CHAPTER III

RESULTS

3.1 Investigation of the glypican-3 in the serum

3.1.1 Optimization of glypican-3 assay

Double antibody sandwich ELISA was developed to detect GPC3 levels in human serum. Initially the assay was optimized to give the highest sensitivity, whist retaining a sufficient range of absorbance to produce a good standard curve. This was achieved by increasing the concentration of antibody coated onto the plate at different dilution of biotinylated antibody.

The optimal concentration and dilution of anti-GPC3 and biotinylated anti-GPC3 were determined by performing a chequerboard titration. It was found that the optimal concentration of anti-GPC3 for the coating plate was 1.6 µg/ml (Figure 9) and the optimal dilution for biotinylated anti-GPC3 was 1:500 (Figure 10). By this assay, the undiluted serum could be used to detect GPC3 and used 6%BSA (w/v) as a standard diluent (Figure 11).

Atypical GPC3 standard curve is showed in figure 12, which the range of detectable concentration was 0.050-5 µg/ml. The intra and inter assay precision are 13.13% and 20.26% (Table 4), respectively and the recovery of GPC3 is 106.4% when a known amount of standard was added (Table 5).

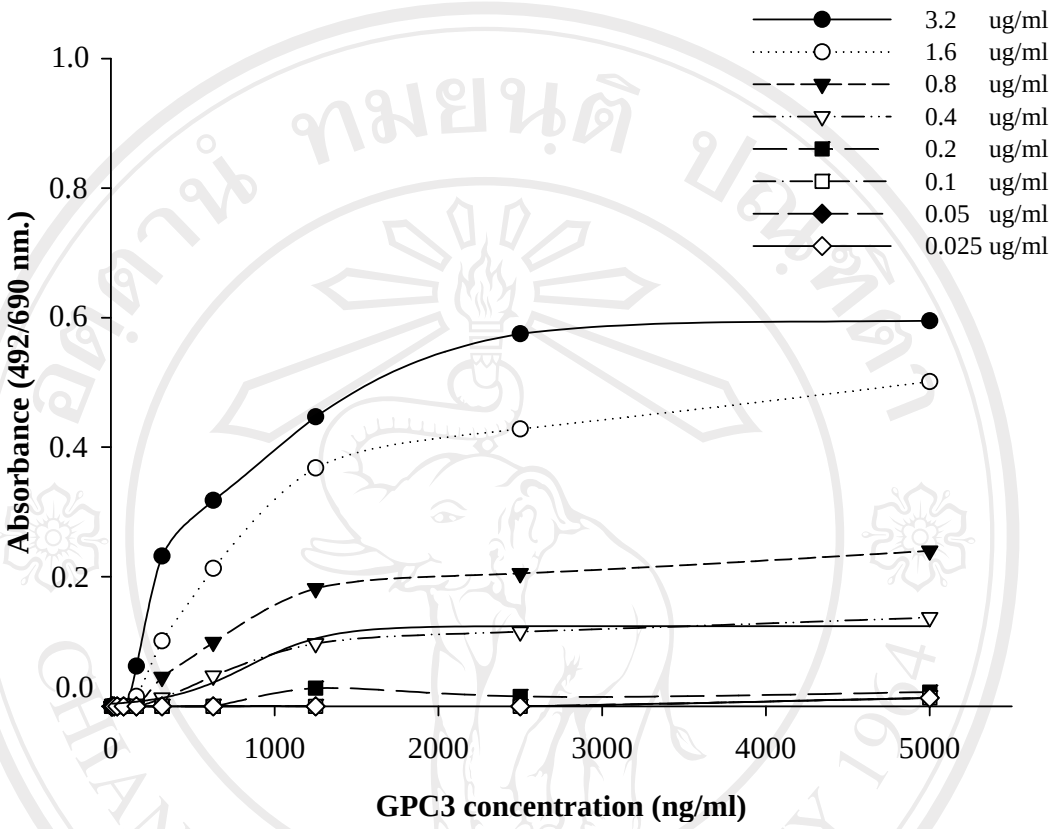

Figure 9 Determination of the optimum coating concentration of anti-human glypican3 at different dilution of recombinant human glypican-3. Peroxidase-mouse monoclonal anti-biotin (1:2,000) was used as a probe, which was demonstrated by an enzyme substrate. Absorbance was measured at 492/690 nm. Each point represented mean of experiment performed in duplicate.

