Analysis of Serum Haptoglobin Fucosylation in Hepatocellular Carcinoma and Liver Cirrhosis of Different Etiologies

Supporting Information

Glycan composition confirmed by MS/MS analysis

The glycan composition was confirmed by MS/MS analysis which can provide valuable information of MS/MS fragmentation to assist sequence assignment. At low energy CID, the predominant fragments are *y*-ions resulting from the cleavage of labile GlcNAc-Hex glycosidic bond; therefore, the oligosaccharide composition can be inferred from mass differences of fragment ions.[1] In addition, permethylation allows us to obtain branching information because only unoccupied hydroxyl groups can be permethylated.[2] Fucose is most commonly linked to the innermost core GlcNAc via α 1–6 linkage to form core fucosylation. Additionally, in certain cases, fucose is attached to the subterminal GlcNAc via α 1–3 or α 1–4 linkage resulting in antennary fucosylated glycans. While the fragmentation from CID could not provide detailed linkage information, we utilized characteristic CID fragment ions to discriminate between core and antennary fucosylation.

The representative CID MS/MS spectra of fucosylated glycans (*m/z* 2244.13, 2693.40, 2867.48, and 3142.69, respectively) are shown in Figure S3. The fucosylated biantennary glycan (*m/z* 2244.13) is confirmed as core fucosylated because of a diagnostic fragment at 1317.63 which corresponds to a fucosylated pentasaccharide core (Figure S3A). This conclusion is also supported by an ion at 1329.79 resulting from loss of core Fuc-GlcNAc. The bifucosylated triantennary glycan (*m/z* 2867.46) is determined as a triantennary structure with both core and antennary fucosylation (Figure S3B). The fragment ion at *m/z* 1940.65 is the product after loss of two nonreducing terminal Gal-GlcNAc residues, with two attached fucose residues. For the majority of the glycans, one of the two fucoses must be

attached to the core GlcNAc. Core fucosylation is also confirmed by the presence of a fragment ion at *m/z* 1303.76 corresponding to a fucosylated pentasaccharide core structure. A peak at m/z 1489.52 resulting from loss of core Fuc-GlcNAc unambiguously indicates antennary fucosylation.

Similarly, the fucosylated tri-antennary glycan (*m/z* 2693.40) is confirmed as antennary fucosylated (Figure S3C). A diagnostic peak at *m/z* 1952.83 is the cleavage product of peak at 2230.40 after loss of core GlcNAc, indicating that there is no core fucose attached originally. The fucosylated tetra-antennary glycan (*m/z* 3142.69) is also antennary fucosylated due to a diagnostic peak at *m/z* 1938.83, corresponding to product after loss of core GlcNAc from the peak at 2216.15 (Figure S3D). Fragments at *m/z* 2679.20, 2216.15, and 1752.73 correspond to products after loss of one, two, and three nonreducing terminal Gal-GlcNAc, respectively, from the parent ion.

Validation of total fucosylation degree by AAL lectin blot

AAL, a fucose-specific lectin, has a high affinity for fucose residues in all binding positions (α1–2, α1–3, α1–4, α1–6), which is widely used as a carbohydrate probe for fucose. Therefore, AAL lectin blot was performed to validate the total fucosylation level in patients of HCC and cirrhosis induced by HBV, HCV, and ALC, respectively.

The result of AAL blot analysis is shown in Figure S6. First, haptoglobin was immunoprecipitated from serum samples of 6 HCC and 6 cirrhosis patients, respectively. Then, aliquots of purified haptoglobin were separated by 4–15% SDS-PAGE gels in duplicate. One gel was used for AAL lectin blot analysis after transferring onto a PVDF membrane. The other was evaluated by silver staining, confirming equal amounts of haptoglobin loaded on the gel for each sample. The results of the AAL blot verified that the total fucosylation level of haptoglobin was significantly increased in HCC patients with HBV and ALC etiologies compared to their corresponding cirrhosis, which was consistent with the MS result.

