

RESEARCH ARTICLE

Glutathione S-Transferase Expression in Upper Urinary Tract Urothelial Carcinomas: a Taiwan Study

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Abstract

Objectives: Glutathione S-transferase (GST) isoenzymes play important roles in resistance to cell apoptosis and carcinogenesis. We aimed to establish the relationship between GST expression and the prognosis of upper urinary tract urothelial carcinoma (UTT-UC) in Taiwan. **Methods:** This study retrospectively reviewed 46 patients with pathologically confirmed UUT-UC at Kaohsiung Medical University Hospital. In each patient, expression of GSTT1 and GSTP1 was compared between urothelial carcinoma and normal urothelial cells by Western blotting. **Results:** GSTP1 expression in the UUT-UC cells was significantly higher than that in normal urothelial cells (1.6 fold, $p < 0.001$). Expression of GSTT1 was significantly associated with the invasiveness of the carcinoma ($p = 0.006$). **Conclusions:** In UUT-UC, GSTP1 might be a potential tumor marker, whereas high GSTT1 expression could be used as an indicator of cancer progression. This study is the first to demonstrate potential applications of different GST isoenzymes for biomolecular analysis of UUT-UCs in Taiwan.

Keywords: Upper urinary tract - urothelial carcinoma - GSTP - GSTT - protein expression

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Introduction

Renal pelvis cancer comprises only 5% of all urothelial tumors, and ureteral cancer is even less common. The reported ratios of ureteral cancer to renal pelvis cancer cases range from 1:3 to 1:4. However, the ratio of upper urinary tract urothelial carcinoma (UUT-UC) to lower urinary tract urothelial carcinoma (LUT-UC) cases is 3.08:6.72 in Taiwan, even in non-endemic areas of blackfoot disease (Yang et al., 2002). Besides, UUT-UC had more malignant behavior than LUT-UC (Catto et al., 2007). Contributing factors in the development of urothelial carcinoma (UC) include genetic characteristics, exposure to toxicants, occupations, and diets. Environmental carcinogens such as cigarette smoking, aromatic amines, and polycyclic hydrocarbons are also known risk factors (Cohen et al., 2000; Pavanello et al., 2010).

Our previous reports, based on immunochemistry experiments, revealed the roles of cyclooxygenase-2 (Ke et al., 2012), osteopontin (Aminsharifi et al., 2011), hypoxia-induced factor 1 α (Ke et al., 2008), and nuclear factor- κ B (Yeh et al., 2010) as prognostic predictors in UUT-UC. However, the exact molecular mechanisms of

tumor invasion, recurrence, and prognosis of UUT-UC are not yet clear. There are no clinical applicable biomarkers for diagnosis, outcome prediction, or treatment effect monitoring for UUT-UC.

Glutathione S-Transferases (GSTs) are enzyme groups that biotransform several compounds known to be risk factors for urothelial carcinoma. By catalysis to the -SH group of the antioxidant glutathione, they protect normal cells from potentially harmful environmental substances such as carcinogens and xenobiotics. Detoxification of these compounds accelerates their dissolution in aqueous cells and their excretion from the body. The GSTs are classified according to their primary structures. Well-characterized classes include GST Alpha (GSTA), GST Mu (GSTM), GST Pi (GSTP), and GST Theta (GSTT), each of which includes several different isoenzymes. Since different GST gene polymorphisms have different effects on cancer susceptibility, the role of GSTs in cancer has been studied intensively. For example, several studies have explored the role of GST enzyme expression in urologic oncology, especially GSTP and GSTT, but most these studies have focused on bladder cancer. Although GST isoenzymes are not an effective marker of malignancy

