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Serial lectin affinity chromatography demonstrates altered asparagine-linked sugar chain structures of γ-glutamyltransferase in human renal cell carcinoma

Ken-ichiro Yoshida[a,*], Shuhei Sumi[a], Mikihiko Honda[a], Yoshikatsu Hosoya[a], Masataka Yano[a], Kyoko Arai[a], Yoshihiko Ueda[b]

[a]*Department of Urology, Dokkyo University School of Medicine, 880 Kobayashi, Mibu-cho, Shimotsuga-gun, Tochigi 321-02, Japan*

[b]*Department of Pathology, Dokkyo University School of Medicine, 880 Kobayashi, Mibu-cho, Shimotsuga-gun, Tochigi 321-02, Japan*

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Abstract

Differences between human renal cortex and human renal cell carcinoma (RCC) in asparagine (Asn)-linked sugar chain structures of γ-glutamyltransferase (GGT) were investigated by using a serial lectin affinity chromatographic technique. The relative amounts of GGT which passed through the concanavalin A (Con A) column but bound to the phytohaemagglutinin E column, were significantly decreased in RCC, but there were significant increases in the relative amounts of GGT which bound weakly to the Con A column and passed through the pea lectin (PSA) column, and bound strongly to the Con A column and bound to the wheat germ agglutinin column in RCC compared with those of the normal renal cortex. A significant correlation was observed in RCC between nuclear grade and relative amount of GGT which bound weakly to the Con A column and passed through the PSA column. The findings indicate that Asn-linked sugar chain structures are altered in RCC and suggest that studies of qualitative differences of sugar chain structures of GGT might lead to a useful diagnostic tool for human RCC.

1. Introduction

γ-Glutamyltransferase (GGT, EC 2.3.2.2) is a plasma-membrane glycoprotein that is linked to the γ-glutamyl cycle and regulates the glutathione-to-glutamic acid and cysteinylglycine step [1]. This enzyme, widely distributed in mammalian organs, exhibits the highest activities in the kidney and liver [2]. The enzyme is presumed to have five N-glycosylated sugar chains [3], which are known to have organ specificity [4] and to be altered during malignant alternations [5–8]. Sugar chains of GGT are of great interest as a marker of human renal cell carcinoma (RCC). Although it is well known that asparagine (Asn)-linked sugar chains of GGT in the kidney are composed mostly of complex sugar chains containing bisection N-acetylglucosamine (GlcNAc) [8–10], there are few studies of changes in the sugar chain structures of GGT in

* Corresponding author.

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human RCC. In the present study, using concanavalin A (*Canavalia ensiformis*, Con A)–sepharose, phytohaemagglutinin E (*Phaseolus vulgaris*, PHA E)–agarose, pea lectin (*Pisum sativum*, PSA)–agarose and wheat germ agglutinin (*Triticum vulgaris*, WGA)–agarose columns, we undertook to separate GGT into six fractions and to compare their relative amounts in human renal cortex and RCC.

2. Experimental

2.1. Reagents

The following reagents were purchased: L-γ-glutamyl-3-carboxy-4-nitroanilide from Sigma (St. Louis, MO, USA), Con A–sepharose from Pharmacia (Uppsala, Sweden) and PHA E–agarose, PSA–agarose and WGA–agarose from E.Y. Labs. (San Mateo, CA, USA). All other chemicals were of analytical-reagent grade and were obtained from Wako (Osaka, Japan).

2.2. Samples

Five samples of normal renal cortex and nine of RCC were obtained from patients, aged 36–71 years, undergoing total nephrectomy for RCC. RCC tissues were carefully dissected to exclude both necrotic areas and surrounding normal renal tissues. Dissection of the samples was carried out in ice-cold 100 mmol/l Tris–HCl buffer (pH 7.4) containing 1 mmol/l benzamidine chloride and 0.3 mmol/l phenylmethylsulfonyl fluoride. All patients consented to the use of their renal tissues for this study. After isolation of the tissues, all samples were immediately frozen in the operating room and kept at −80°C until used.

2.3. Enzyme preparations

Tissues obtained from each sample were subjected to the following preparation. First, the tissues were homogenized with an ultradisperser (Yamato, Tokyo, Japan) in 5 volumes of ice-cold 100 mmol/l Tris–HCl buffer (pH 8.0) containing 0.1% Triton X-100 and 150 mmol/l sodium chloride. The homogenate was stirred for 2 h and centrifuged at 20 000 *g* for 20 min at 4°C to obtain a crude extract. The resulting supernatant was further centrifuged at 105 000 *g* for 60 min at 4°C to obtain a solubilized fraction. The solubilized fraction was further fractionated between 40 and 70% saturation of ammonium sulfate and the precipitate was suspended in ice-cold 100 mmol/l Tris–HCl buffer. The resulting preparation was dialysed against 100 mmol/l Tris–HCl buffer (pH 8.0). The resulting enzyme preparations were used for serial lectin affinity chromatography.