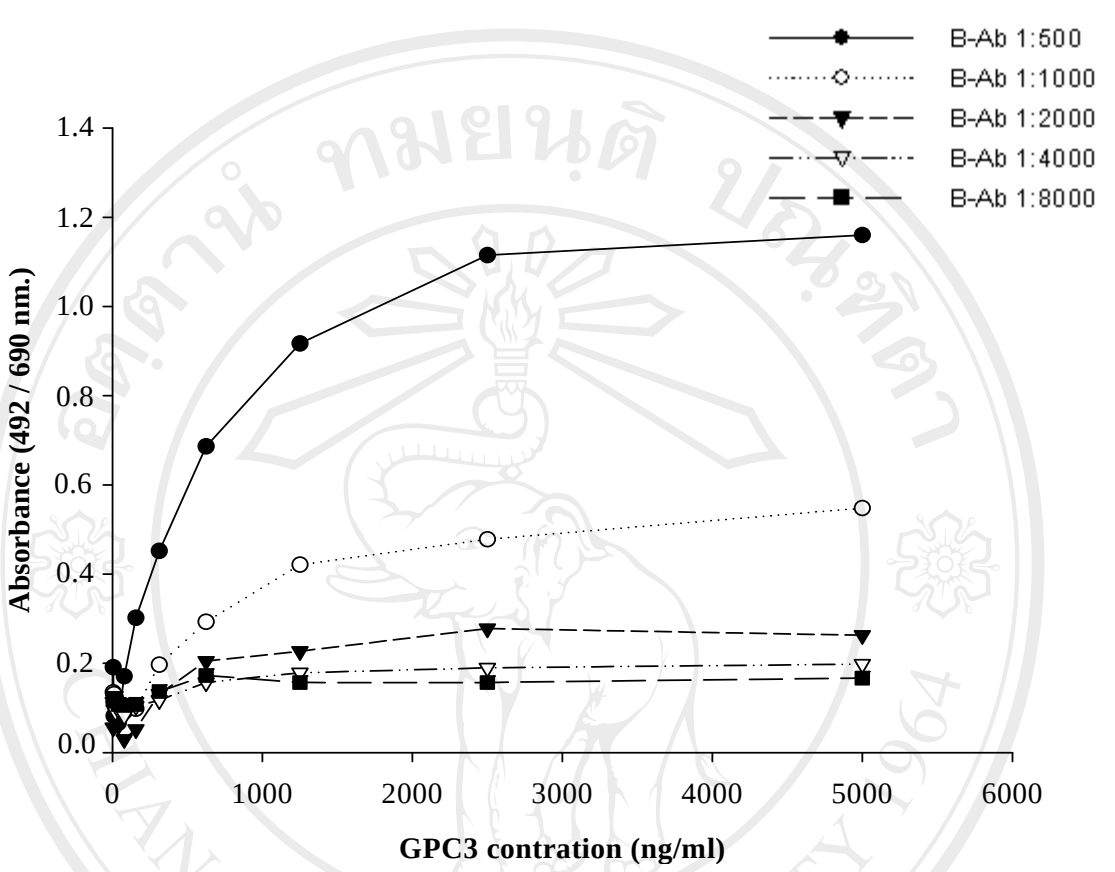

Figure 10 Determination of the optimum concentration of biotinylated anti-glypican-3, which was used as a secondary antibody. Peroxidase-mouse monoclonal anti-biotin (1:2,000) was used as a probe, which was demonstrated by an enzyme substrate. Absorbance was measured at 492/690 nm. Each point represented mean of experiment performed in duplicate.

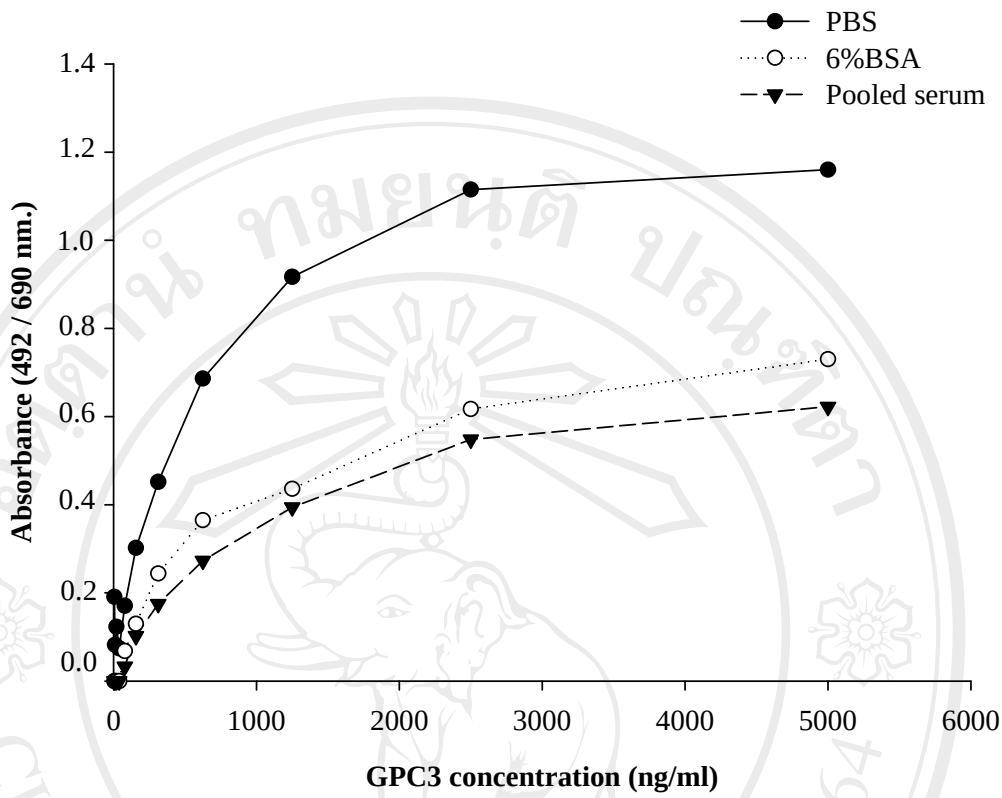

Figure 11 Determination of the optimal diluent for standard glypican-3. Standard human GPC3 was diluted in PBS, 6%BSA (w/v) and pooled serum.