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Table 1. Comparisons of Patient with Tumor-pairs GSTT1 and GSTP1 Expressions According to T Stage

| | GSTT1 expression | | P | GSTP1 expression | | P |
|-----------------------|------------------|------------------|-------|------------------|------------------|--------|
| | Tumor (Mean±SD) | Normal (Mean±SD) | | Tumor (Mean±SD) | Normal (Mean±SD) | |
| Disease age, years | 70.64±9.79 | | | | | |
| Male, n (%) | 23 (50.0) | | | | | |
| All Expression (n=46) | 0.24±0.33 | 0.22±0.36 | 0.338 | 0.94±0.34 | 0.58±0.21 | <0.001 |
| T | | | | | | |
| a (n=6) | 0.12±0.19 | 0.20±0.38 | 1 | 0.99±0.45 | 0.52±0.27 | 0.13 |
| 1 (n=12) | 0.12±0.19 | 0.06±0.10 | 0.188 | 0.89±0.37 | 0.53±0.16 | 0.007 |
| 2 (n=17) | 0.29±0.30 | 0.36±0.47 | 0.638 | 0.91±0.35 | 0.63±0.23 | 0.007 |
| 3 (n=7) | 0.25±0.23 | 0.15±0.20 | 0.271 | 1.08±0.18 | 0.57±0.25 | 0.005 |
| 4 (n=4) | 0.61±0.75 | 0.27±0.34 | 0.193 | 0.87±0.28 | 0.67±0.17 | 0.406 |
| T1 | | | | | | |
| a+1 (n=18) | 0.12±0.19 | 0.11±0.23 | 0.313 | 0.93±0.39 | 0.53±0.19 | 0.002 |
| 2 (n=17) | 0.29±0.30 | 0.36±0.47 | 0.638 | 0.91±0.35 | 0.63±0.23 | 0.007 |
| 3 (n=7) | 0.25±0.23 | 0.15±0.20 | 0.271 | 1.08±0.18 | 0.57±0.25 | 0.005 |
| 4 (n=4) | 0.61±0.75 | 0.27±0.34 | 0.193 | 0.87±0.28 | 0.67±0.17 | 0.406 |
| Tumor | | | | | | |
| Sup.(n=18) | 0.12±0.19 | 0.11±0.23 | 0.313 | 0.93±0.39 | 0.53±0.19 | 0.002 |
| Inv.(n=28) | 0.32±0.37 | 0.29±0.40 | 0.61 | 0.95±0.31 | 0.62±0.22 | <0.001 |
| Grade | | | | | | |
| Low (n=13) | 0.10±0.16 | 0.16±0.30 | 1 | 0.92±0.42 | 0.58±0.20 | 0.037 |
| High (n=33) | 0.30±0.36 | 0.25±0.38 | 0.35 | 0.94±0.32 | 0.58±0.22 | <0.001 |

SD, standard deviation; Sup, superficial; Inv, invasive; Comparisons of patient tumor-pairs GSTT1 and GSTP1 expressions were carried out by means of analyses of variance, Wilcoxon signed-rank tests, or paired t tests, as appropriate

in urinary cytology, they can be used to construct the molecular biologic database for use in further research (Oğuztüzin et al., 2011). This study compared GSTT1 and GSTP1 expressions in UUT-UC tissue and normal appearing urothelium.

Materials and Methods

Surgical specimens and clinicopathologic data

This study analyzed UUT-UC cells and pathologically normal urothelial cells from 46 patients who had received nephroureterectomy and bladder cuff excision for UUT-UC at the Department of Urology, Kaohsiung Medical University Hospital from 1997-2006. The specimens were collected during surgery, put into cryotubes, submerged in liquid nitrogen for transferring, and stored in refrigerator with the temperature at -80 °C. The pathologic grade was classified according to World Health Organization histologic criteria (Epstein et al., 1998), and tumor stage was determined according to the International Union Against Cancer tumor-node-metastasis classification (Greene et al., 2002). The study protocol was reviewed and approved by the Institutional Review Board of Kaohsiung Medical University Hospital (KMUH-IRB-20120138) (KMUH-IRB-20120164).

