CHAPTER IV

DISCUSSION AND CONCLUSION

In 1996, Pilia *et al.* reported that GPC3, encoding one member of the glypican family, is mutated in patients with Simpson–Golabi–Behmel syndrome (SGBS) (Pilia *et al*., 1996). SGBS is an X-linked disorder characterized by pre- and postnatal overgrowth, and a broad spectrum of clinical manifestations which vary from a very mild phenotype in carrier females to infantile lethal forms in some males (Neri *et al.*, 1998). The list of clinical manifestations of SGBS includes a distinct facial appearance, cleft palate, syndactyly, polydactyly, supernumerary nipples, cystic and dysplastic kidneys, congenital heart defects. Most GPC3 mutations are point mutations or small deletions encompassing a varying number of exons. Given the lack of correlation between patient phenotype and location of the mutations, it has been proposed that SGBS is caused by the lack of a functional GPC3 protein, with additional genetic factors being responsible for the intra- and interfamilial phenotypic variation (Hughes-Benzie *et al.*, 1996).

Since GPC3 is an inhibitor of cell proliferation and can induce apoptosis in certain types of tumor cells (Gonzalez *et al*., 1998) thus, the reports indicating that GPC3 expression is down-regulated in tumors of different origin were not surprising. Lin *et al*. showed that, although GPC3 was expressed in the normal ovary, the expression was undetectable in any significant proportion of ovarian cancer cell lines. In all cases where GPC3 expression was lost, the GPC3 promoter was

hypermethylated, and mutations were nil in the coding region. GPC3 expression was restored by treatment with a demethylating agent.

GPC3 messenger RNA (mRNA) was expressed in 143 of 191 (75%) primary and recurrent HCCs, but in only 5 of 154 (3%) normal livers. In addition, no GPC3 expression was detected in six cholangiosarcomas (Hsu *et al.*, 1997).

The other reports confirmed the over-expression of GPC3 mRNA in HCC (Zhu *et al*., 2001, Zhou *et al.*, 2000). One of these studies the authors compared the levels of GPC3 mRNA in HCC versus normal liver, focal nodular hyperplasia (a benign lesion), and liver cirrhosis. They found that GPC3 mRNA values in focal nodular hyperplasia and liver cirrhosis were similar to those of normal liver, and that levels of GPC3 in HCCs were above the mean value of the non-malignant groups in 25 of 30 (83%) patients. Based on these results the investigators proposed that GPC3 could be a useful marker to differentiate between benign and malignant liver tissue (Zhu *et al.*, 2001).

By immunostaining paraffin-embedded tissue sections with an mAb against C-terminus of GPC3, Capurro *et al*. (2003) shown that GPC3 is expressed in 72% of HCCs, whereas it was undetectable in hepatocytes from benign liver diseases, reactive liver, and normal liver. Another interesting aspect of the immunohistochemical study was the finding that GPC3 was expressed in small tumors (<3cm), a property that was very important for a tumor marker to be used for diagnostic purposes.

In this study, we decided to investigate the expression of serum GPC3 in HCC patients. Thus, we established a sandwich ELISA technique for quantification of GPC3 in the serum. The technique was based on the polyclonal antibody which

coated directly onto the microtiter wells and used biotinylated polyclonal antibody against GPC3 as the secondary antibody.

The biotinylation of antibody is a commonly used tool for increasing the sensitivity in ELISA assay. It takes advantage of the highly specific interaction between avidin/streptavidin or anti-biotin antibody. Protein biotinylation utilizes biotin containing an active ester group which reacts with primary amine of protein, particularly with the N-terminus. There by a novel amide bond is formed and the biotin moiety is covalently attached to the respective amino group. With increasing concentrations of the biotin in the biotinylation reaction, the numbers of biotinylated peptides increased. In this study, biotinylation reaction for the antibody we used anti-GPC3 and *N*-hydroxysuccinimidobiotin at a ratio of 2:1, at this concentration contained more biotinylated residues. After removing unbound biotin by a Sephadex G-25 column, we collected the biotinylated polyclonal antibody as a stock solution for optimized an ELISA assay. The optimal dilution of biotinylated anti-GPC3 was determined by varying the dilution from 1:500 to 1:8,000. We found that at dilution 1:500 gave the good standard curve compared with the other one.

The sandwich assay optimization was performed by optimized amount of antibody which can bound to a plate. The amount of antibody adsorbed has been proportionally increased and related with the concentration of the coated protein on the well. Thus, the coated antibody generated increasing signal related the varying concentration of coating antibody which are 0.1µg/ml to 10µg/ml. As the amount of antibody bound reaches saturation, it appears to form a monolayer on the surface of the plate. It has been observed that further increasing the amount of antibody added leads to an unstable condition in which sensitivity actually begins to decrease. In this

study, we optimized anti-GPC3 for coating plate by titrating added antibody from 0.025 µg/ml up to 3.2 µg/ml. We found the optimal concentration of anti-GPC3 for the coating plate was 1.6 µg/ml.

Using this assay to dertermine the serum GPC3 concentration, the diluent for standard GPC3 should be optimized, thus PBS, 6% BSA (w/v) and pooled human serum were selected as the candidate for standard GPC3 diluent. We found that 6% BSA (w/v) gave a GPC3 standard curve similar to pooled human serum. Thus, 6% BSA (w/v) was selected for diluting GPC3 standard, undiluted serum could be used for detecting GPC3 by these assay, since it has a little interfere from non-specific binding in this optimized condition. Of note, in a higher concentration of serum GPC3 should be diluted with 6% BSA (w/v) before used.

