LC-MS Profiling of N-Glycans Derived from Human Serum Samples for Biomarker Discovery in Hepatocellular Carcinoma

H. W. Ressom*, M. Wang, Y. Zhao, T.-H. Tsai, C. Di Poto, Y. Hu, S. Zhou, M. G. Tadesse, D. H. Ziada, K. Shetty, Y. Mechref
Lombardi Comprehensive Cancer Center, Georgetown University, Washington, DC *Corresponding author (hwr@georgetown.edu)

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Objective

The objective of this study is to identify candidate N-glycan biomarkers by comparing the levels of permethylated N-glycans in sera of hepatocellular carcinoma (HCC) patients with those of cirrhotic patients using liquid chromatography-mass spectrometry (LC-MS).

Study Population

The participants in this study consist of 89 subjects (40 HCC cases and 49 cirrhotic controls) from Tanta University, Tanta, Egypt (Egyptian cohort, **Table I**) and 94 subjects (48 HCC cases and 46 cirrhotic controls) from Georgetown University Hospital, Washington, DC (US cohort, **Table II**). Controls were required to be HCC free for at least 6 months from the time of study entry.

Table I: Characteristics of the Egyptian cohort

		HCC (n=40)	CIRR (n=49)	p-value
Age	Mean(SD)	53.2 (3.9)	53.8 (7.6)	0.3530
Gender	Male (%)	77.5%	67.3%	0.3474
HCV Serology	HCV Ab (+)	100.0%	100.0%	1.0000
HBV Serology	HBsAg (+)	0.0%	6.1%	0.2492
MELD	Mean (SD)	18.6 (7.7)	18.9 (7.1)	0.1328
	MELD ≤ 10	20.0%	12.2%	0.3863
AFP	Median (IQR)	275.9 (1244.3)		
HCC Stage	Stage I	72.5%		
	Stage II	15.0%		
	Stage III	5.0%		
	Unknown	7.5%		

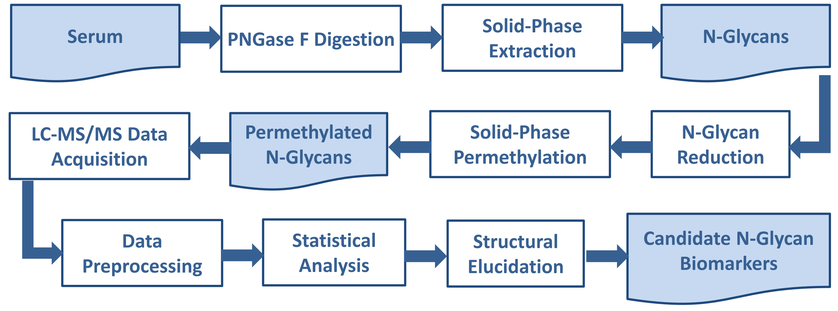
Table II: Characteristics of the US cohort

		HCC (n=48)	CIRR (n=46)	p-value
Age	Mean (SD)	60.2 (6.0)	58.9 (7.1)	0.3443
Gender	Male (%)	77.1%	73.9%	0.8121
Ethnicity (%)	Caucasian	50.0%	63.0%	0.4566
	African American	33.3%	26.1%	
	Others	16.7%	10.9%	
HCV Serology	HCV Ab (+)	68.8%	41.3%	0.0033
	HCV RNA (+)	62.5%	39.1%	0.0385
HBV Serology	Anti-HBV (+)	45.8%	26.1%	0.0554
	HBsAg (+)	8.3%	2.2%	0.3619
MELD	Mean (SD)	11.3 (4.1)	17.3 (16.1)	0.0190
	MELD ≤ 10	47.9%	10.9%	0.0042
AFP	Median (IQR)	38.8 (91.1)	4.5 (11.85)	0.0001
HCC Stage	Stage I	54.2%		
	Stage II	22.9%		
	Stage III	6.3%		
	Unknown	16.7%		