Western blot

Tissues of upper urinary tract urothelial carcinoma and normal appearing urothelial epithelium from 46 UUT-UC patients were extracted using lysis buffer (50mM Tris-HCl, pH7.4; 1% NP-40; 0.25% sodium deoxycholate; 150mM NaCl; 1mM EDTA; 1mM PMSF; 1µg/ml each aprotinin, leupeptin, pepstatin; 1mM Na₃VO₄; 1 mM NaF). The protein concentration of these samples was measured by Bradford method. Fifty micrograms of protein lysate was analyzed by 10% SDS-polyacrylamide

gel electrophoresis. After transfer of the proteins from the gel to a nitrocellulose membrane (Amersham Pharmacia Biotech, Freiburg, Germany), the membranes were non-specifically blocked in 1% skim milk solution and incubated with the primary antibodies anti-GSTP1 (sc-66000, Santa Cruz biotechnology, CA, USA), anti-GST theta 1 (ab96592, abcam, UK), anti-β-actin (MAB1501, clone C4, Millipore Corporation, CA, USA), and with the horseradish peroxidase(HRP)-conjugated secondary antibodies (Santa Cruz biotechnology, CA, USA). Followed by reaction with HRP-conjugated mouse anti-mouse antibody, the immunoreactive bands were visualized using an enhanced chemiluminescence kit (Perkin-Elmer Life Sciences, Boston, MA, USA). Protein expressions were normalized using β-actin as internal control.

Statistical Analysis

In each patient, comparisons of tumor-paired GSTT1 and GSTP1 expressions were performed by analyses of variance, Wilcoxon signed-rank tests, or paired t tests, as appropriate. The data set for normal and tumor tissues from the same individual was obtained by a repeated-measure design. Repeated (PROC MIXED) or random (PROC GLIMMIX) statements were used to compare GSTT1 and GSTP1 expressions in normal and tumor tissues, which were taken from the same individual. The GLIMMIX procedure fits statistical models to correlations in the data even if the response is not normally distributed. Therefore, the repeated measure data were compared with quantitative data by mixed linear model/generalized linear mixed model, and conclusions were drawn according to the parameter and standard error. Resampling was performed to limit post hoc test results (Dunnnett test results) to only interesting comparisons to avoid unduly sacrificing statistical power. Dunnnett test was also used to

Table 2. Association Between GSTT1 or GSTP1 Expression and T Stage in 46 Patient with Tumor-normal Pairs

| | Tumor-pairs GSTT1 expression | | Tumor-pairs GSTP1 expression | |
|-------------|------------------------------|-----------|------------------------------|-----------|
| | LSmeans±SE | P | LSmeans±SE | P |
| T | | | | |
| a (n=6) | 0.16±0.09 | Reference | 0.75±0.10 | Reference |
| 1 (n=12) | 0.09±0.07 | 0.896 | 0.71±0.07 | 0.988 |
| 2 (n=17) | 0.32±0.05 | 0.356 | 0.77±0.06 | 0.999 |
| 3 (n=7) | 0.20±0.09 | 0.993 | 0.83±0.09 | 0.938 |
| 4 (n=4) | 0.44±0.11 | 0.167 | 0.77±0.12 | 1 |
| T1* | | | | |
| a+1 (n=18) | 0.11±0.05 | Reference | 0.73±0.05 | Reference |
| 2 (n=17) | 0.32±0.05 | 0.022 | 0.77±0.06 | 0.911 |
| 3 (n=7) | 0.20±0.09 | 0.75 | 0.83±0.09 | 0.693 |
| 4 (n=4) | 0.44±0.11 | 0.029 | 0.77±0.12 | 0.98 |
| Tumor | | | | |
| Sup. (n=18) | 0.11±0.05 | Reference | 0.73±0.06 | Reference |
| Inv. (n=28) | 0.31±0.04 | 0.006† | 0.78±0.04 | 0.412 |
| Grade | | | | |
| Low (n=13) | 0.13±0.07 | Reference | 0.75±0.07 | Reference |
| High (n=33) | 0.27±0.04 | 0.085 | 0.76±0.04 | 0.939 |