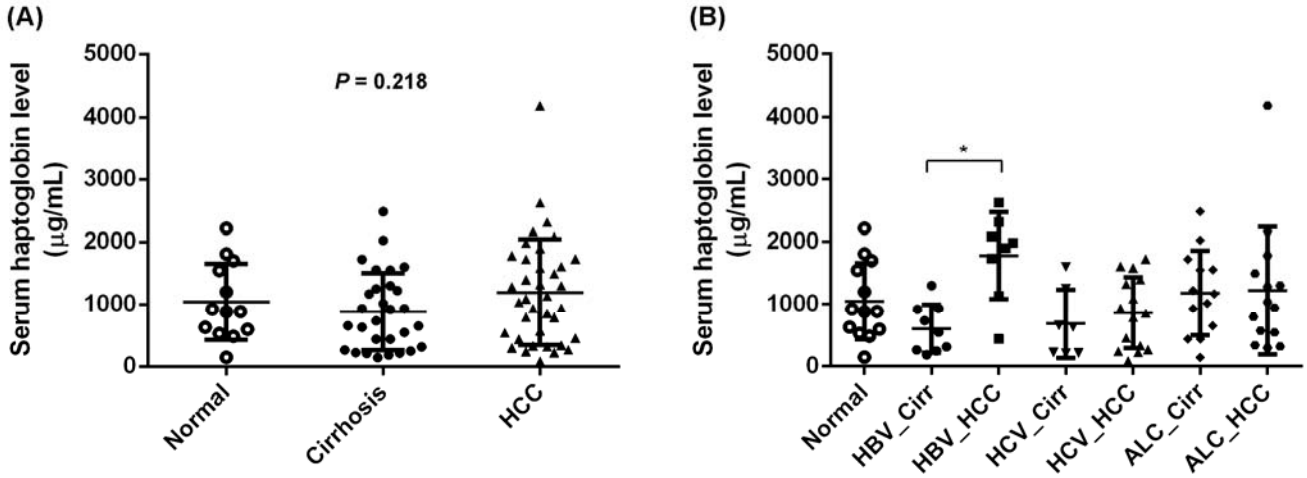

Figure S1. Scatter plot of serum Hp concentration measured by ELISA assay in patients of HCC, cirrhosis and normal groups (A) and in the subgroups divided according to the etiology, HBV, HCV, and ALC, respectively (B). (HBV_Cirr: HBV-related cirrhosis; HCV_Cirr: HCV-related cirrhosis; ALC_Cirr: ALC-related cirrhosis; HBV_HCC: HBV-related HCC; HCV_ HCC: HCV-related HCC; ALC_ HCC: ALC-related HCC)

