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ELECTROPHORETIC AND IMMUNOLOGICAL ANALYSIS OF GLUTATHIONE TRANSFERASE ISOENZYMES OF HUMAN KIDNEY CARCINOMA

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Abstract—By sequential use of GSH-affinity chromatography and chromatofocusing, the isoenzymes of glutathione transferase from tumor and non-tumor kidney tissues have been purified and their properties compared. On the basis of electrophoretic mobilities on SDS/polyacrylamide gel, substrate specificities toward the diagnostic substrates cumene hydroperoxide and ethacrynic acid and immunoreactivity with antisera raised against alpha, mu and pi class glutathione transferases, it was found that most of the isoenzymes purified from both tumor and non-tumor kidney can be identified as members of either alpha or pi classes. All the samples investigated lacked mu class glutathione transferase. In addition, we could identify in tumor samples two transferases GST-7.6 and GST-5.8/5.9 which on the basis of immunological properties cannot be related to any of the members of the three major classes of glutathione transferases. The latter do not appear to have corresponding forms in non-tumor tissues. It was suggested that specific transferases can be selectively expressed by tumor kidney carcinoma.

Glutathione transferases (GST; EC 2.5.1.18) comprise a group of abundant and widely distributed catalytic and binding proteins that facilitate the conjugation of GSH with the electrophilic centre of a large spectrum of hydrophobic molecules [1]. Multiple forms of GST have been purified and characterized from a variety of sources [1]. Each GST isoenzyme is composed of two subunits that are either homo- or heterodimeric [1]. On the basis of their structural and immunological properties and by substrate specificities the human GST can be assigned to three distinct classes, alpha, mu and pi [1] which are considered to be products of at least three gene loci GST2, GST1 and GST3 [2, 3].

Numerous toxic chemicals, including substances considered to be carcinogens and mutagens, were found to be substrates of GST [4, 5]. In addition, GST activity can be enhanced in the cytosol of many organs of different animals by compounds considered to be anti-carcinogenic [6, 7]. Furthermore, GST, a pi class enzyme, has been found to be overexpressed in a number of human tumor tissues [8–15] suggesting that it may be a good tumor marker. It has also been demonstrated that GST P (GST 7-7; P-26) which is not expressed to any great extent in normal rat liver, significantly increases in the hepatocyte nodules induced by chemical carcinogens [16–18]. Human GST and rat GST 7-7 are immunologically identical [19]. Recently, GST has also been implicated in the development of cellular resistance to several classes of compounds with anti-cancer properties [20, 21]. GST can also bind a wide range of drugs and foreign chemicals via "suicidal binding" thereby preventing genotoxic substances from interacting with vital macromolecules [22]. These findings have lead to the suggestion that GST may play a critical role in carcinogenesis [4, 23]. However, despite their presumptive role in carcinogenesis, relatively little is known of the distribution of molecular forms of GST in human tumor tissues. The previous report [10] indicating that the isoenzyme profile of GST in human tumor kidney is markedly different from that of non-tumor kidney prompted us to characterize the tumor kidney GST in more detail.

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MATERIALS AND METHODS

Materials. Human kidney specimens of tumoral, peritumoral and distant regions were obtained from three patients (two male and one female) at time of surgery. The three tumors investigated were found, upon histopathological examination, to be renal cell carcinoma of the clear cell type. The samples were immediately transferred to cold saline solution, washed exhaustively and stored at −20° until used.

Purification of glutathione transferases. The cytosolic GST of human tumor and non-tumor kidney were purified by affinity chromatography and chromatofocusing [24]. Briefly, the samples of kidney tissue were minced and then homogenized in 5 vol. 10 mM potassium phosphate buffer, pH 7.0 supplemented with 1 mM EDTA and 1 mM dithiothreitol by using a Waring blender at 15,000 rpm for 2 min. The homogenate was centrifuged at 105,000 *g* for 60 min and the supernatant subjected to an affinity chromatography column (1 × 10 cm) of GSH linked to epoxy activated Sepharose 6B [25]. The column was pre-equilibrated with 10 mM potassium phosphate buffer at pH 7.0. After washing the column with equilibration buffer supplemented with 50 mM KCl, the GST pools were eluted with 50 mM Tris–HCl buffer pH 9.6, containing 5 mM GSH at flow rate of 10 ml/hr. The fractions containing GST