SE, standard error; LS-means, least squares means; Least squares means and the P-value for the correlation with Dunnett's post hoc test are estimated by repeated models (PROC MIXED and PROC GLIMMIX statements); *We used repeated models by PROC MIXED and PROC GLIMMIX to estimate the P-trends (0.0173 and 0.0112); †A generalized linear mixed model (PROC GLIMMIX) is estimated the P-value (0.009).

compare each group against the control or reference group. Statistical analysis was performed with SAS version 9.3 for Windows (SAS Institute Inc, Cary, North Carolina, USA).

Results

The UUT-UC specimens were obtained from 46 patients (mean age, 70.64±9.79 years). The male-to-female ratio was 0.23. The cancer stage was superficial in 18 patients and invasive in 28 patients: 17 were T2, 7 were T3, and 4 were T4. Cancer grade was low in 13 patients and high in 33 patients. Table 1 shows the clinical and pathologic characteristics of the UUT-UC patients.

The GSTP1 activities were significantly higher in UC tissues compared to normal urothelial tissues (1.62-fold higher, $p<0.001$). Patients with high-grade cancer or invasive-stage cancer had a higher expression of GSTP1 compared to normal urothelium ($p<0.001$).

The GSTT1 expression in UC tended to increase with cancer progression. Cancer tissues and normal tissues showed similar frequency of GSTT1 expression. However, the cancer tissues showed a significant positive association between GSTT1 expression and invasiveness ($p=0.006$) (Table 2) with OR 1.29 compared to superficial urothelial carcinoma (Figure 1), and also increased with UUT-UC grade but not significant ($P=0.085$).

Discussion

Compared to normal uroepithelial cells, GSTP1 expression was significantly higher in UUT-UC cells.

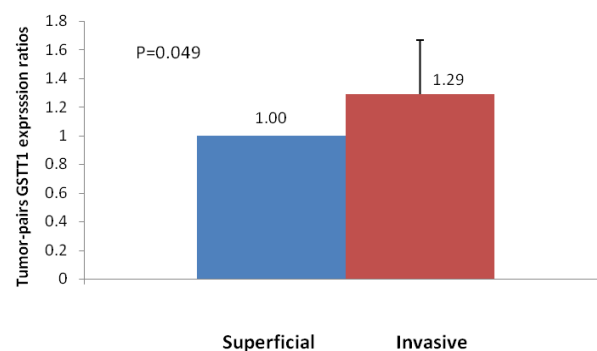


Figure 1. Association of T Stage and Tumor-pairs GSTT1 Expression (≥ 0.11 / <0.11) in 46 Patients. The odds ratio [OR] were estimated from the logistic regression model with the generalized estimating equation (GEE) repeated-measures analysis method. In the presence of tumor-pairs GSTT1 expression, invasive showed a significant association (OR=1.29, 95% confidence interval (CI)= 1.00-1.67; $p=0.0493$)

This functional enzyme may be useful for early detection (i.e., before morphological change) of cancerous cells. However, GSTT1 expression tends to increase with advanced tumor stage. Therefore, GSTT1 may play an important role in tumor progression.

An important enzymatic function of GSTs is detoxifying harmful environmental substances, xenobiotics, and carcinogenic compounds in substrates. Since UC is often associated with exposure to carcinogens, researchers have evaluated the use of the GST genotype for stratifying UC risk (Kempkes et al., 1996). Other studies have investigated either GST genetic polymorphisms or tissue-specific GST expression in bladder cancer (Giralt et al., 1993; Pljesa-Ercegovac et al., 2010; Pljesa-Ercegovac et al., 2011). Although the GST isoenzyme pattern in UC resembles that of the corresponding normal uroepithelium, the increased expressions of all GST subtypes reportedly correspond with progression of the cancer (Simic et al., 2005). In addition to their catalytic activity, GSTs independently regulate stress signaling and resistance to apoptosis. Analyses of the effects of increased Glutathione(GSH) levels suggest that the oxidant/antioxidant balance in UC tends to favor a reduced state (Giralt et al., 1993). Antioxidant enzyme activity and GSH-replenishment activity are also higher in UC tissues compared to normal uroepithelial tissues (Savic-Radojevic et al., 2007). Glutathione content in tumors is also positively associated with proliferation rate and decreases apoptosis by reduction of free radical-induced cell damage (Yang et al., 1997).