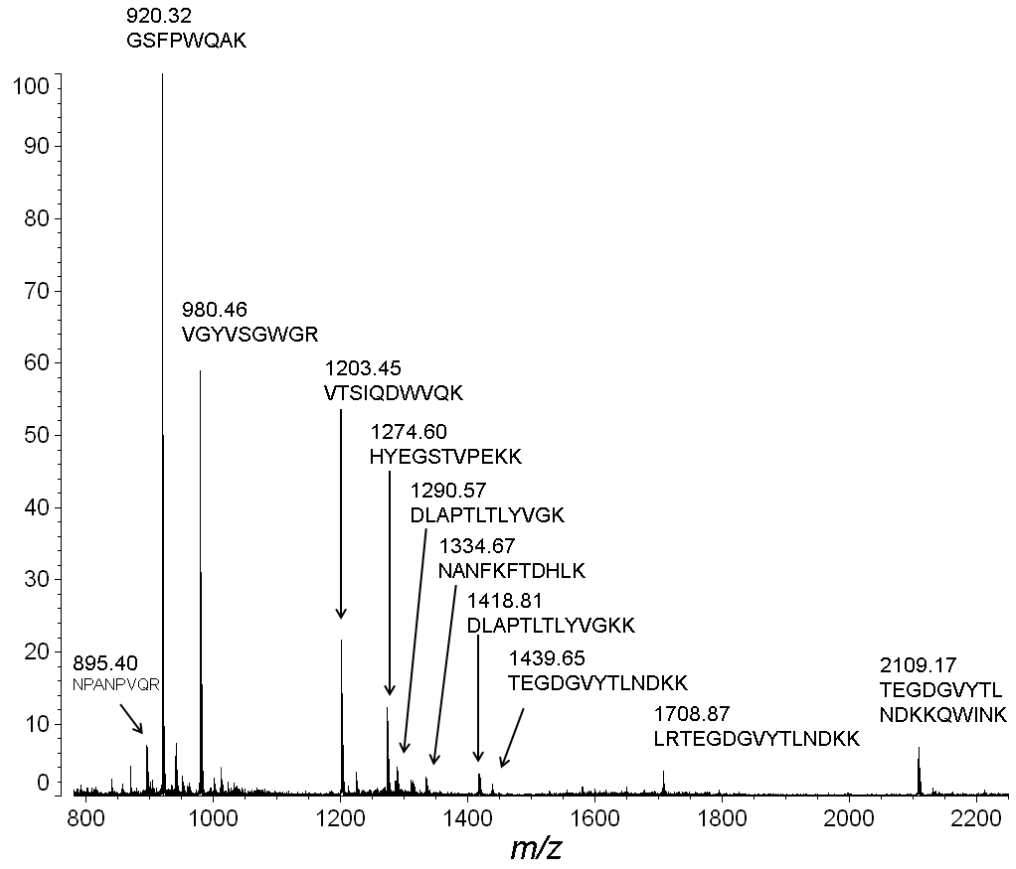

Figure S2. Representative MALDI-QIT-TOF MS spectrum of tryptic peptides of haptoglobin resulting from 10-min on-plate digestion. The spectrum was searched in the Mascot database and returned human haptoglobin as the only significant protein with 11 matched peptides.

