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Alternations of N-glycans recognized by Phaseolus vulgaris leucoagglutinin in the saliva of patients with breast cancer

Jiajun YANG[1], Tianran MA[1], Hanjie YU[1], Mengqi YIN[1], Yan QIAO[2], Lili NIU[3], Fuquan YANG[3], Jianjun HE[2,*], Zheng LI[1,*]

[1]Laboratory for Functional Glycomics, College of Life Sciences, Northwest University, Xi'an, China; [2]Department of Surgical Oncology, The First Affiliated Hospital of Xi'an Jiaotong University, Xi'an, China; [3]Laboratory of Protein and Peptide Pharmaceuticals and Laboratory of Proteomics, Institution of Biophysics, Chinese Academy of Sciences, Beijing, China

**Correspondence: chinahjj@163.com; zhengli@nwu.edu.cn*

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Breast cancer is the most frequently diagnosed cancer in most countries. Early diagnosis of breast disease is necessary for its prognosis and treatment. Altered protein glycosylation has been shown to be expressed in precursor lesions of breast cancer, making them powerful early diagnostic biomarkers. The present study validated alterations of the N-glycan profiles of their salivary glycoproteins isolated by the Phaseolus vulgaris leucoagglutinin (PHA-E+L)-magnetic particle conjugates from 141 female subjects (66 healthy volunteers (HV), and 75 patients with breast disease including breast benign cyst (BB) or breast cancer in stage I/II (BC-I/II)) were analyzed and annotated by MALDI-TOF/TOF-MS. The results showed that there were 11, 20, 16, and 17 N-glycans recognized by PHA-E+L identified and annotated from the pooled salivary samples of HV, BB, BC-I, and BC-II, respectively. There were 3 N-glycans peaks (m/z 2459.8799, 2507.9139, and 2954.0547), 2 N-glycans peaks (m/z 1957.7265 and 2794.0427), and 2 N-glycans peaks (m/z 1866.6608 and 2240.8056) recognized by PHA-E+L that existed only in BB, BC-I, and BC-II, respectively. The present study compared the alternations of N-glycans from the salivary proteins isolated by PHA-E+L-magnetic particle conjugates among HV, BB, BC-I, and BC-II, which could provide information on N-glycans during the development of breast cancer in saliva to promote the study of its biomarkers.

Key words: saliva, breast cancer, N-glycans, PHA-E+L, MALDI-TOF/TOF-MS

Breast cancer is the most frequently diagnosed cancer in most countries and the leading cause of cancer death in over 100 countries, and breast cancer cases accounted for almost a quarter of all female cancer cases in 2018 [1]. The incidence and mortality rates of breast cancer have been rising for most of the low and middle-income countries [2, 3]. These trends may reflect those demographic factors related to social and economic development, breast cancer screening, and awareness [4]. Whereas the incidence and mortality have been continuously declining for the past decade in several developed countries, including the United States, Canada, the United Kingdom, France, and Australia [5, 6]. At least 20 percent of the observed reductions were benefited from mammographic screening, indicating the necessity of early detection for breast lumps [7, 8]. Nevertheless, mammographic screening may not be the most appropriate diagnostic method for early detection in low and middle-income countries because of its high-cost burden and adverse effects [9]. Currently, biopsy and histopathology are applied to the definitive diagnosis of breast cancer, and the best-validated markers include estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor-2 (HER-2), urokinase plasminogen activator (uPA), and plasminogen activator inhibitor-1 (PAI-1). Therefore, the current researches are focused to explore the molecular changes in body fluid during the early stage of breast lesions, in order to find biomarkers for clinical decision-making.

The glycan chains are usually linked to their polypeptide backbone by nitrogen of asparagine (N-glycans) or oxygen of serine or threonine (O-glycans) [10]. The expressions of altered glycans are related to numerous pathologies and even affect the susceptibility of certain diseases [11, 12] so that they have the potential to both respond and reflect changes in the microenvironment of the organism [13]. Different glycans are expressed by malignant tumor cells, including truncated simple O-glycans, changes in N-glycan branching, increased sialylation, fucosylation, and altered glycosaminoglycans [14, 15]. N-glycosylation of integrins is necessary for cell and cell-extracellular matrix interaction, and both alterations in integrins and glycosylation pattern are considered

as an early event in breast cancer progression and metastasis [16]. Glycoconjugates, such as alpha-N-acetylgalactosamine, the epitopes of Tn and Thomsen-Friedenreich (T), and CA15-3, are reported to be involved in the development of breast cancer [17–20].

The alternations of glycosylated molecules during the malignant transformation may be utilized as cancer biomarkers for patient diagnosis and prognosis [21, 22]. The most widely used cancer tests are glycoproteins, mainly mucin 1 (MUC1), MUC16 (also known as CA125), prostate-specific antigen (PSA), and carcinoembryonic antigen (CEA) [23]. Recent research in the biomarkers is focused on the multiplexed analysis of numerous proteins with or without post-translational modifications and exclusively glycans due to the advances in technologies, such as mass spectrometry for glycan analyses and lectin-antibody array methodologies [24]. Non-invasive clinical samples are also considered the general trend. Saliva not only contains molecules that can reflect the state of body but also is non-invasive and easy to collect [25]. The potential of saliva in detecting disease has been demonstrated, such as lung cancer [26], breast cancer [27], type II diabetes [28], and gastric cancer [29]. Our previous study evaluated alternations in salivary glycosylation and their potential as biomarkers for early-stage breast cancer diagnosis [30]. The results showed that salivary glycopatterns recognized by Phaseolus vulgaris leucoagglutinin (PHA-E+L) exhibited significantly increased expression levels in BB, BC-I, and BC-II compared with HV, which achieved the potential to distinguish them. In addition, our previous study demonstrated the potential of the galactosylated-glycans recognized by Bandeiraea simplicifolia Lectin I to identify early-stage breast cancer [31].