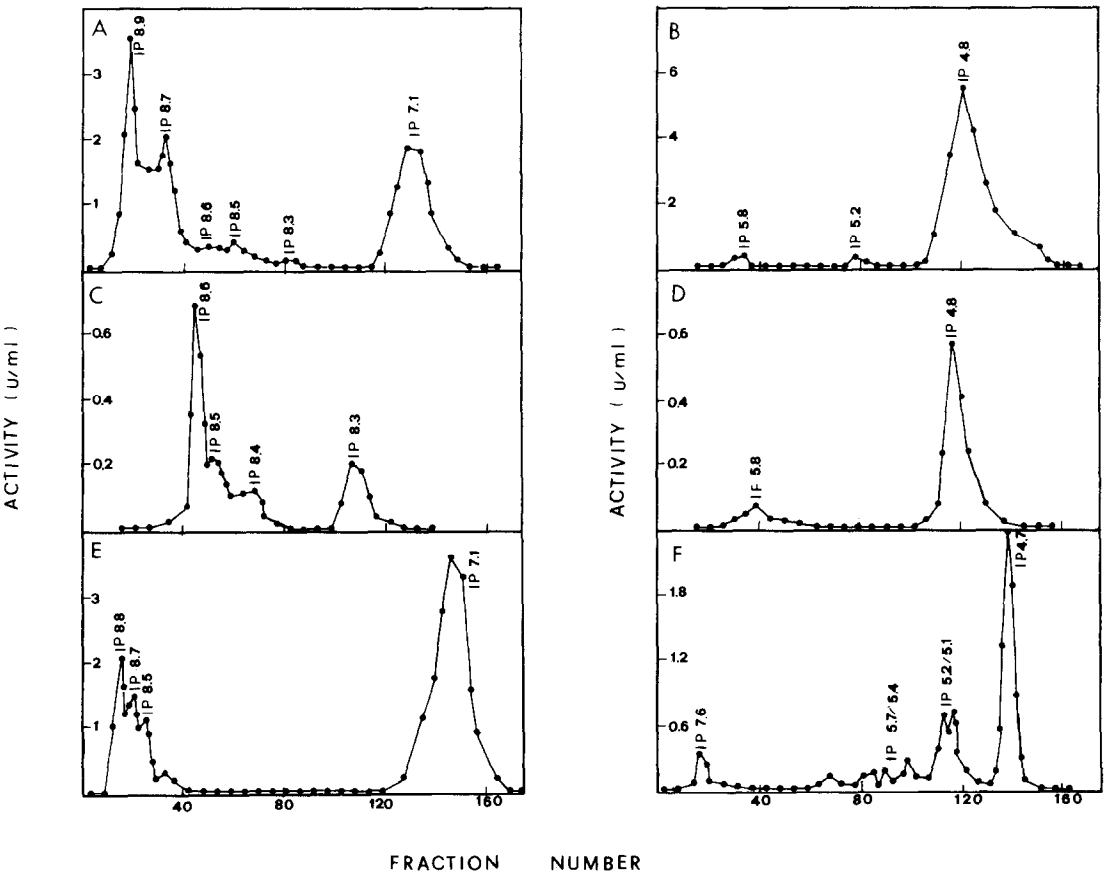

Fig. 1. Renal isoenzyme patterns of tumor and non-tumor glutathione transferases after affinity chromatography on GSH linked to epoxy-activated Sepharose 6B and chromatofocusing [23]. The GST purified by affinity chromatography were applied to a column (1 × 30 cm) of chromatofocusing gel PBE 118. The enzymes were eluted with pH gradient 10.5–7. The glutathione transferases still bound to the column were first eluted with 1 M NaCl and then applied to a column (1 × 30 cm) of chromatofocusing gel PBE 74. The enzymes were eluted with pH gradient 7–4. For further details see Materials and Methods section. In panels A, B and I, J are the chromatofocusing activity profiles from basic (10.5–7) to acid (7–4) range obtained from non-tumor (distant) region of human kidney. In panels C, D are the chromatofocusing profile from non-tumor (peritumoral) region. In panels E, F; G, H and K are the chromatofocusing profiles from tumor kidney. Glutathione transferase activity was measured with 1-chloro-2,4-dinitrobenzene as second substrate. The profiles in panels A, B, C, D, E, F are from tumor and non-tumor kidney samples of a 46-year-old man, those in panels G, H are from the tumor kidney samples of a 63-year-old man, those in panels I, J, K are from the tumor (K) and non-tumor (I, J) kidney samples of a 62-year-old female.