2.4. Enzyme assays

The enzyme assay was carried out as described previously [11]. In brief, 1.0 ml of the reaction mixture contained 3.0 mmol/l L-γ-glutamyl-3-carboxy-4-nitroanilide, 100 mmol/l glycylglycine, and 100 mmol/l Tris–HCl buffer (pH 8.25). One unit of enzyme activity was defined as that which released 1 μmol of *p*-nitroanilide per minute at 25°C.

Protein concentrations were determined according to Bradford's method [12].

2.5. Serial lectin affinity chromatography

A column (10 × 0.5 cm I.D.) of Con A–sepharose or PSA– or WGA–agarose, 2 ml, was equilibrated with 10 mmol/l Tris–HCl-buffered saline (TBS) (pH 8.0) supplemented with 1 mmol/l each of calcium chloride, magnesium chloride and manganese chloride and 10 μmol/l of zinc chloride. A column of PHA E–agarose (10 × 0.5 cm I.D.; 2 ml) was equilibrated with phosphate-buffered saline (PBS) (pH 7.5) containing the same metal ions as in TBS. Serial lectin affinity chromatography was carried out by the method of Koyama et al. [13], with slight modification. First, an enzyme preparation with a specific activity of ca. 100 mU/mg of protein was applied to the Con A column and allowed to stand at room temperature for 3 h. The column was then washed with TBS containing 0.1% Triton X-100 (TBS-T) supplemented with the

above metal ions until the activity of GGT in the fraction became undetectable. The initial elution was carried out with 10 mmol/l α-methyl-D-mannoside (α-MM) in TBS-T, and subsequent elution was carried out with 500 mmol/l α-MM in TBS-T. Three fractions were obtained by use of the two different concentrations of α-MM: an unbound fraction (fraction I), a weakly bound fraction (fraction II) and a strongly bound fraction (fraction III). Fractions I, II and III were further applied to the PHA E, PSA and WGA columns, respectively. The unbound and bound fractions, designated A and B, were separated on their separate columns by use of 500 mmol/l GlcNAc, 500 mmol/l α-MM and 500 mmol/l GlcNAc, respectively, as the elution buffers. The relative amount of GGT was calculated by dividing the GGT activity in each fraction by the sum of the GGT activities in all six fractions.

2.6. Histopathological examination of RCC

Microscopic sections were evaluated by a pathologist. All RCC were classified by growth pattern, cell type and nuclear grade [14]. RCC containing cells of mixed grades were designated as the higher grade.

2.7. Statistical analysis

Values were expressed as means ±S.D. Statistical analyses were performed using the Mann–Whitney U test and the Spearman rank correlation test. A level of $P < 0.01$ was considered statistically significant.

3. Results

3.1. Histopathological examination of RCC

Histopathological classifications of nine RCC are listed in Table 1. Seven out of the nine were classified as common growth pattern and two as cystic growth pattern. Four were of clear cell type, three were of mixed cell type (mixed clear and granular cells) and two were of granular cell type. Four were nuclear grade I, two were grade II and three were grade III.

3.2. GGT activity in normal renal cortex and RCC tissues

The mean specific GGT activity in the normal renal cortex was 55.3 ± 7.1 mIU/mg of protein (range 44.0–62.7 mIU/mg of protein) (Table 2) and, in RCC, 3.5 ± 1.8 mIU/mg of protein (range 1.4–6.0 mIU/mg of protein). The GGT activity in RCC was significantly lower than that in the normal renal cortex ($P < 0.0001$). No significant differences in GGT activity were observed for different histopathological classifications of RCC.

3.3. Serial lectin-binding studies

The six fractions separated by serial lectin affinity technique were as follows: IA, the fraction that passed through both the Con A and the PHA E columns; IB, the fraction that passed through the Con A column, but was bound to the PHA E column; IIA, the fraction that was bound weakly to the Con A column and passed through the PSA column; IIB, the fraction that was bound weakly to the Con A column and bound to the PSA column; IIIA, the fraction that was bound strongly to the Con A column and passed through the WGA column; and IIIB, the fraction that was bound strongly to the Con A column and bound to the WGA column (Fig. 1). The total activity recovered after separation by the serial lectin affinity technique was more than 95% of the initial activity added.

