours, it is possible that some were misclassified. Further differences between the studies include use of a monoclonal antibody at high dilution (1:300) on a tissue array in our study, whereas others have used polyclonal antibodies at low dilution (1:30 to 1:50) on arrays or whole sections. The main limitations of all the studies are the use of tissue arrays rather than whole sections and the lack of genetic evidence to support the diagnosis of the tumour type. Nevertheless, compared with other antibodies commonly used to differentiate these tumours, GST- $\alpha$  appears more highly specific than vimentin, mRCC

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and CD10 as a marker for **primary conventional** RCC (31, 32). This gives this antibody immediate utility in differentiating **conventional** RCC from other types of RCC, a finding of clinical importance as some of the new treatments available for RCC are licensed for **conventional** RCC only.

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In contrast with our observations made for GST- $\alpha$ , we did not see major differences in GST- $\pi$  expression patterns, or levels, for the different types of RCC although a trend towards significance was achieved for papillary tumours. Our observations therefore suggest that GST- $\pi$  possesses neither the sensitivity, nor the specificity necessary to confer diagnostic utility.

The second important finding is that loss of GST- $\alpha$  immunoreactivity predicts poor survival in **conventional** RCC. This has not been reported before and appears related to the strong association of GST expression with better differentiated tumours ( $p=0.005$  after correction for multiple analyses) with less well differentiated tumours losing this specific marker of proximal tubular differentiation. However, when tested in multivariate analysis, this loss of proximal tubular phenotype is not an influential predictor of survival if grade is included in the analysis. This limits its value as a prognostic marker. However, it might have some use in small biopsy material. **Furthermore**, if prognostic models are constructed that include all available information, a strong GST- $\alpha$  staining **tends to** support the allocation of a low grade-tumour, grading being a procedure subject to substantial inter- and intra-observer variation. The link with differentiation and prognosis also provided clues about the function of GSTs in tumour progression.

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The loss of GST- $\alpha$  expression in poor prognosis conventional RCC may have implications in basic cancer biology and in considering treatment options (5). Loss of GST- $\alpha$  would be accompanied with persistence of oxidative stress, which in turn would result in an increase in genetic instability and clonal selection (33), hereby significantly increasing the risk of tumour progression. Furthermore, as the GST pathway is the main pathway to detoxify carcinogens and drugs, it is possible that changes in GST- $\alpha$  expression may alter the sensitivity of tumours to various drugs (12). A better understanding of how GST- $\alpha$  expression is regulated

might go some way to explain how certain factors can promote malignant progression and the therapeutic responsiveness to anti-cancer drugs. Manipulation of such an enzyme expression and/or function could contribute to altering disease outcome for conventional RCC.

Our study also investigated the expression of GST- $\pi$ , which has been reported to be the predominant GST isoenzyme in RCC (21, 34). In the current study, GST- $\pi$  was expressed in the cytosol, and occasionally the nucleus, of all the specimens examined, observations which are consistent with previous reports (21). GST- $\pi$  is a known endogenous inhibitor of the MAPK pathway. It binds c-Jun N-terminal Kinase (JNK) (36), which is activated in response to oxidative stress. Thus, the high levels of expression of GST- $\pi$  could account for the increased resistance to apoptotic cell death (37).

In conclusion, future studies are required to explore further the intra-renal GST isoenzyme distribution and their relative expression levels. Such information is required to test whether renal GSTs have any power as diagnostic tools to help sub-classify the different types of RCC, to explain differential prognosis and therapeutic responsiveness and as potential drug targets. Finally, given the availability of commercial kits to measure urinary levels of GST- $\alpha$ , further studies are necessary to determine if loss of GST- $\alpha$  in the parenchyma of more aggressive forms of conventional RCC is associated with an increase in its shedding into the urine. Such an intriguing possibility would elect GST- $\alpha$  as a non-invasive, leaky biomarker to assist in the diagnosis of this type of tumour, as well as other benign conditions associated with proximal tubular damage.