activity were pooled, concentrated to 4–5 ml by ultrafiltration, equilibrated with 25 mM triethylamine–HCl, pH 11, and then applied to a column (1 × 30 cm) of chromatofocusing gel PBE 118 equilibrated with the same buffer. Elution was performed with 400 ml Pharmalyte (pH 8.0–10.5) which was diluted 80-fold with deionized water and adjusted to pH 7.0 with HCl. The GST which were not eluted with this solution, were eluted with 1 M NaCl. This latter fraction of GST was desalted, dialyzed against 25 mM imidazole/HCl buffer pH 7.4 and then applied to a column (1 × 30 cm) of chromatofocusing gel PBE 94 equilibrated with the same buffer. Elution was carried out with 400 ml of polybuffer 74 HCl (pH 4.0) diluted 8-fold with deionized water. Eluted enzymes were separately pooled, concentrated on a Diaflo PM 10 filter, dialyzed against 10 mM potassium phosphate buffer, pH 7.0, supplemented with 1 mM dithiothreitol and used for further studies.

SDS/polyacrylamide gel electrophoresis. Subunit molecular weight of glutathione transferase were determined by SDS/polyacrylamide slab gel electrophoresis as described by Laemmli [26]. SDS concentration was 0.1% (w/v), the spacer gel and the separating gel were 3% and 12.5% respectively. Albumin (66,000 M_r), ovalbumin (45,000 M_r), glyceraldehyde-3-phosphate dehydrogenase (36,000), carbonic anhidrase (29,000 M_r), trypsinogen (24,000), trypsin inhibitor (20,100), and α-lactalbumin (14,200) were used as standards for characterization of subunits molecular size.

Immunological studies. The immunological studies were carried out using antisera prepared against GST pI 8.5 of human skin (class alpha) [27], GST V of

human uterus (class Pi) [28], and GST III of human uterus (class mu) [28]. These antisera recognize GST isoenzymes belonging to the same class but do not recognize members of other classes. Ouchterclony immunodiffusion experiments were performed at 4° in 1% agarose gels containing 50 mM potassium phosphate (pH 6.9) and 0.9% NaCl.

Assays. GST activity towards 1-chloro-2,4-dinitrobenzene and ethacrynic acid was determined at 24° in a Beckman model spectrophotometer according to the method of Habig *et al.* [29], whereas the activity towards cumene hydroperoxide was measured as previously reported [30]. Protein concentration was determined with the method of Bradford using γ-globulin as standard [31].

RESULTS

Purification of glutathione transferases

The GST isoenzymes purified from tumor and peritumor region as well as from distant region of human kidney by affinity chromagraphy on GSH linked to epoxy-activated Sepharose 6B were subjected to chromatofocusing in the pH interval 10.5–7.0. Other GST still bound to the column were eluted with 1 M NaCl and then separated by chromatofocusing in the pH interval 7.0–4.0. The chromatofocusing profiles from basic to acid range obtained from non-tumor (distant) regions of two different samples of human kidney are reported in Fig. 1 (panels A, B and I, J). Nine activity peaks at IP 8.9, 8.7, 8.6, 8.5, 8.3, 7.1, 5.8, 5.2 and 4.8 were resolved from the first sample (panels A and B), whereas six activity peaks at IP 8.6, 8.5, 8.4, 8.3, 5.8 and 4.8 appear from the second sample (panels I and J). The GST of (non-tumor) peritumoral regions resolved into six peaks of enzyme activity corresponding to a IP values of 8.6, 8.5, 8.4, 8.3, 5.8 and 4.8 (Fig. 1; panels C and D). The chromatofocusing profile obtained from three different samples of human tumor kidney are reported in Fig. 1 (panels E, F; G, H; and K). At least six active fractions at IP 8.8/8.5, 7.1, 7.6, 5.7/5.4, 5.2/5.1 and 4.7 can be separated from the first sample (panels E and F). Eight active fractions at IP 8.3, 8.2, 7.9, 7.7, 7.6, 5.6, 5.4 and 4.8 appeared from the second sample (panels G and H). Chromatofocusing analysis in the pH 7.0–4.0 region of the third kidney, showed up to three separate peaks at IP 7.6, 5.9/5.8 and 4.8 (panel K) were. Owing to the paucity of the material available, the chromatofocusing of third kidney in the basic region of gradient was not performed. Several of the forms of glutathione transferase were subjected to a second chromatofocusing analysis, each gave essentially a single peak of activity with an apparent isoelectric point identical to that of the first run.