The Michaelis constant (K_m) of GGT obtained from six fractions ranged from 0.93 to 1.02 mmol/l for the substrate, which was identical with that of the enzyme preparations (0.95–1.01 mmol/l). The relative amounts of GGT activity of the six fractions in human renal cortex and human RCC are shown in Tables 1 and 2. In the normal renal cortex, the relative amounts of activity of the six fractions were similar in each case, viz., either fraction IA or IB showed the greatest amount of activity, whereas fractions IIB, IIIA, and IIIB showed the smallest amounts. The relative amounts of GGT in fractions IA and

Table 1
Histopathological classification, absolute GGT activity and relative amounts of activity of six types of sugar chains of GGT in human renal cell carcinoma

Case No.	Growth pattern	Cell type	Nuclear grade	Specific activity (mIU/mg of protein)	Relative amount (%)					
					IA	IB	IIA	IIB	IIIA	IIIB
1	Cystic	Granular	I	1.4	26.6	29.8	21.8	3.7	8.9	9.2
2	Common	Granular	III	1.6	2.1	32.3	35.4	2.3	7.8	20.1
3	Common	Mixed	II	2.3	11.1	7.0	29.1	10.3	19.7	22.8
4	Common	Mixed	III	2.6	13.8	9.0	41.3	0.2	11.3	24.4
5	Cystic	Clear	II	4.6	18.5	33.1	29.5	8.0	2.4	8.5
6	Common	Clear	I	4.9	75.0	1.1	14.9	0.3	2.1	6.7
7	Common	Clear	I	5.8	48.2	15.5	22.8	3.9	5.4	4.6
8	Common	Clear	I	6.0	52.9	8.9	25.0	4.3	4.8	4.1
9	Common	Mixed	III	2.2	17.8	2.1	31.5	6.7	22.1	19.8
Mean ±S.D.				(3.5 ± 1.8)	$29.6 \pm 23.8^{a,d}$	$15.4 \pm 13.0^{a,b,d}$	27.9 ± 7.9^{a}	4.4 ± 3.4^{b}	$9.4 \pm 7.2^{b,c,d}$	13.4 ± 8.3^{d}

Mean values without a common superscript differ significantly ($P < 0.01$).

Table 2
Absolute GGT activity and relative amounts of activity of six types of sugar chains of GGT in normal human renal cortex

Case No.	Specific activity (mIU/mg of protein)	Relative amount (%)					
		IA	IB	IIA	IIB	IIIA	IIIB
1	59.5	42.6	32.9	13.1	1.5	3.4	6.5
2	55.4	53.8	37.8	4.6	0.2	0.2	3.4
3	62.7	50.4	31.1	11.8	1.6	2.4	2.7
4	44.0	37.7	40.7	7.4	8.4	5.2	0.6
5	55.1	35.0	42.2	10.1	6.4	3.0	3.3
Mean ± S.D.	(55.3 ± 7.1)	43.9 ± 8.1[a]	36.9 ± 4.8[a]	9.4 ± 3.4[b]	3.6 ± 3.6[b,c]	2.8 ± 1.8[c,d]	3.3 ± 2.1[c,d]

Mean values without a common superscript differ significantly ($P < 0.01$).

IB were significantly greater than those in other fractions ($P < 0.001$), but the amounts in fractions IA and IB did not differ significantly from one another. The relative amount of GGT in fraction IIA was significantly greater than those in fractions IIIA ($P = 0.0079$) and IIIB ($P = 0.0079$). In contrast, the relative amounts of GGT of the six fractions in RCC varied among the cases: fraction IA was extremely high in case 6, but much lower in case 2; fraction IB was higher in cases 1, 2 and 5, but lower in cases 6 and 9. Statistically, however, fraction IA showed the greatest relative amount of activity (29.6 ± 24.0%), followed by fraction IIA (27.9 ± 7.9%). The lowest relative amount of activity was observed in fraction IIB (4.4 ± 3.3%). The relative amount of GGT in fraction IA was significantly greater than that of fraction IIB ($P = 0.0012$), and that of fraction IIA was significantly greater than that of fraction IIB ($P < 0.0001$), IIIA ($P = 0.0002$) or IIIB ($P = 0.0019$). The relative amount of GGT in fraction IIB was significantly lower than that in fraction IIIB ($P = 0.0078$). There was no significant correlation between histopathological classification and relative amount of GGT in the fractions, except between nuclear grade and relative amount of GGT in fraction IIA ($r = 0.94$, $P = 0.0002$).