However, GST expression is rarely reported in UUT cancer. A literature review shows that the only other published report is Marija et al. (2010), which GST isoenzyme profiles showed significantly higher mean GSTP1 and GSTT1 activities (2- and 3.6-fold higher, respectively) in UUT-UC tissues compared to normal urothelial tissues in 20 cases analysis. Nevertheless, our study identifies GSTP1 overexpression in 46 cases of UUT-UC. Although GSTT1 expression dose not significantly differ between normal tissues and UUT-UC tissues, GSTT1 expression correlates positively with tumor behavior. These results are different from earlier

study of bladder cancer by Simic et al. (2005), which reported significantly higher mean GSTP1 and GSTT1 levels in UC compared to normal uroepithelial cells. Several studies agreed that up-regulation of GSTP1 is a hallmark of bladder cancer (Berendsen et al., 1997; Wang et al., 2001). An interesting finding is that the inhibition of cell apoptosis can be influenced by inhibiting c-Jun NH₂-terminal kinase, and this putative apoptosis-inhibiting mechanism depends on the cellular redox state (Simic et al., 2009). The reduced cellular environment stimulates monomeric GSTP1 protein-protein interactions with c-Jun NH₂-terminal kinase (Wang et al., 2001; Townsend and Tew, 2003), then decreases the JNK-induced degradation of Bcl-2. As Bcl-2 increases, caspase 3 activity decreases. The resulting inhibits cell apoptosis (Karam et al., 2007; Yanamadala et al., 2007; Pljesa-Ercegovac et al., 2011), which promotes carcinogenesis, and reduces tumor suppression (Lowe and Lin, 2000).

In our study, GSTT1 upregulation in the malignant phenotype of UUT-TCC is related to tumor malignancy behavior. GSTT1 has strong hydroperoxidase activity and shifts the oxidant/antioxidant balance toward a reduced state (Hurst et al., 1998; Hayes and Strange, 2000). The antioxidant activity of GSTT1 suggests that it mutually supports and coordinates GSTP1 in inhibiting apoptosis (Matic et al., 2010). Besides, when combined with high reduced GSH in cancer cell, it might trigger redox-sensitive mitogenic pathways, resulting in greater cancer proliferation (Simic et al., 2005). This is compatible to our result that GSTT1 correlate to cancer progression and malignancy behavior. The study of Simic et al. (2005) had similar result showing GSTT1 may correlate to transition cell carcinoma stage and increase in cancer progression, though it focus on bladder cancer.

This study has some limitations. First, this was a retrospective study, and the case number was small. Further studies are needed to confirm the findings of this study in a larger population. Additionally, the normal tissues used for the comparisons in this study were still sampled from cancer patients. Although the tissue samples were pathologically normal, molecular-biological abnormalities could have influenced the results. Finally, the comparison was limited to T stage rather than lymph node or distal metastasis. Further studies are needed for a detailed analysis of tumor stages, even UUT-UC recurrent rate and clinical outcome in long-term follow-up.

In conclusions, this study showed that the GSTP1 enzyme is overexpressed in UUT-UC but not in a normal urothelium. Expression of GSTT1 has a strong association with the progression of cancer. In contrast with other studies, which mostly surveyed bladder cancer, this study is the first to distinguish GST activity in a Taiwan population with a high prevalence of UUT-UC.

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