Immunological studies

The immunological properties of the enzymes in the different peaks identified by chromatofocusing was studied by Ouchterlony double-immunodiffusion techniques using the antisera directed against pi, mu and alpha GST. The kidney GST cross-reacted either with antibodies raised against pi class or alpha classes GST (Tables 1–3) but no cross-reaction was observed with antisera against mu class GST. It is, however, evident from both the results of chromatofocusing analyses and the immunological studies that there is a different expression of some isoenzymes in tumor as compared with non-tumor (peritumor and distant region) kidney tissues. In particular, a form of glutathione transferase (GST-7.6) that does not cross-react with antisera raised against any of the three major classes of human GST is present, though in trace amounts, in all the samples of tumor kidney investigated.

SDS/polyacrylamide gel electrophoresis

Subunit composition of the isoforms isolated were studied by SDS/polyacrylamide slab gel electrophoresis and a representative set of data from both tumor and non-tumor kidney is presented in Figs 2A and B. The isoenzymes studied were found to be homodimers of either 23,000 M_r or 24,000 M_r subunits as compared with standard proteins marker. It should be noted that all kidney isoenzymes which have a subunit M_r of 23,000 cross-reacted with the antibodies raised against pi class GST, whereas, those having a subunit M_r of 24,000 cross-reacted with the antibodies raised against alpha class GST.

Substrate specificity

In addition to 1-chloro-2,4-dinitrobenzene the activity of kidney GST was also measured with the cumene hydroperoxide and ethacrynic acid which are the diagnostic substrates of GST of alpha class and pi class respectively [1] (Tables 1–3). The similarities in the immunological and structural properties in the isoenzymes of same group are also reflected in their substrate specificities. Members of alpha class GST of human kidney have in general the capacity to reduce cumene hydroperoxide, whereas the members of pi class have a relatively good activity with ethacrynic acid.

DISCUSSION

The results of the present investigation show that most of the GST separated in both tumor and non-tumor kidney can be identified as members of pi and alpha classes. All the GST with cationic behavior, from both tumor and non-tumor kidney, belong to alpha class, even though GST 5.2/5.1 (Fig. 1; panel F), as well as GST 5.8 (Fig. 1; panels B, D, J) eluted in the a[illegible]ange of chromatofocusing can be considered al[illegible]class transferases. These conclusions were supported by immunodiffusion and SDS/polyacrylamide analyses and by substrate specificities. Alpha class GST which elute in the acidic pH range of chromatofocusing were also found in other human tissues [8, 28, 32]. The most acidic transferase (GST 4.8) appears to be identical to GST first described in human placenta [33] but also present in the majority of human tissues so far analyzed [1, 10, 13, 27, 28, 34–36]. This isoenzyme has been shown to be a good tumor marker in a number of human tissues [8–15] including kidney [10]. All kidney samples investigated were devoid of mu class transferases consistent with the results of Takeoka *et al.* [32] but not with those of Singh *et al.* [36]. These researchers, however, studied GST isoenzymes in

Table 1. Characteristic features of GST isoenzymes from non-tumor (distant region) kidney

Apparent isolectric point (IP)	Specific activity (U/mg)			Subunit molecular weight (M_r)	Immunoreactivity with antibodies to class:		
	CDNB	EA	CHP		Alpha	Mu	Pi
GST-8.9	48	ND	3.6	24,000	+	–	–
GST-8.7	39	ND	3.3	24,000	+	–	–
GST-8.6	14	ND	1.2	24,000	+	–	–
GST-8.5	12	ND	1.1	24,000	+	–	–
GST-8.4	6	ND	0.7	24,000	+	–	–
GST-8.3	10	ND	1.1	24,000	+	–	–
GST-7.1	29	ND	7.8	24,000	+	–	–
GST-5.8	1.3	ND	0.3	24,000	+	–	–
GST-5.2	6.4	ND	4.3	24,000	+	–	–
GST-4.8	25	0.8	ND	23,000	–	–	+

One unit of enzyme utilized 1 μmol/min at 24°. Abbreviations: CDNB, 1-chloro-2,4-dinitrobenzene; EA, ethacrynic acid; CHP, cumene hydroperoxide. ND, not detectable (<0.06 unit/mg).