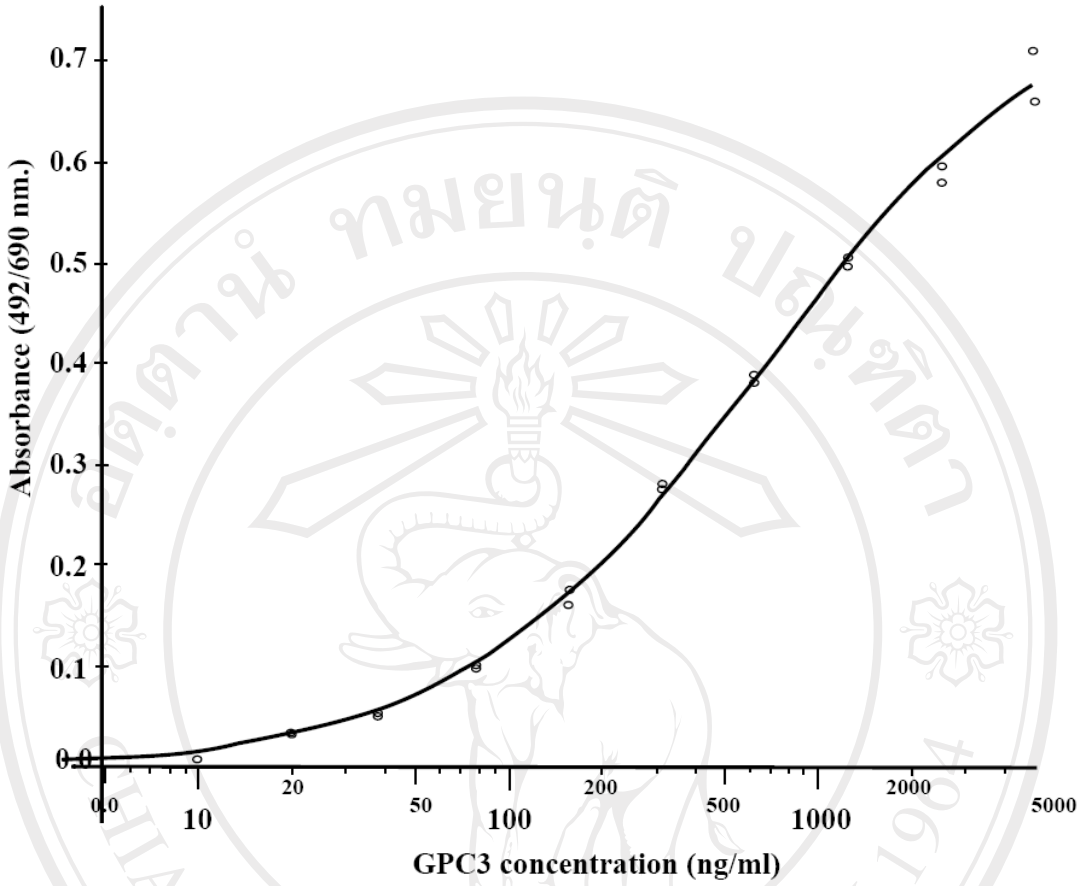

Figure 12 A calibration curve for the quantitation of glypican-3 in human serum using ELISA method. The calibrator was standard GPC3 dissolved in 6%BSA in PBS, pH7.4.

Table 4 The intra- and inter assay coefficient of variation for glypican-3 determination

Assay	Number of samples	Mean ± SD	%CV
Intra assay	20	168.97 ± 22.19	13.13
Inter assay	8	159.13 ± 32.24	20.26

Table 5 The recovery of glypican-3 at various concentrations. Standard GPC3 at various concentration were diluted in normal serum, then it was determined using ELISA assay.

Sample number	GPC3 content (ng/ml)	%Recovery*
1	3129.3	100
2	1564.6	114.6
3	782.3	105.1
4	391.25	109.6
5	195.625	114.4
6	97.81	118.6
7	48.9	82.34
Mean		106.4

* The data was showed as percent recovery of GPC3 content. Each point represented mean of experiment performed in duplicate.

3.1.2 Determination of glypican-3 in healthy and patient serums

As GPC3 is a GPI-anchored membrane protein and can be secreted. To investigate whether GPC3 could be detected in the serum of patients with HCC, a sandwich ELISA was developed.