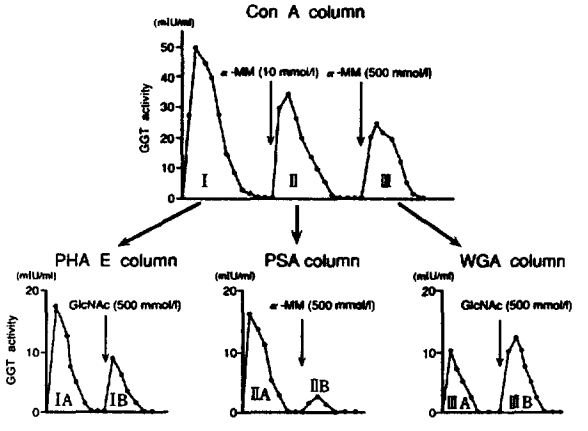

Fig. 1. Separation of the enzyme preparations into six fractions by serial lectin affinity chromatography. Fractions I, II and III obtained on the Con A column were subsequently applied to PHA E, PSA and WGA columns, respectively, as described under Experimental. α-MM = α-methyl-D-mannoside; GlcNAc = N-acetylglucosamine. Arrows indicate the starting points of elution.

When normal human renal cortex and human RCC were compared for the relative amount of GGT in each fraction, that in fraction IB of RCC was significantly smaller than that in fraction IB of normal cortex ($P = 0.004$). In contrast, fractions IIA and IIIB of RCC showed significant increases compared with normal renal cortex ($P = 0.001$ and $P = 0.004$, respectively) (Fig. 2).

4. Discussion

GGT has an extremely high molecular multiplicity, caused by the different number of sialylated complex sugar chains in the enzyme [15,16]. The sugar chain structure of GGT is considered to have organ specificity [4], viz., the

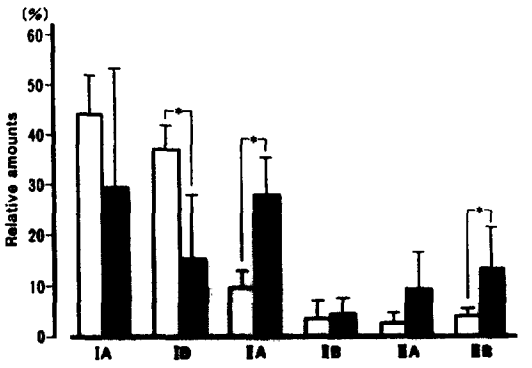

Fig. 2. Comparison between human renal cortex and human RCC of relative amounts of six types of sugar chains of GGT. Columns and bars represent means and standard deviations, respectively. Open column, normal renal cortex; closed column, RCC. Asterisks indicate $P < 0.01$. The relative amounts of fraction IB were significantly decreased in RCC. In contrast, the relative amounts of fractions IIA and IIIB were significantly increased in RCC.

sugar chains of human kidney have structural characteristics similar to those of mouse, rat or bovine kidney GGT in that they contain bisecting GlcNAc residue [9,10], whereas liver GGT in both human and rat contains mainly the biantennary complex sugar chains and never contains bisecting GlcNAc residue [8]. Recently, alterations in the organ- and species-specific sugar chain structures of GGT have been found in carcinoma tissues. For instance, bisecting GlcNAc residue has been found in rat hepatoma GGT [8] and also in the sera of hepatoma patients [17].

In this study, to determine whether there are any structural alterations in sugar chains of GGT during carcinomatous transformation of the human kidney, we compared normal renal cortex and RCC for the relative amounts of GGT in six fractions separated by serial lectin affinity chromatography. No significant difference in K_m values for the substrate was observed between the enzyme preparations and six fractions separated by lectin affinity chromatography, suggesting that the active site of GGT for the substrate was unaffected by the lectin treatments. Based on reports of serial lectin affinity chromatographic studies [13,18], sugar chain structures of those six fractions are proposed as follows: IA, multiantennary complex type; IB, complex type with bisecting GlcNAc; IIA, biantennary complex type without fucose linkages to the innermost GlcNAc; IIB, biantennary complex type with fucose linkages to the innermost GlcNAc; IIIA, high mannose type or hybrid type with fucose linkages to the innermost GlcNAc; and IIIB, hybrid type without fucose linkages to the innermost GlcNAc. In the normal renal cortex, the multiantennary complex type and the complex type with bisecting GlcNAc were suggested to be the major sugar chains of GGT in all cases. In contrast, the nature of the major sugar chains in RCC varied from case to case, suggesting that alterations in the sugar chain structures of RCC GGT differed from those of normal renal cortex, and also from each other. However, when the six types of sugar chains in the two groups were compared, it was observed that the relative amount of fraction IB was significantly reduced in RCC GGT, whereas both fractions IIA and IIIB were significantly increased. The former finding suggests that N-acetylglucosaminyl-transferase III, which adds the bisecting GlcNAc residue to sugar chains, is less expressed in RCC, and the latter finding suggests that fucosyl-transferase, which adds the fucose residue to sugar chains, is less expressed in RCC. Hence sugar chains of GGT might lose their own organ-specific structures by altered expression of N-acetylglucosaminyl- and fucosyl-transferase during malignant transformation. It may even be that the more malignant the RCC, the greater is the decrease in fucosyl-transferase in RCC GGT, because a significant correlation was found between the relative amount of GGT in fraction IIA and nuclear grade.