Study Design

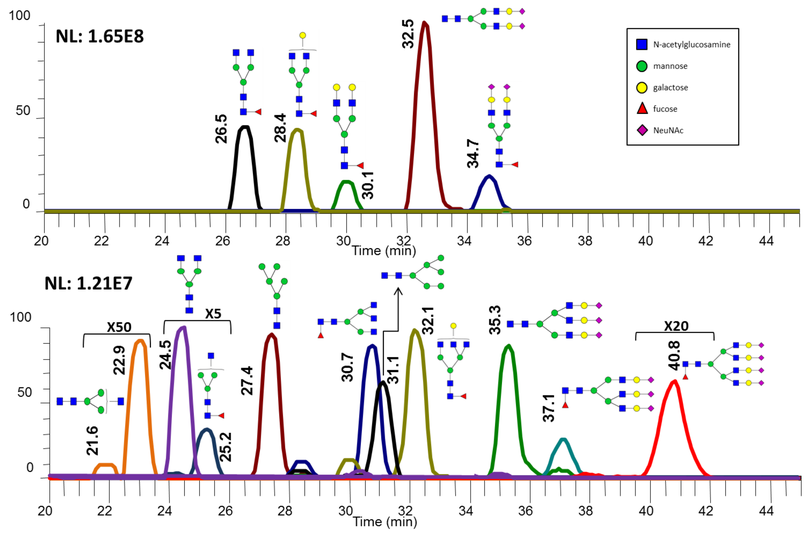
- Each cohort was analyzed in four batches (n≈24): E1/E2/E3/E4 in the Egyptian cohort; U1/U2/U3/U4 in the US cohort.
- Balanced assignment of HCC cases and cirrhotic controls into each batch in terms of age, race, gender, smoking, alcohol, and BMI.

Analyzing Workflow



LC-MS/MS Data Acquisition

- Thermo Scientific LTQ Orbitrap Velos mass spectrometer coupled to the Ultimate Dionex 3000 HPLC system (Nano LC 350 nL/min)
- Five MS/MS scans per MS scan on positive mode



LC-MS Data Preprocessing

- Main preprocessing steps:
  1. Deisotoping of mass spectra by DeconTools [PMID: 19292916]
  2. Peak detection using an in-house developed algorithm
  3. Peak alignment & peak matching by SIMA [PMID: 21296750]
- 2333, 2817, 2656, and 2725 peaks were detected in E1, E2, E3, and E4, respectively.
- 2770, 2597, 2683, and 3375 peaks were detected in U1, U2, U3, and U4, respectively.

Statistical Analysis

- Parametric (*t*-test and ANOVA) and nonparametric (Wilcoxon rank-sum test) methods were applied.
- Peaks with *p*-values < 0.05 and the same regulation direction in a least two out of four batches by the same test were selected as statistically significant.

Candidate N-Glycan Biomarkers

- 14 candidate N-glycan biomarkers were identified with putative structure.
- Six of them were previously reported in the literature (**Table III**).
- These glycans represent possible modifications including hyperfucosylation, increased branching and bisecting N-acetylglucosamine.
- The majority of these glycans was down-regulated in HCC versus cirrhosis.

Table III: List of candidate N-glycan biomarkers

Structure	Cohort	Mass	RT	Batch	FC	Literature
[4,3,2,0,0]	Egypt	2063.077	31.4	E3	↓1.51	
			32.1	E4	↓1.73	[PMID: 19223512]
	US	2063.077	29.9	U3	↓1.65	[PMID: 17683101]
			29.9	U4	↓1.87	[PMID: 18619944]
[4,3,0,1,0]	Egypt	1828.967	25.8	E1	↓2.43	[PMID: 17683101]
			25.7	E4	↓1.35	[PMID: 18619944]
[5,3,0,1,0]	Egypt	2074.093	29.9	E1	↓2.41	[PMID: 17683101]
			29.8	E4	↓1.39	[PMID: 18619944]
[5,3,1,0,1]	US	2465.278	29.2	U3	↓2.59	[PMID: 19223512]
			29.1	U4	↓2.61	[PMID: 18189345]
[4,3,1,1,0]	US	2033.067	25.5	U1	↑1.98	[PMID: 17683101] [PMID: 18189345]
			25.4	U4	↑1.63	[PMID: 18619944] [PMID: 19764807]
[5,3,2,1,1]	US	2843.467	35.9	U3	↓2.71	[PMID: 19764807]
			36.0	U4	↓2.15	

Symbols: N-acetylglucosamine ■, mannose ●, galactose ○, fucose ▲, NeuNAc ◆

Summary

- Permethylation of glycans enzymatically removed from serum proteins allows relative quantification of hundreds of oligosaccharides.
- The global profiling workflow presented here allows identification of reliable candidate biomarkers for HCC.

Future Work

- **Targeted quantitation** of candidates from this study by multiple reaction monitoring (MRM) method on TSQ Vantage is on the way.
- **Stratified analysis** to evaluate the effect of age, gender, race, viral infection, etc.
- Multivariate analysis to identify a **panel of biomarkers**

Acknowledgements

This work was supported by NIH Grants R01CA143420 and R01GM086746.