Table 2. Characteristic features of GST isoenzymes from non-tumor (peritumoral region) kidney

Apparent isoelectric point (IP)	Specific activity (U/mg)			Subunit molecular weight (M_r)	Immunoreactivity with antibodies to class:		
	CDNB	EA	CHP		Alpha	Mu	Pi
GST-8.6	74	ND	1.4	24,000	+	–	–
GST-8.5	38	ND	4.7	24,000	+	–	–
GST-8.4	34	ND	9.5	24,000	+	–	–
GST-8.3	18	ND	1.4	24,000	+	–	–
GST-5.8	13	ND	0.4	24,000	+	–	–
GST-4.8	40	0.5	ND	23,000	–	–	+

One unit of enzyme utilized 1 μmol/min at 24°. Abbreviations: CDNB, 1-chloro-2,4-dinitrobenzene; EA, ethacrynic acid; CHP, cumene hydroperoxide. ND, not detectable (<0.06 unit/mg).

Table 3. Characteristic features of GST isoenzymes from tumor kidney

Apparent isoelectric point (IP)	Specific activity (U/mg)			Subunit molecular weight (M_r)	Immunoreactivity with antibodies to class:		
	CDNB	EA	CHP		Alpha	Mu	Pi
GST-8.8	75	ND	29	24,000	+	–	–
GST-8.7	58	ND	19	24,000	+	–	–
GST-8.5	47	ND	13	24,000	+	–	–
GST-8.3	60	ND	11	24,000	+	–	–
GST-8.2	19	ND	3.1	24,000	+	–	–
GST-7.9	21	ND	13	24,000	+	–	–
GST-7.7	15	ND	4.2	24,000	+	–	–
GST-7.1	22	ND	12	24,000	+	–	–
GST-7.6	2.3	ND	0.3	24,000	–	–	–
GST-5.9/5.8	—	—	—	—	–	–	–
GST-5.7/5.4	3.1	ND	ND	—	–	–	+
GST-5.6	19	0.2	0.2	23,000	–	–	+
GST-5.4	2.5	0.4	ND	23,000	–	–	+
GST-5.2/5.1	2.2	ND	1.2	—	+	–	–
GST-4.8	58	0.5	ND	23,000	–	–	+

One unit of enzyme utilized 1 μmol/min at 24°. Abbreviations: CDNB, 1-chloro-2,4-dinitrobenzene; EA, ethacrynic acid; CHP, cumene hydroperoxide. ND, not detectable (<0.06 unit/mg).