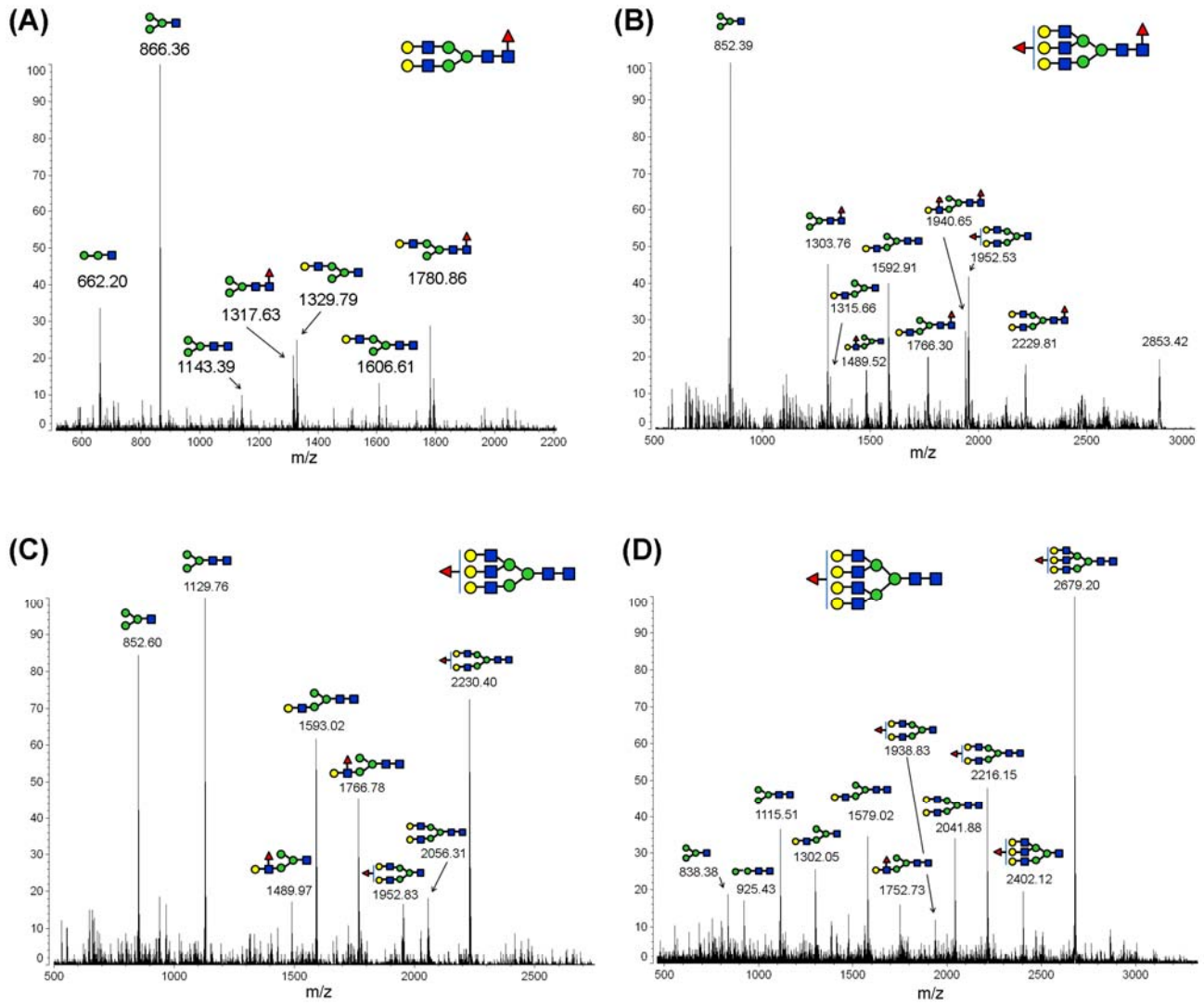

Figure S3. MALDI-QIT-TOF MS/MS spectra of glycans at (A) *m/z* 2244.13, (B) *m/z* 2867.48, (C) *m/z* 2693.40, and (D) *m/z* 3142.69. Potential structures of fragment ions are labeled. Fragment ions at 1317.63 and 1329.79 (A) imply core fucosylation for glycan at *m/z* 2244.13. Fragment ions at *m/z* 1303.76, 1489.52, and 1940.65 (B) indicate both core and antennary fucosylation for glycan at *m/z* 2867.48. Fragment ions at 1489.97 and 1952.83 (C) confirm antennary fucosylation for glycan at *m/z* 2693.40. Fragment ion at *m/z* 1938.83 (D) implies antennary fucosylation for glycan at *m/z* 3142.69.