A sandwich ELISA assay has demonstrated on intra- and inter- assay coefficient of variation of 13.13% and 20.26%, respectively. The percentage of recovery in this assay was 104.6%, which has been proven to be an accuracy assay.

Using this assay we found that GPC3 was undetectable in serum of healthy donors and pathological samples including CCA, hepatitis, liver cirrhosis and benign liver masses, whereas, 40% HCC patients (28 of 70 patients) could be detected in serum. In the Capurro *et al.* study, the sensitivity of the GPC3 test was somewhat higher than in our study (53%). However, it has to be noted that Capurro *et al*. used a monoclonal antibodies against C-terminus of GPC3 for GPC3 ELISA assay which is more specific. Thus, the specificity of the serological GPC3 test for the diagnosis of HCC in a population of patients with chronic liver disease was very high. On the other hand, the sensitivity was limited.

The monoclonal antibody WF6, which recognizes small native epitope on chondroitin sulfate chain has been useful for investigation the pathology changes. Pothacharoen *et al*. reported that serum WF6 epitope levels were elevated in 88% of all patients with ovarian epithelial cancer. The accumulation of chondroitin sulfate proteoglycans especially chondroitin 6-sulfate proteoglycans, which are likely to be rich in WF6 epitope, may be synthesized by the tumor cells themselves. Many studies have also reported the elevation of chondroitin sulfate proteoglycan levels in hepatocellular carcinoma, focal nodular hyperplasia and cirrhosis (Kovalszky *et al.*, 1990, Koshiishi *et al*., 1999, Kojima *et al*., 1975) but the levels of WF6 epitope has never been reported. In this study, analysis in the patients serum showed the significant decreasing of the WF6 epitope concentration in hepatocellular carcinoma, cholangiocarcinoma, hepatitis and cirrhosis when compare to the control (p<0.001). Moreover, HPLC analysis of chondroitinase ABC or AC digested tissue chondroitin sulfate showed a 58 fold increase in Δ-Di-0S (GlcUAβ1-3GalNAc) disaccharides in hepatocellular carcinoma, indicating the significant level of the undersulfated chondroitin sulfate, whereas in focal nodular hyperplasia and adenoma, dermatan sulfate (IdoAα1-3GalNAc) was the predominant glycosaminoglycan (Kovalszky *et al*., 1990). In the Koshiishi *et al*. study, they also demonstrated the increasing of Δ-Di-4S [GlcUAβ1-3GalNAc(4-*O*-sulfate)] in liver cirrhosis (Koshiishi *et al.*, 1999). Both evidences indicated that specific changes of glycosaminoglycan composition occured in hepatocellular carcinoma, focal nodular hyperplasia, adenoma and liver cirrhosis suggested that proteoglycan metabolism in the liver tissue is also altered during the developing of the diseases. With complementary to those evidence, therefore, the levels of serum WF6 epitope which recognized Δ-Di-6S [GlcUAβ1-3GalNAc(6-*O*-

sulfate)] and Δ-Di-S_D [GlcUA(2-*O*-sulfate)β1-3GalNAc(6-*O*-sulfate)] sequences (Pothacharoen *et al*., 2007) was decreased by increasing of Δ-Di-0S and Δ-Di-4S liver chondroitin sulfate proteoglycan composition.

Not only the group of proteoglycan changed during cancer development, the previous studies also reported that in neoplasms the concentration of sialic acid on tumor cell surface often increased, and sialoglycoprotein were shred or secreted by some of these cells which increased in blood concentration.

In this study, we employed periodate-resorcinol microassay for detection of serum total sialic acid. We found that the mean serum total sialic acid level in patients with HCC was significantly increased compare to those with hepatitis, cirrhosis, benign liver masses and healthy donors (p<0.01). Notably, the mean concentration of serum TSA in patients with CCA was substantially higher than that in those with HCC (p<0.001). The data of the present study are in agreement with those previously reported which used serum TSA to differentiate CCA from HCC (Kongtawelert *et al.*, 2003). The mechanisms underlying the substantial difference in levels of TSA between patients with CCA and HCC are unclear. One possible explanation is that, in certain cancers, increased activity of sialytransferase might lead to spontaneous shedding of aberrant sialic acid-containing cell surface glycoconjugates into the circulation (Singhal and Hakomori, 1990). Alternatively, since TSA has been well described as being associated with the acute-phase protein response (Crook *et al.,* 1994), increased activity of serum or tissue sialidase in combination with inflammatory response could considerably elevate serum TSA levels in patients with CCA.

In conclusion, the present study revealed that serum GPC3 was overexpressed in 40% of HCC samples whereas serum GPC3 was undetectable in all normal, benign liver, CCA, cirrhosis and hepatitis indicating that the upregulation of GPC3 expression in HCC was unique. Based on these findings, we concluded that the GPC3 could serve as a potential diagnostic marker that may enable the differential diagnosis between hepatocellular carcinoma and the other types of liver diseases. The measurement of serum WF6 epitope and TSA might provide complementary information with serum GPC3 for early diagnosis of HCC patients.