The serum GPC3 protein quantitation of 70 HCC, 57 CCA, 76 hepatitis, 8 cirrhosis, 7 benign liver masses and 24 healthy individuals is shown in figure 13. It was found that GPC3 was undetectable in all healthy individuals, 40% of patients with HCC (28/70) have significantly elevated levels of serum GPC3 with values range from 35.49 to 6547.9 ng/ml (Table 6, Figure 13). In addition, GPC3 was undetectable in patients with CCA, hepatitis, cirrhosis and benign liver mass. Statistical analysis using the Mann-Whitney U test showed that the distribution of serologic level of GPC3 in patients with HCC is significantly higher than those with CCA, hepatitis, cirrhosis, benign liver masses and healthy donor, whereas there is no significant difference between the last 5 groups.

Table 6 Serum concentration of glypican-3, total sialic acid and WF6 epitope in patient with HCC

Patients no.	GPC3 (ng/ml)	Sialic acid (mg%)	WF6(ng/ml)
1	6547.9	89.6	77.6
2	6350.0	75.0	34.4
3	2462.5	69.2	78.7
4	1459.9	60.25	166.1
5	1253.9	77.4	24.4
6	1250.0	58.8	68.6
7	399.2	61.5	23.5
8	330.2	59.0	195.0
9	315.3	53.1	23.8
10	277.6	48.4	208.7
11	210.1	35.7	105.6
12	198.6	47.5	51.4
13	169.6	34.8	37.3
14	133.5	37.5	106.9
15	127.5	45.4	78.0
16	107.5	32.4	84.7
17	107.5	35.9	16.7
18	107.3	50.3	7.7
19	104.6	35.3	59.4
20	104.6	33.9	65.5
21	91.4	64.7	61.3
22	74.4	35.0	164
23	65.4	59.5	50.7
24	59.3	40.6	55.5
25	47.2	41.8	169.0
26	41.8	37.0	56.3
27	41.8	34.6	72.6
28	35.5	26.8	132.2
29	0	42.8	60.8
30	0	20.6	25.3
31	0	20.1	223.4
32	0	42.1	129.6
33	0	9.5	50.6
34	0	28.6	ND
35	0	29.5	ND
36	0	24.3	126.3
37	0	38.1	121.5
38	0	43.2	91.1
39	0	38.7	ND
40	0	35.1	78.0
41	0	33.9	21.5
42	0	61.5	ND

Patients no.	GPC3 (ng/ml)	Sialic acid (mg%)	WF6(ng/ml)
43	0	47.5	63.95
44	0	38.7	37.3
45	0	30.2	31.28
46	0	33.8	109.8
47	0	26.4	182.8
48	0	53.1	164.9
49	0	28.4	27.0
50	0	45.4	23.4
51	0	33.3	256
52	0	32.7	59.9
53	0	53.1	23.8
54	0	19.9	ND
55	0	32.9	84.6
56	0	43.6	103.1
57	0	16.7	23.9
58	0	28.4	102.7
59	0	37.5	ND
60	0	46.8	191.4
61	0	32.5	138.2
62	0	43.4	ND
63	0	23.0	31.49
64	0	36.1	208.8
65	0	34.0	593.7
66	0	31.0	ND
67	0	51.8	71.5
68	0	32.5	91.2
69	0	22.1	70.97
70	0	13.6	ND

* ND; not done (no left of serum to analyse)