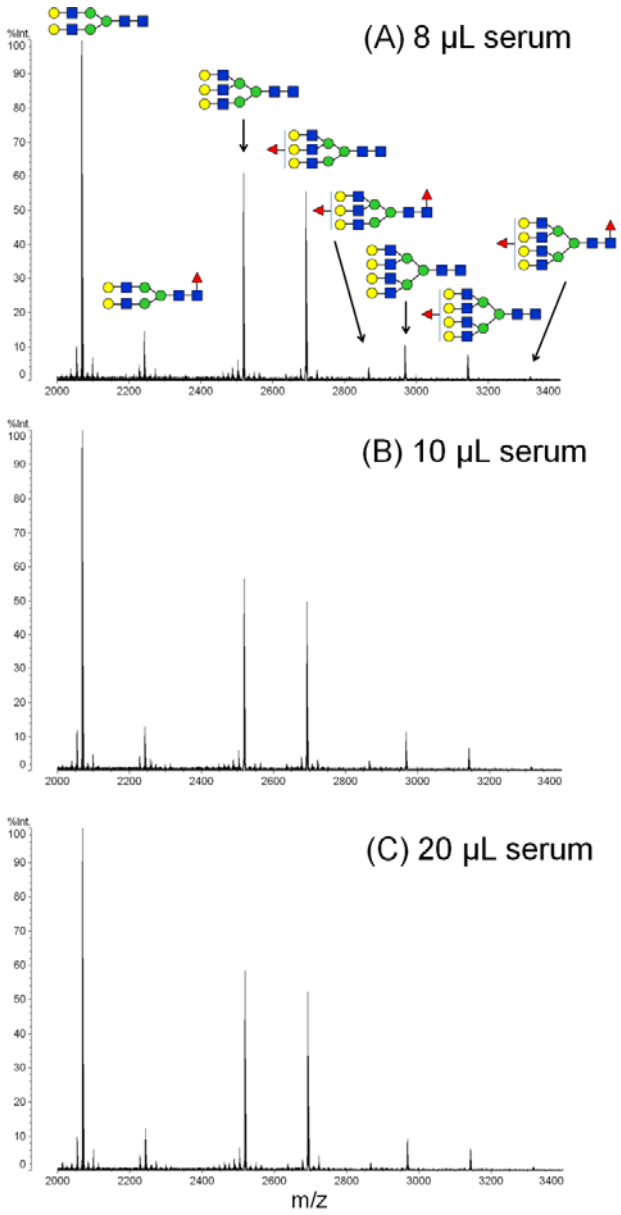

Figure S4. MALDI-QIT-TOF MS spectra of *N*-glycans from an ALC-related HCC serum sample evaluated at three different volumes, 8, 10 and 20 µL, respectively. No differences were observed in the spectra at these 3 different volumes.