### Acknowledgements

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For Peer Review



### Figure legends

**Figure 1: Expression of GST- $\alpha$  and GST- $\pi$  in normal adult kidney tissue.** Representative immunoblots showing 10  $\mu$ g of kidney homogenate (C: cortex; M: medulla; P: papilla) per lane probed with anti-mouse GST- $\alpha$  (A) or anti-mouse GST- $\pi$  (D) antisera. Specific immunoreactivities are observed at ~25 kD (GST- $\alpha$ ) and at ~45kD and ~25kD (GST- $\pi$ ). GST- $\alpha$  specific immunoreactivity is found within the cytoplasm of cells of the proximal convoluted (B, cortex) and straight tubules (C, outer medulla). Note the absence of GST- $\alpha$  immunoreactivity from the glomeruli (g) and the distal convoluted tubule (d). Cellular GST- $\pi$  immunoreactivity is more widespread and can be observed in the cytoplasm, apically and basolaterally in cells of both the collecting duct (cd) and loop of Henle (lh) of both the renal cortex (E) and outer medulla (F). Note that  $\pi$ -GST is absent in the glomerulus (g). Scale bar: 200  $\mu$ m (B and E) or 100  $\mu$ m (C and F).

**Figure 2: GST- $\alpha$  immunostaining in clinically confined RCC.** Representative TMA of clinically confined RCC showing GST- $\alpha$  protein pattern of expression and intensity of (A) mouse IgG (negative control); (B) RCC with GST- $\alpha$  score 1+; (C) RCC with GST- $\alpha$  score 2+; (D) RCC with GST- $\alpha$  score 3+. Note that GST- $\alpha$  is completely absent in papillary (E) and chromophobe (F) RCCs, for which GST- $\alpha$  score is 0. Scale bar: 200  $\mu$ m.

**Figure 3: GST- $\pi$  immunostaining in clinically confined RCC.** Representative TMA of clinically confined RCC showing GST- $\pi$  protein pattern of expression and intensity of (A) mouse IgG (negative control); (B) RCC with GST- $\pi$  score 1+; (C) RCC with GST- $\pi$  score 2+; (D) RCC with GST- $\pi$  score 3+; (E) Papillary RCC with GST- $\pi$  score 2+; (F) Chromophobe RCC with GST- $\pi$  score 2+. Scale bar: 200  $\mu$ m

**Figure 4: Kaplan-Meier disease-free survival plot of GST- $\alpha$  score.** Clear cell RCC patients that were positive for GST- $\alpha$  (Score 1) had a mean disease-free survival of 6.0 years (95% CI 5.33 – 6.63), compared with those patients whose tumours were negative for GST- $\alpha$  and for which mean disease-free survival was 4.7 years (3.54 – 5.90) (Score 0;  $p = 0.011$ , log rank test).

**Table 1: Cross tabulation of GST- $\alpha$  grade of staining with the conventional histological prognostic covariates.** For contingency tables of the individual prognostic and GST- $\alpha$  covariates, the p values from chi-square testing are as follows: grade,  $p < 0.001$ ; stage  $p = 0.88$ ; size  $> 7\text{cm}$ :  $p = 0.75$ ; vascular invasion:  $p = 0.45$ ; capsular invasion:  $p = 0.84$ ; tumour type:  $p < 0.001$ .

**Table 2: Cross tabulation of GST- $\pi$  grade of staining with the conventional histological prognostic covariates.** For contingency tables of the individual prognostic and GST- $\pi$  covariates, the p values from chi-square testing are as follows: grade,  $p = 0.10$ ; stage  $p = 0.40$ ; size  $> 7\text{cm}$ :  $p = 0.17$ ; vascular invasion:  $p = 0.77$ ; capsular invasion:  $p = 0.15$ ; tumour type:  $p = 0.049$ .

**Table 3: Cox regression analysis of GST- $\alpha$  score, vascular invasion and capsular invasion.** GST- $\alpha$  is not influential if grade is included in the Cox regression analysis however, if it is omitted, it has influence at the 0.1 level.