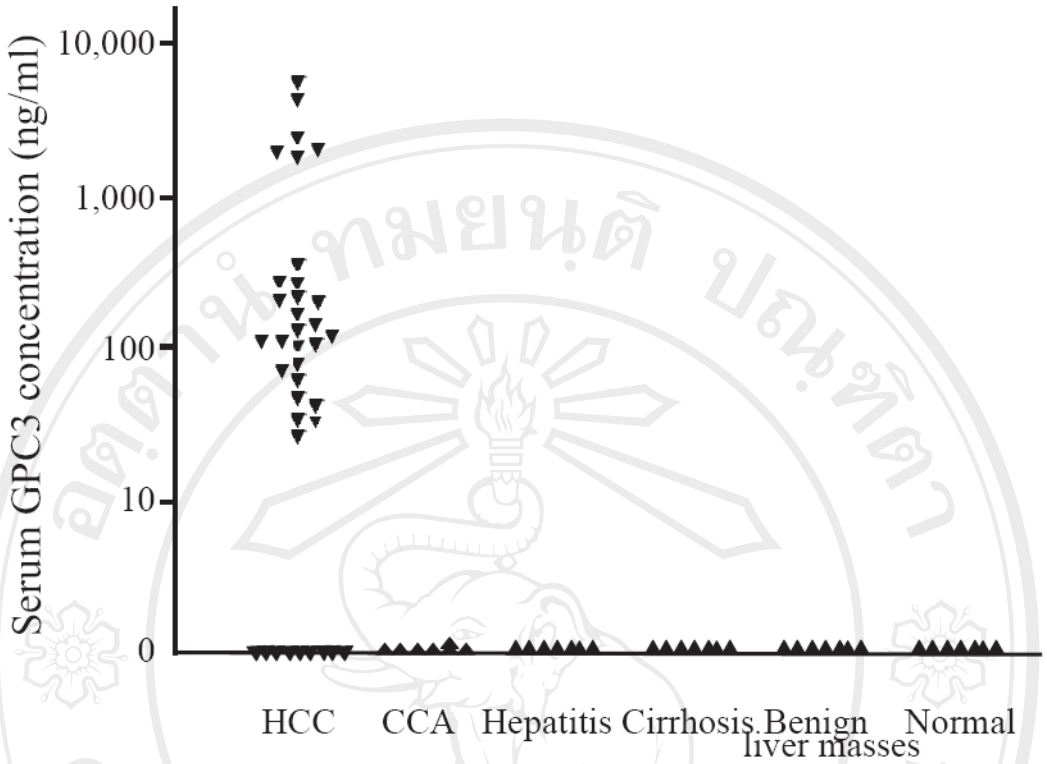

Figure 13 Serum concentration of the glypican-3. Comparison to the value obtained from normal human serum (n=24), HCC (hepatocellular carcinoma) serum (n=70), hepatitis serum (n=76), CCA (cholangiocarcinoma) serum (n=57), cirrhosis serum (n=8) and benign liver masses serum (n=7). Serologic levels of GPC3 in serum were determined by ELISA, and the results shown represent the average of duplicate.

3.2 Determination of WF6 epitope in healthy and patient serums

Monoclonal antibody WF6 recognizes an epitope in native chondroitin-6 sulfate and competitive ELISA was developed to detect the WF6 epitope in human serum using aggrecan (A1D1 fraction) as a standard (Pothacharoen *et al*., 2006).

A typical WF6 standard curve is showed in figure 14, which the range of detectable concentration was 0.0019-10µg/ml. It was found that the serum WF6 epitope in healthy individuals was 182.22±76.11ng/ml. In contrast in patients with HCC, hepatitis, CCA and cirrhosis the serum levels of WF6 epitope were much lower, 96.66 ± 88.27, 92.66 ± 70.75, 56.42 ± 82.77 and 51.38 ± 29.55 ng/ml, respectively. As illustrated in figure 15 and table 7, serum WF6 epitope levels were significantly lower in HCC, CCA, hepatitis and cirrhosis compared with normal subjects. These results indicated that WF6 epitope was markly decreased in patients serum with HCC, CCA, hepatitis and cirrhosis. Notably, the mean concentration of serum WF6 epotope in patients with HCC was substantially higher than that in those with CCA (p<0.001).

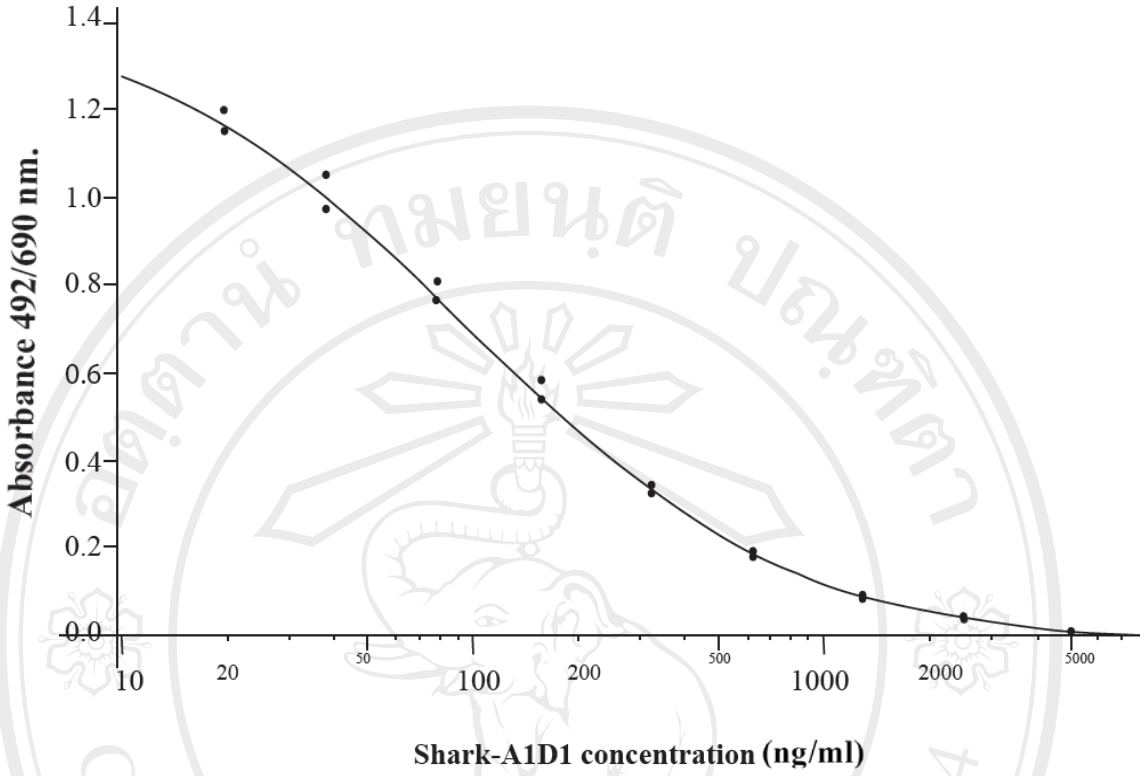

Figure 14 A calibration curve for the quantitation of chondroitin sulfate WF6 epitope in human serum using competitive immunoassay.

