CHAPTER II

MATERIALS AND METHODS

2.1 Materials

All reagents and instruments used in the study are summarized in appendix.

2.2 Specimen collection

Healthy samples. Serum were collected from 24 apparently healthy individuals. Serum was stored at -20 ^{0}C before used.

Pathological samples. Serum samples were obtained from 70 patients with HCC, 76 patients with chronic hepatitis, 57 patients with CCA and 7 patients with benign liver masses. This serum was examined and diagnosed by medical doctor at Chulalongkorn University. The serum was collected and divided into aliquots and stored at –20^{0}C until analysis. This studies was passed qualification of research ethics committee.

2.3 Biotinylation of Antibodies

Biotinylation of anti-glypican-3 (R&D systems) was performed by a standard method (Kongtawelert, 2000). Anti-glypican-3 was dissolved in 0.1 M sodium hydrogen carbonate buffer pH 8.5 and mixed at a ratio of 2:1 (w/w) with *N* - hydroxysuccinimidobiotin (1 mg/mL in DMSO) at 4^{0}C overnight. The mixture was applied to a Sephadex G-25 column, which was eluted with PBS, pH

7.4. The excluded protein peak was collected, aliquoted and stored at -20^{0}C as a stock solution of the biotinylated anti-glypican-3.

2.4 Analytical methods

2.4.1 Enzyme-linked immunosorbent assay (ELISA)

2.4.1.1 Optimization of ELISA procedures

All reagents were prepared in PBS and the concentrations and conditions were changed according to the experimental procedures. All incubations were carried out at 37^{0}C for 60 min. Every condition was tested in duplicate. For coating of the wells the dilutions of anti-glypican-3 (3.2, 1.6, 0.8, 0.4, 0.2, 0.1, 0.05, 0.025 µg/ml) were prepared in carbonate-bicarbonate buffer pH 9.6. After overnight incubation, the wells were blocked with 1% BSA-PBS (150µl/well). After blocking, the wells were washed three times with the washing buffer (PBS-0.05% Tween 20) and added 100 µl of recombinant human glypican-3 (standard; 9.78-5000 ng/ml). After washing the biotinylated anti-glypican-3 (100 µl/well; 1:500, 1:1,000, 1:2,000, 1:4,000, 1:8,000) were added. Again, washed three times and the peroxidase-mouse monoclonal anti-biotin (1:2,000) was added to detect the biotin label. To detect the enzyme label, OPD substrate was used and the reaction was stopped after 15-20 min by the addition of 4 M sulfuric acid. The plates were measured at 492 nm with background subtraction at 620 nm.

2.4.1.2 Double antibody sandwich ELISA for glypican-3 determination in pathological serum

Microtiter plates (Maxisorp, Nunc) were coated at 4^0C overnight with 1.6 µg/ml anti-human glypican-3 (100 µl/well) in the coating buffer. Uncoated area was then blocked with 1% (w/v) BSA (150 µl/well) for 60 min at 37^0C. After washing, 100 µl of sample were added. After incubation for 60 min at 37^0C, plates were washed and added the biotinylated anti-glypican-3 (100 µl/well; 1:500) and incubated for 60 min at 37^0C. After washing the peroxidase-mouse monoclonal anti-biotin (100 µl/well; 1:2,000) was added and incubated for 60 min at 37^0C. The plates were washed again and then the peroxidase substrate (OPD; 100µl/well) was added and incubated at 37^0C for 15-20 min to allow the color developed. The reaction was stopped by addition of 50 µl of 4 M H_2SO_4. The absorbance at 492 nm was measured using the Titertek Multiskan M340 multiplate reader.

2.4.2 Micromethod periodate-resorcinol assay for total sialic acid (TSA)

Forty µl of samples or standard sialic acid (2-10 µg /well) solution were added to the wells of a 96-well microtiter plate. 50 µl of 1.3 mM periodic acid was added to each well and mixed by shaking the plate for 5 minutes on a microplate shaker at room temperature. The plate was placed in an icebox for 60 minutes, then 100 µl of 0.6 g% of resorcinol reagent was added and mixed by shaking as described above. The plate was covered with a glass placed and heated at 80^0 C for 60 minutes in hot water bath then it was removed and placed on the shaker for about 2 minutes mixing as well as cooling the contents down to room temperature. 100 µl of 95 % tert-butyl alcohol was added to each well and the mixture was mixed once again as described

above. The absorbance at 620 nm was measured immediately by microtiter plate reader.

2.4.3 Competitive immunoassay using monoclonal antibody WF6

A quantitative ELISA was modified from a previous study (Teingburanathum 1996) for the epitopes recognized by monoclonal antibody WF6. The standard used was shark cartilage aggrecan (A_1D_1 fraction) at concentrations 19-10,000 ng/ml in 6% BSA in incubation buffer (0.1M Tris HCl, pH 7.4 containing 0.15M sodium chloride, 0.1% Tween 20 and 0.1% BSA). Diluted human serum samples (1:5 in 6% BSA-in incubation buffer) were added to 1.5 ml plastic tubes containing an equal volume of WF6 (culture medium, 1:100 diluted in incubation buffer). They were incubated at 37^{o}C for 1 h, and then added to the microtitre plate, which was pre- coated with shark aggrecan (A_1 fraction). Non-specific protein binding was blocked with 1%BSA. Blank (incubation buffer only) and reference wells (WF6 antibody, no competing antigen) were incubated on every plate. After incubated at 37^{o}C for 1 h, the remaining steps were as above. The concentration of WF6 epitope in serum samples was calculated by reference to a standard curve.

2.5 Evaluation of precision and accuracy of the assay

Precision:

Twenty aliquots of pooled human serum which has been spiked with known amount of standard GPC3 were used to assess the precision of the assay. Intra-assay precision: determined by using 20 replicated analysis in 20 serum pools. Inter-assay precision: determined by using duplicate measurement on different plates.

Recovery:

The analytical recovery of serum GPC3 was determined by using pooled serum which has been spiked with known amount of standard GPC3. The GPC3 concentration in samples was measured and compared with the determinated, theoretical concentration.

2.6 Statistical method

Statistical analysis. Concentration of analytes was determined by reference to a standard curve, using genesis software using the absorbance values of the standard curve. The ELISA data were analyzed using the statistical program SPSS.

To analyze the data, we divided patients into different groups according to clinical and pathologic parameters. The Mann-Whitney U-test was used for comparison of different serum components. In all cases, *p* values less than 0.05 were considered significant. All results compared are from assays performed at the same time and carried out at least in duplicate.