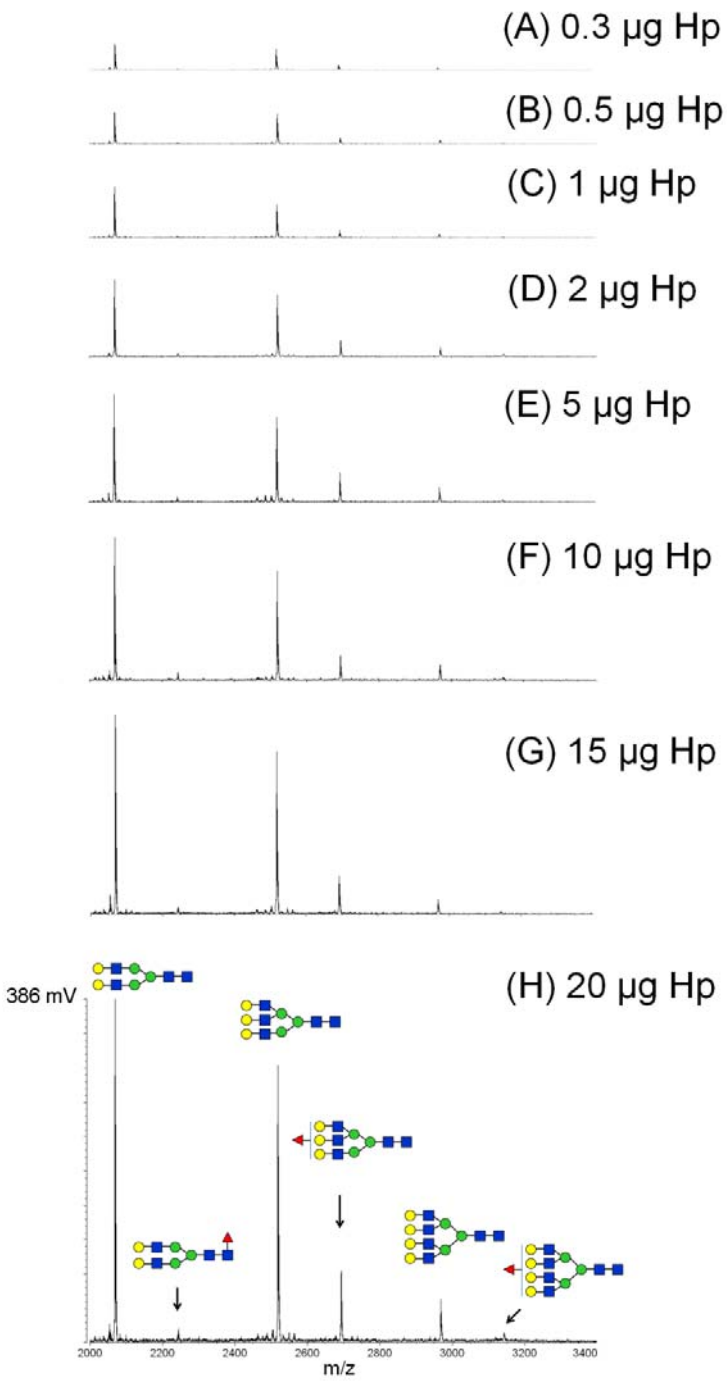

Figure S5. MALDI-QIT-TOF MS spectra of *N*-glycans from a human haptoglobin standard processed in sequential aliquots of 0.3, 0.5, 1, 2, 5, 10, 15, and 20 µg, respectively.

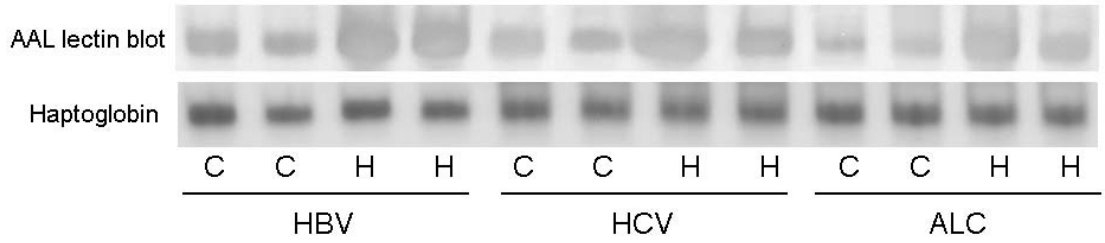

Figure S6. AAL lectin blot analysis of fucosylation level of haptoglobin in patients of HCC and cirrhosis induced by HBV, HCV, and ALC, respectively. Haptoglobin was immunoprecipitated from serum samples of 6 HCC and 6 cirrhosis patients, respectively. Aliquots of purified haptoglobin were separated by 4–15% SDS-PAGE gels in duplicate. One gel was used for AAL lectin blot analysis after transferring onto a PVDF membrane (upper panel). The other was evaluated by silver staining, confirming equal amounts of haptoglobin loaded on the gel for each sample (lower panel). AAL blot verified that the total fucosylation level of haptoglobin was significantly increased in HCC patients with HBV and ALC etiologies, compared to their corresponding cirrhosis. (C: cirrhosis; H: HCC)

Table S1. (A) Reproducibility test of four aliquots of an HCV-related HCC sample. (B) Influence of sample amount on quantification of total fucosylation and bifucosylation degrees of an ALC-related HCC serum sample evaluated at three different volumes, 8, 10 and 20 μL, respectively.

			(A)		
	aliquot 1	**aliquot 2**	**aliquot 3**	**aliquot 4**	**RSD**
Total fucosylation degree	0.4175	0.4330	0.4070	0.4487	4.3%
Bifucosylation degree	0.0612	0.0604	0.0696	0.0708	8.3%
			(B)		
	8 μL serum	**10 μL serum**	**20 μL serum**	**RSD**	
Total fucosylation degree	0.6811	0.6574	0.7103	3.9%	
Bifucosylation degree	0.1398	0.1264	0.1460	7.3%	

References

1. Harvey, D. J.; Martin, R. L.; Jackson, K. A.; Sutton, C. W. Fragmentation of N-linked glycans with a matrix-assisted laser desorption/ionization ion trap time-of-flight mass spectrometer. *Rapid Commun Mass Spectrom* 2004, *18* (24), 2997-3007.

2. Yu, S. Y.; Wu, S. W.; Khoo, K. H. Distinctive characteristics of MALDI-Q/TOF and TOF/TOF tandem mass spectrometry for sequencing of permethylated complex type N-glycans. *Glycoconj J* 2006, *23* (5-6), 355-369.