Increased GGT activity has been found in the tissues of hepatoma, mammary carcinoma and colon carcinoma [5,16,19]. In this study, the GGT activity in RCC was significantly lower than that in normal renal cortex, suggesting that quantitative evaluation of GGT in human body fluids is unlikely to become a tumour marker for RCC. The determination of qualitative differences in sugar chain structures of GGT between normal renal cortex and RCC tissues may perhaps lead

to the development of a useful diagnostic tool for RCC.

So far, there has been no useful clinical marker for detecting RCC. Studies of detailed structural alternations of sugar chains of RCC-produced GGT may provide a new route to the diagnosis of RCC and to the development of a biochemical index of malignancy.

References

[1] A. Meister and S.A. Tate, Annu. Rev. Biochem., 45 (1976) 559.
[2] J.A. Gaoldbarg, O.R. Friedman, E.P. Pineda, E.E. Smith, R. Chatterji, E.H. Stein and A.M. Rutenburg, Arch. Biochem. Biophys., 91 (1960) 61.
[3] Y. Laperche, F. Bulle, T. Aissani, M.-N. Chobert, M. Aggerbeck, J. Hanoune and G. Guellaën, Proc. Natl. Acad. Sci. U.S.A., 83 (1986) 937.
[4] K. Yamashita, A. Hitoi, N. Tateishi, T. Higashi, Y. Sakamoto and A. Kobata, Arch. Biochem. Biophys., 225 (1983) 993.
[5] S. Jaken and M. Mason, Proc. Natl. Acad. Sci. U.S.A., 75 (1978) 1750.
[6] H. Yamamoto, K. Sumikawa, T. Hada, K. Higashino and Y. Yamamura, Clin. Chim. Acta, 11 (1981) 229.
[7] P. Selvaraj and K.A. Balasubramanian, Eur. J. Biochem., 153 (1985) 485.
[8] K. Yamashita, A. Hitoi, N. Taniguchi, N. Yokosawa, Y. Tsukada and A. Kobata, Cancer Res., 43 (1983) 5059.
[9] K. Yamashita, Y. Tachibana, H. Shichi and A. Kobata, J. Biochem., 93 (1983) 135.
[10] K. Yamashita, A. Hitoi, N. Tateishi, T. Higashi, Y. Sakamoto and A. Kobata, Arch. Biochem. Biophys., 240 (1985) 573.
[11] L.M. Show, J.W. London, D. Fetterolf and D. Garfinkel, Clin. Chem., 23 (1977) 79.
[12] M. Bradford, Anal. Biochem., 72 (1976) 248.
[13] I. Koyama, M. Miura, H. Matsuzaki, Y. Sakagishi and T. Komoda, J. Chromatogr., 413 (1987) 65.
[14] S.A. Fuhrman, L.C. Lasky and C. Limas, Am. J. Surg. Pathol., 6 (1982) 655.
[15] S.S. Tate and A. Meister, Proc. Natl. Acad. Sci. U.S.A., 73 (1976) 2599.
[16] S. Tsuchida, K. Hoshino, T. Sato, N. Ito and K. Sata, Cancer Res., 39 (1979) 4200.
[17] K. Yamashita, K. Totani, Y. Iwaki, I. Takamisawa, N. Tateishi, T. Higashi, Y. Sakamoto and A. Kobata, J. Biochem., 105 (1989) 728.
[18] K. Arai, K-I. Yoshida, T. Komoda, N. Kobayashi, H. Saitoh and Y. Sakagishi, Clin. Chim. Acta, 184 (1989) 75.
[19] S. Fiala, A.E. Fiala, W.R. Keller and E.S. Fiala, Arch. Geschwulstforsch., 47 (1977) 117.