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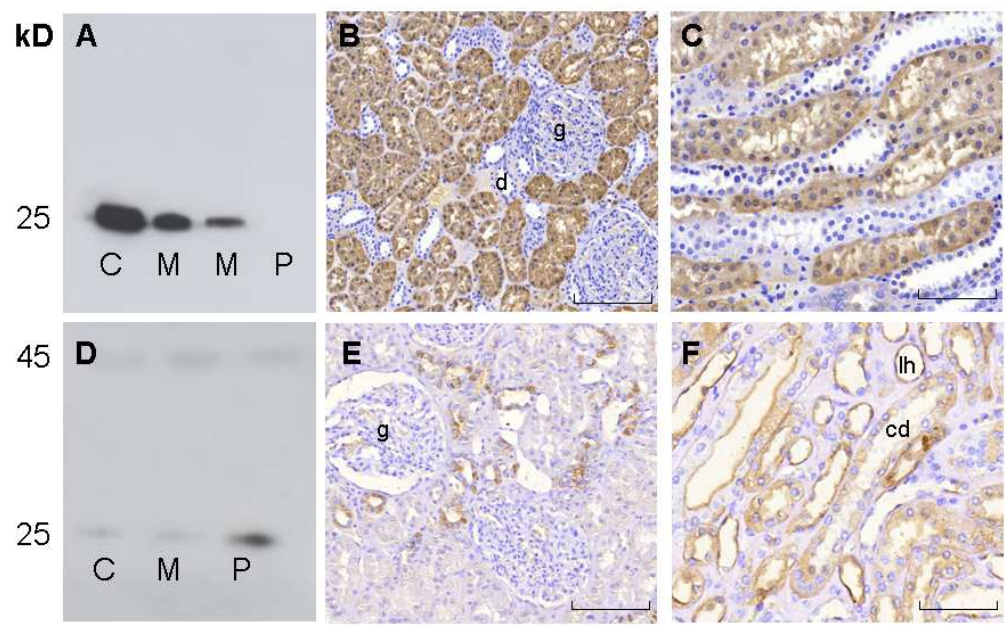


Figure 1: Expression of GST-α and GST-n in normal adult kidney tissue. Representative immunoblots showing 10 µg of kidney homogenate (C: cortex; M: medulla; P: papilla) per lane probed with anti-mouse GST-α (A) or anti-mouse GST-n (D) antisera. Specific immunoreactivities are observed at ~25 kD (GST-α) and at ~45kD and ~25kD (GST-n). GST-α specific immunoreactivity is found within the cytoplasm of cells of the proximal convoluted (B, cortex) and straight tubules (C, outer medulla). Note the absence of GST-α immunoreactivity from the glomeruli (g) and the distal convoluted tubule (d). Cellular GST-n immunoreactivity is more widespread and can be observed in the cytoplasm, apically and basolaterally in cells of both the collecting duct (cd) and loop of Henle (lh) of both the renal cortex (E) and outer medulla (F). Note that n-GST is absent in the glomerulus (g). Scale bar: 200 µm (B and E) or 100 µm (C and F).  
74x50mm (300 x 300 DPI)