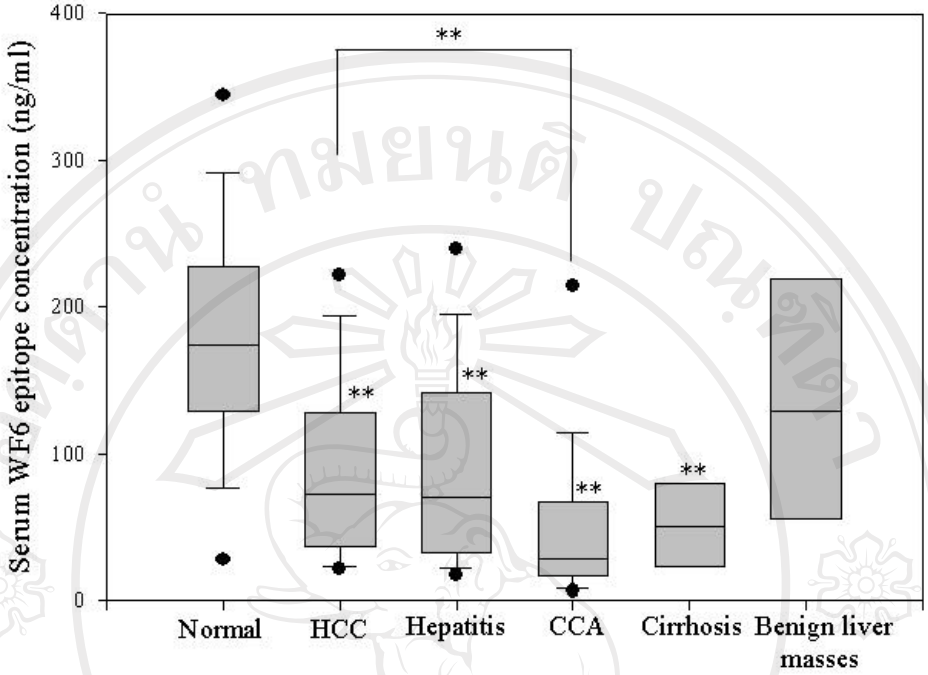

Figure 15 Serum concentration of the WF6 epitope. Comparison to the value obtained from normal human serum (n=24), HCC (hepatocellular carcinoma) serum (n=61), hepatitis serum (n=67), CCA (cholangiocarcinoma) serum (n=57), cirrhosis serum (n=8) and benign liver masses serum (n=7). Boxes represent median and the interquartile range, between 5th and 95th quartile with error bars. Statistically significant difference (p<0.001 shown with an double asterisks) relative to the median of the normal serum.

Table 7 Statistical data of WF6 epitope concentration from normal and patients serum.

	Normal	Hepatocellular carcinoma	Hepatitis	Cholangio carcinoma	Cirrhosis	Benign liver masses
Number (n)	24	61	67	57	8	7
Mean (ng/ml)	182.22	96.66[a]	92.66[a]	56.42[a]	51.38[a]	178.87
Standard deviation (ng/ml)	76.11	88.27	70.75	82.77	29.55	90.38
Max (ng/ml)	354.21	593.70	307.05	527.1	97.98	293.56
Min (ng/ml)	15.36	7.69	6	3.69	16.27	45.7

Significant values (againt normal): a; $p<0.001$

3.3 Determination of total sialic acid in healthy and patient serums

Periodate-resorcinol microassay have been developed to detect total sialic acid in the serum (Kongtawelert *et al.*, 2003). This assay has several advantages over the conventional methods. These include use of smaller samples (5 µl), a larger number of samples simultaneously analyzed, and a greater speed in measuring absorbance by a microtiter plate spectrophotometer.

Periodate-resorcinol assay was based on the principle that periodate oxidation of glycosidically bound sialic acid gave a chromogen which contained an aldehyde group that extremely reacted with the tresorcinol reagent.

A typical total sialic acid standard curve is showed in figure16. Total sialic acid was determined among healthy and pathological samples. The distribution of serum TSA levels in each group are shown in figure 17 and table 8. The mean serum TSA concentration in patients with HCC (44.25±15.23 mg%) was significantly higher than hepatitis (33.95±9.71 mg%), cirrhosis (28.74±2.73 mg%), benign liver masses (28.82±5.58mg%) and normal individuals (28.27±6.22 mg%). Notably, the mean concentration of serum TSA in patients with CCA (55.40±20.25 mg%) was substantially higher than that in those with HCC ($p<0.001$).