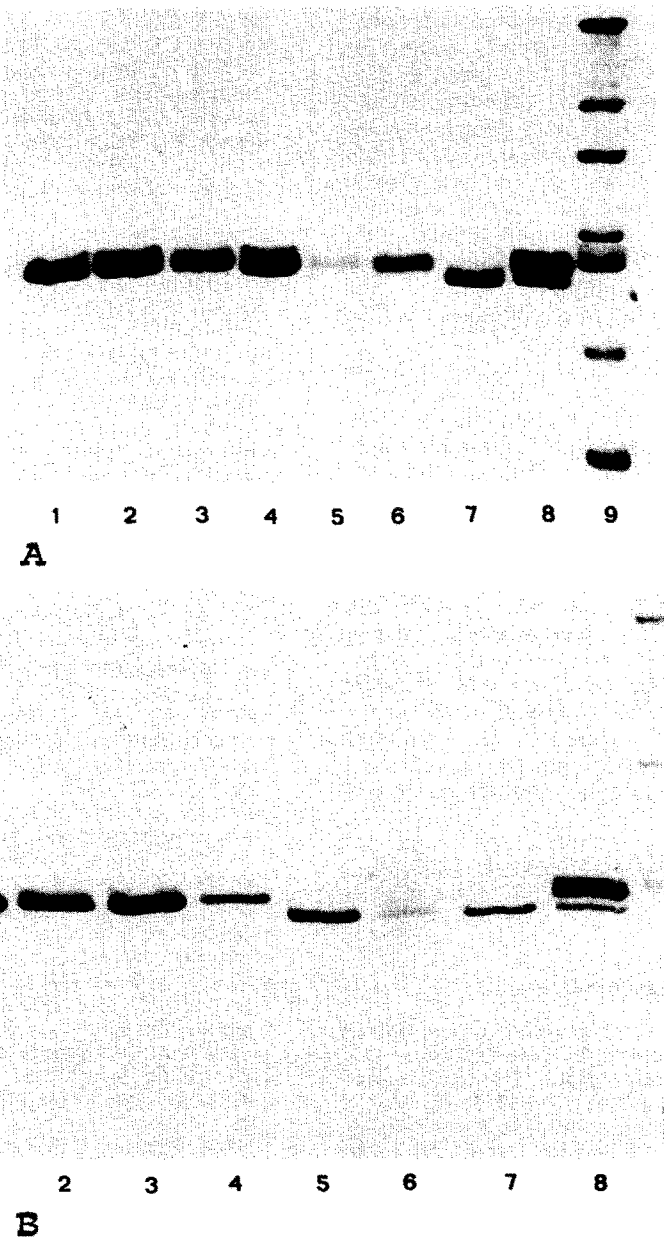

Fig. 2. SDS/polyacrylamide slab gel electrophoresis of the purified isoenzymes of non-tumor (A) and tumor (B) human kidney. (A) non-tumor glutathione transferases: lane 1, GST 8.9; lane 2, GST 8.7; lane 3, GST 8.6; lane 4, GST 7.1; lane 5, GST 5.8; lane 6, GST 5.2; lane 7, GST 4.8; lane 8, GST from affinity; lane 9, standards. (B) tumor glutathione transferases: lane 1, GST 8.3; lane 2, GST 8.2; lane 3, GST 7.9; lane 4, GST 7.7; lane 5, GST 5.6; lane 6, GST 5.4; lane 7, GST 4.8; lane 8, GST from affinity; lane 9, standards. The standards and their molecular masses from top to bottom are albumin (66,000 M_r), ovalbumin (45,000 M_r), glyceraldehyde-3-phosphate dehydrogenase (36,000 M_r), carbonic anhydrase (29,000 M_r), trypsinogen (24,000 M_r), trypsin inhibitor (20,100 M_r), α-lactalbumin (14,200 M_r).

kidney specimens obtained at autopsy from individuals who died from non-renal disease. It must be noted that the GST of mu class, in certain human tissues, is expressed only by about 50% of individuals [1, 37, 38]. Thus, it is necessary to investigate a large number of kidney specimens to ascertain whether or not an association exists between the absence of mu class GST and increased incidence of neoplastic kidney disease. Mu class GST is particularly active against chemical carcinogens and mutagens such as benzo(*a*)pyrene-4,5-oxide and styrene-7,8-oxide [1, 5] suggesting that individuals who do not possess this enzyme may be at a higher risk when exposed to polycyclic hydrocarbons. By comparing the acidic chromatofocusing profiles of tumor and non-tumor kidney samples several differences can be noted. The tumor kidney contains GST 7.6 (in all the samples) and GST 5.9/5.8 (in one sample), not detected in

non-tumor kidney. In addition, immunodiffusion of GST 7.6 and GST 5.9/5.8 with antisera against the three major classes of human GST failed to show any evidence of cross-reactivity, suggesting that they are genetically independent. Even though a limited number of samples were investigated, our results strongly suggest that specific GST can be selectively expressed by tumor tissues. This is further supported by the fact that tumor and non-tumor samples of the same patients were investigated, in this way each patient acts as his own control.

Another tumor feature is the presence of GST forms eluting at 5.4–5.7 pH range (Fig. 1; panels F and H). It is interesting to note that from the immunological and structural criteria these forms appear to be related to GST Pi. At the present, it is difficult to speculate on the interrelationship among these forms and the kidney carcinoma. The possibility that they may have arisen as post-translational modification of the major acidic form (GST 4.8) cannot be excluded.

In conclusion, these results, in conjunction with those previously reported [10], clearly indicate that a qualitative as well as a quantitative [10] change in the GST molecular forms can occur in kidney carcinoma. Furthermore, because of their important role in the resistance to anticancer drugs, understanding of the differences in the isoenzymes expression between tumor and non-tumor tissues may permit a more rational approach to chemotherapy.

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