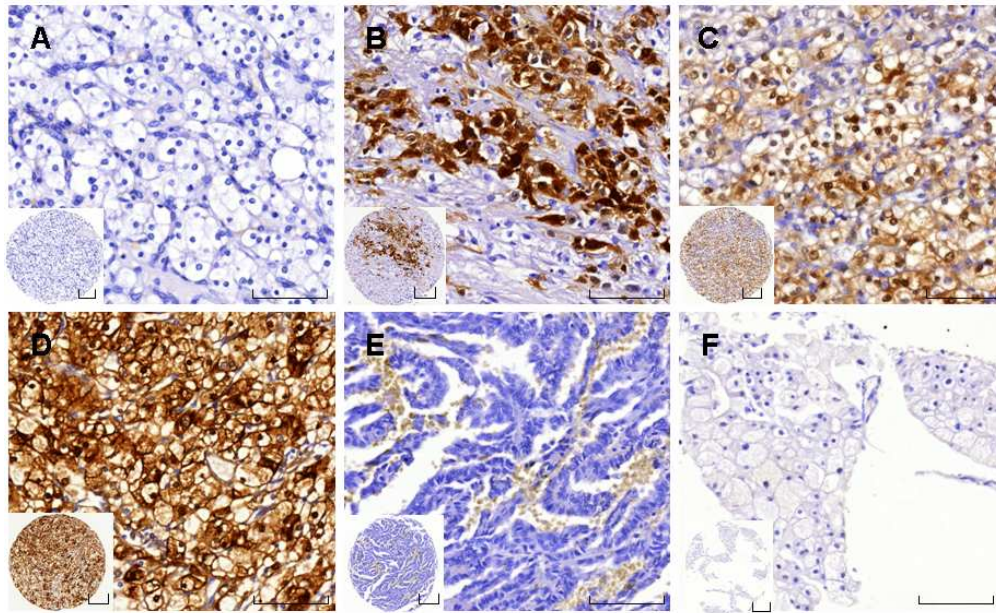


Figure 2: GST- $\alpha$  immunostaining in clinically confined RCC. Representative TMA of clinically confined RCC showing GST- $\alpha$  protein pattern of expression and intensity of (A) mouse IgG (negative control); (B) RCC with GST- $\alpha$  score 1+; (C) RCC with GST- $\alpha$  score 2+; (D) RCC with GST- $\alpha$  score 3+. Note that GST- $\alpha$  is completely absent in papillary (E) and chromophobe (F) RCCs, for which GST- $\alpha$  score is 0. Scale bar: 200  $\mu$ m.  
77x50mm (300 x 300 DPI)



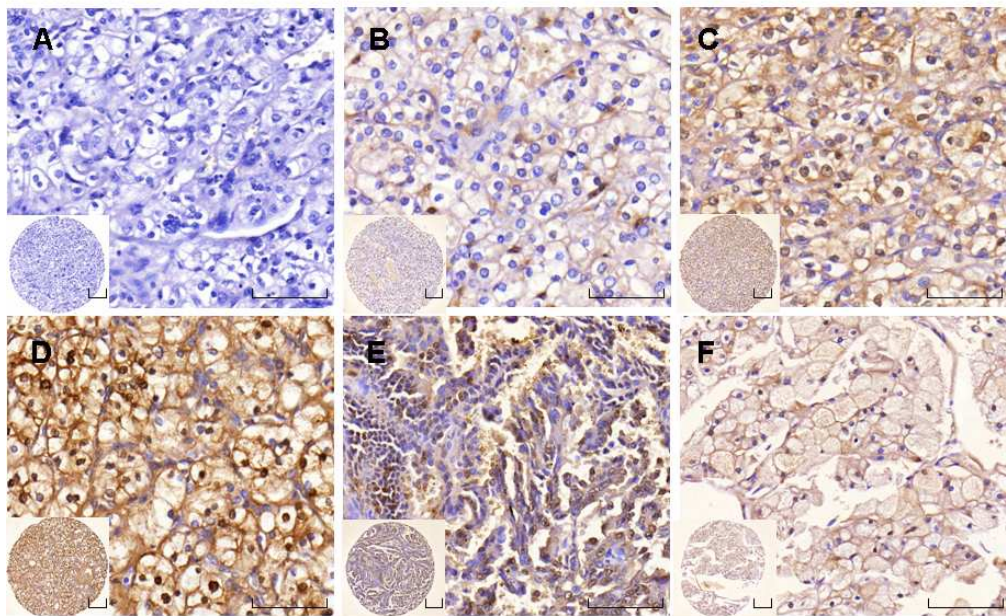


Figure 3: GST-n immunostaining in clinically confined RCC. Representative TMA of clinically confined RCC showing GST-n protein pattern of expression and intensity of (A) mouse IgG (negative control); (B) RCC with GST-n score 1+; (C) RCC with GST-n score 2+; (D) RCC with GST-n score 3+; (E) Papillary RCC with GST-n score 2+; (F) Chromophobe RCC with GST-n score 2+. Scale bar: 200  $\mu$ m  
77x50mm (300 x 300 DPI)