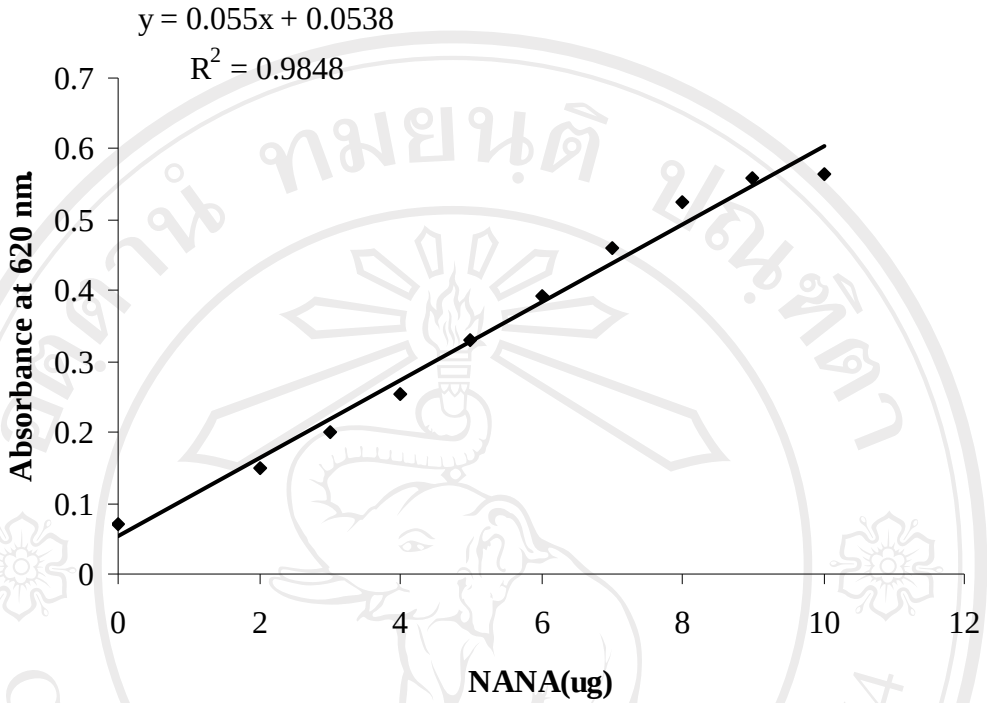

Figure 16 A calibration curve for the quantitation of total sialic acid in human serum using periodate-resorcinol microassay.

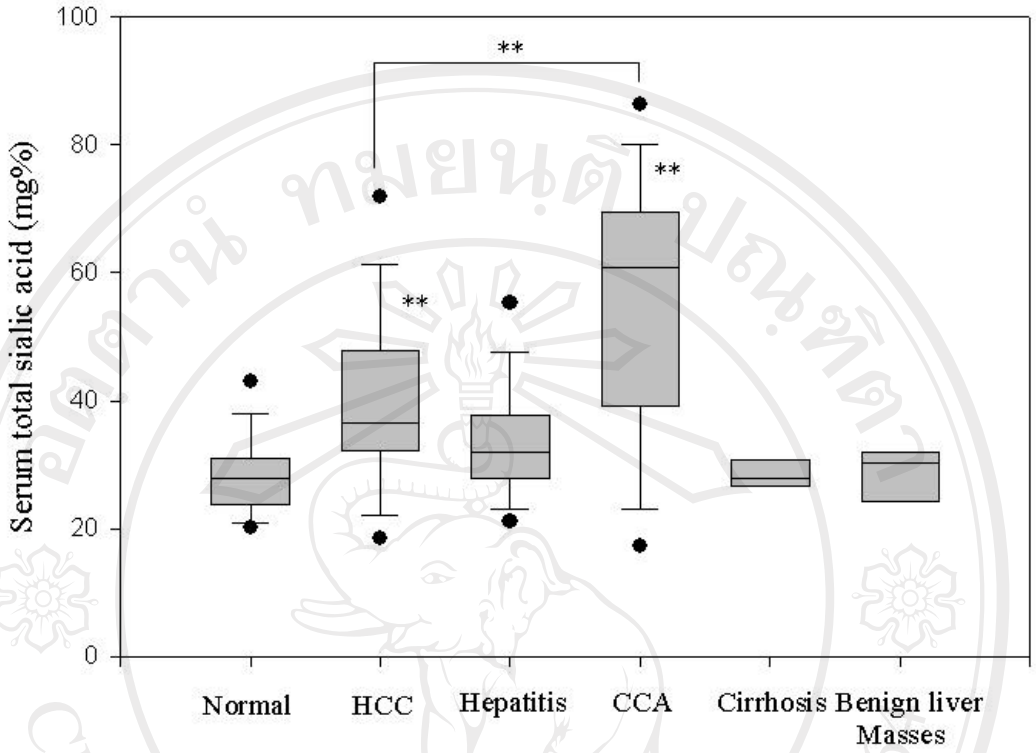

Figure 17 Serum concentration of the total sialic acid. Comparison to the value obtained from normal human serum (n=24), HCC (hepatocellular carcinoma) serum (n=70), hepatitis serum (n=76), CCA (cholangiocarcinoma) serum (n=57), cirrhosis serum (n=8) and benign liver masses serum (n=7). Boxes represent median and the interquartile range, between 5th and 95th quartile with error bars. Statistically significant difference (p<0.001 shown with double asterisks) relative to the median of the normal serum.

Table 8 Statistical data of total sialic acid (TSA) concentration from normal and patients serum.

	Normal	Hepatocellular carcinoma	Hepatitis	Cholangio carcinoma	Cirrhosis	Benign liver masses
Number (n)	24	70	76	57	8	7
Mean (mg%)	28.27	40.25[a]	33.95	55.40[a]	28.74	28.82
Standard deviation (mg%)	6.22	15.23	9.71	20.25	2.73	5.58
Max (mg%)	44.52	89.63	67.34	92.95	33.82	37.94
Min (mg%)	19.96	9.53	15.43	16.81	25.77	24.25

Significant values (againt normal): a; $p<0.001$

3.4 Correlation of GPC3, WF6 epitope and TSA in normal and pathological samples.

Comparison between level of GPC3 positive sample, WF6 epitope and TSA in patients with HCC were showed in Figure 18. It was found that there are no correlation of GPC3 to WF6 epitope and the correlation between GPC3 and TSA was 0.5283.

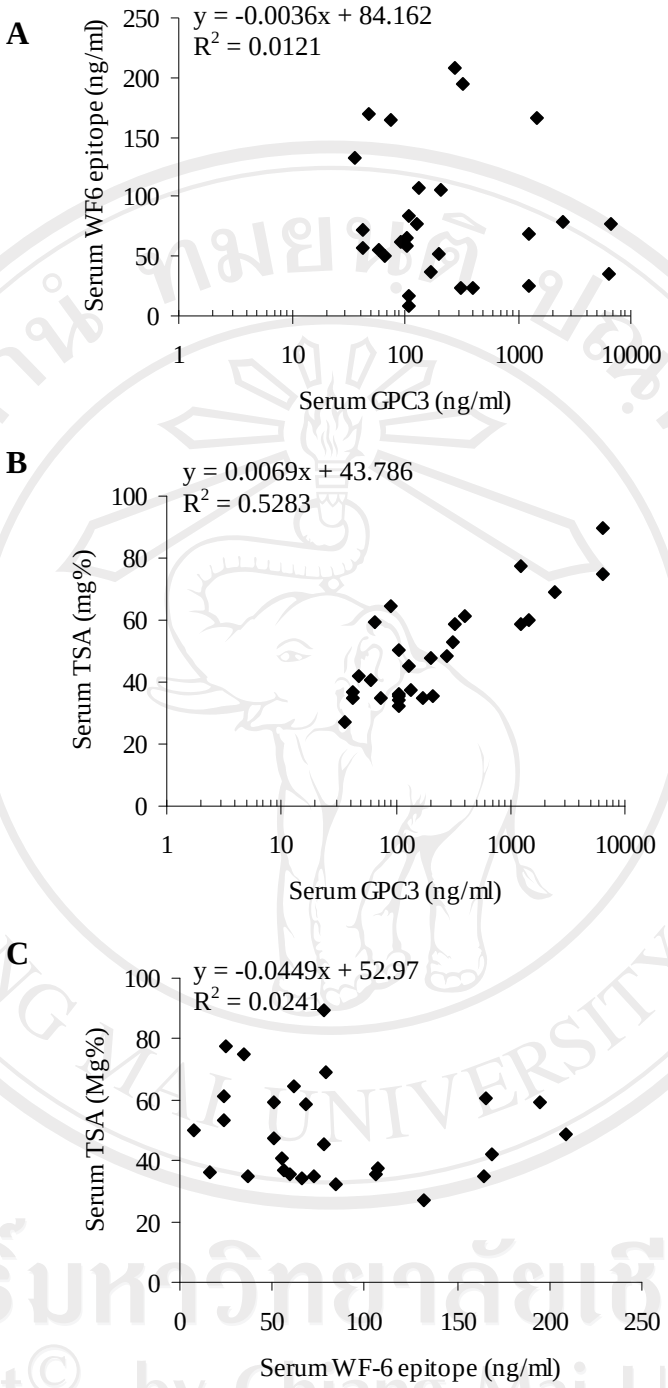

Figure 18 Correlation of GPC3, WF6 epitope and TSA in HCC patients**.** (A) Correlation between concentration of WF6 epitope and GPC3. (B) Correlation between concentration of total sialic acid and GPC3. (C) Correlation between concentration of total sialic acid and WF6 epitope.