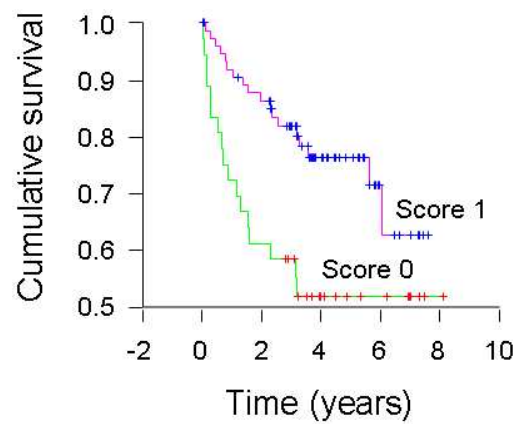


Figure 4: Kaplan-Meier disease-free survival plot of GST- $\alpha$  score. Clear cell RCC patients that were positive for GST- $\alpha$  (Score 1) had a mean disease-free survival of 6.0 years (95% CI 5.33 – 6.63), compared with those patients whose tumours were negative for GST- $\alpha$  and for which mean disease-free survival was 4.7 years (3.54 – 5.90) (Score 0;  $p = 0.011$ , log rank test).

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1

					Total
GST-α Score	0	1	2	3	
Tumour grades 1 & 2*	44	22	32	22	120
Tumour grades 3 & 4	35	4	4	5	48
TNM pT1	33	12	14	11	70
TNM pT2	21	8	10	5	44
TNM pT3 & pT4	25	6	12	11	54
Tumour size ≤7cm	46	16	18	14	94
Tumour size> 7cm	33	10	18	13	74
Vascular invasion absent	48	18	25	14	105
Vascular invasion present	31	8	11	13	63
Capsular invasion absent	65	23	29	23	140
Capsular invasion present	14	3	7	4	28
Conventional type	36	23	32	21	112
Papillary	22	0	0	0	22
Chromophobe	5	0	0	0	5
Unclassified	16	3	4	6	29
Recurrence	31	8	11	3	

2

					<b>Total</b>
<b>GST-<math>\pi</math> Score</b>	<b>0</b>	<b>1</b>	<b>2</b>	<b>3</b>	
<b>Tumour grades 1 &amp; 2*</b>	21	26	36	30	113
<b>Tumour grades 3 &amp; 4</b>	10	3	19	10	42
<b>TNM pT1</b>	15	12	22	11	60
<b>TNM pT2</b>	8	10	12	15	45
<b>TNM pT3 &amp; pT4</b>	31	29	55	40	50
<b>Tumour size <math>\leq 7</math>cm</b>	21	14	31	17	83
<b>Tumour size <math>&gt; 7</math>cm</b>	10	15	24	23	72
<b>Vascular invasion absent</b>	21	19	31	25	96
<b>Vascular invasion present</b>	10	10	24	15	59
<b>Capsular invasion absent</b>	28	27	42	32	129
<b>Capsular invasion present</b>	3	2	13	8	26
<b>Conventional type</b>	21	19	43	22	105
<b>Papillary</b>	4	2	4	11	21
<b>Chromophobe</b>	2	1	2	0	5
<b>Unclassified</b>	4	7	8	7	26
<b>Recurrence</b>	11	8	19	12	

3

Conventional RCC only (112 cases)	Hazard ratio	p	95.0% C.I.
Invasion outside the kidney (21)	4.254	< 0.0001	2.003- 9.031
Any vascular invasion (41)	2.780	< 0.01	1.283- 6.027
Negative or +1 GST- $\alpha$ (91)	5.630	< 0.019	1.327- 23.881