In this study, we further researched the alterations of N-glycans with the structures of bisecting GlcNAc, bi-antennary N-glycans, tri-, and tetra-antennary complex type N-glycans identified by PHA-E+L in saliva from 141 subjects (75 patients with breast benign cyst or breast cancer in stage I/II and 66 healthy volunteers) using MALDI-TOF/TOF-MS. The overall strategy adopted here is presented in Figure 1. This study can provide specifically N-glycans information to distinguish BB, BC-I, and BC-II, and promote the discovery of their noninvasive biomarkers for early detection based on the alternations of salivary glycans.

Patients and methods

Study approval and subjects. The collection and usage of female whole saliva were approved by the Human Research Ethical Committee of Northwest University (Xi'an, China) and First Affiliated Hospital of Xi'an Jiaotong University (Xi'an, China). This study was conducted in accordance with the Ethical Guidelines of the Declaration of Helsinki. Saliva samples were collected from 66 healthy volunteers (HV), 25 benign breast cyst or tumor patients (BB), 25 patients with breast cancer in stage I (BC-I), and 25 patients with breast cancer in stage II (BC-II). Healthy volunteers were non-smoking, non-pregnant, and non-diabetic volunteers. All the patients were diagnosed in the First Affiliated Hospital of Xi'an Jiaotong University. The basic characteristics of healthy volunteers and patients are summarized in Table 1.

Whole saliva collection and preparation. The collection of saliva samples was performed according to the protocol [12]. Unstimulated saliva was collected between 9 a.m. and 10 a.m. at least 2 h after eating. After the mouth was gargled with physiological saline, saliva was collected (about 1 ml), and placed on the ice. A protease cocktail inhibitor (1 μl/ml of the whole saliva) was added to saliva to protect proteins from degradation. The whole saliva was centrifuged at 12,000×g at 4°C for 10 min and the supernatant was collected. Whole saliva samples from healthy volunteers and patients with breast cancer were pooled respectively.

Preparation of glycoprotein from pooled saliva by PHA-E+L-magnetic particle conjugates. The PHA-E+L-magnetic particle conjugates were prepared in accordance with a previous protocol [32]. The epoxy-coated magnetic particles (2 mg) were washed three times with coupling buffer (5 mM NaB_4O_7, 180 mM H_3BO_4, 150 mM Na^+, pH 7.4), then reacted with 0.5 mg/ml PHA-E+L (Vector Laboratory, USA) in coupling buffer at 25°C for 3 h with gentle shaking. The unbound PHA-E+L was removed from the conjugates by washing six times with the coupling buffer. The PHA-E+L-magnetic particle conjugates were blocked by 600 μl blocking buffer (2% ethanolamine, 0.1% BSA, pH 9.0) at 25°C for 1 h under gentle shaking, then rinsed three times with the

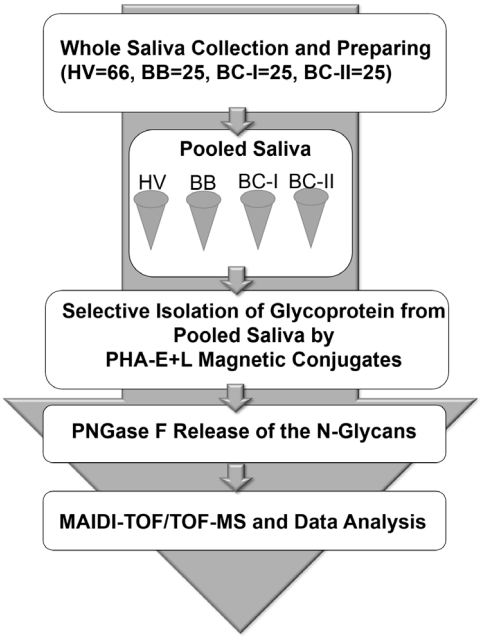


Figure 1. Scheme of the overall study strategy.

Table 1. Baseline characteristics of healthy volunteers and patients with breast cancer.

		Number of study population				Age (mean ± SD)					
Healthy Volunteers (HV)		66				48.37±7.13					
		Benign breast cyst or tumor patients (BB) = 25				44.16±8.77					
Patients		Breast cancer in I stage patients (BC-I) = 25				51.64±10.35					
		Breast cancer in II stage patients (BC-II) = 25				49.20±10.28					
Patient characteristics											
	Grade			**Tumor**		**Lymph node involvement**		**ER/PR receptor**		**HER2 overexpression**	
	G1	G2	G3	T ≤2 cm	T >2 cm	Positive	Negative	Positive	Negative	Positive	Negative
BC-I	0	9	14	25	0	1	24	19	5	14	11
BC-II	1	4	15	4	21	15	10	15	10	14	11

binding buffer (100 mM Tris-HCl, 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM $MnCl_2$, pH 7.4). The pooled saliva samples (2 mg of total protein) were diluted in 600 µl binding buffer and incubated with the prepared PHA-E+L-magnetic particle conjugates at 25 °C for 3 h with gentle shaking. The unbound proteins were removed from the conjugates by washing three times with the washing buffer (0.1% Tween-20, 100 mM Tris-HCl, 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM $MnCl_2$, pH 7.2). Finally, the selectively isolated glycoproteins were eluted with 400 µl eluting buffer (8 M urea, 40 mM NH_4HCO_3) for 1 h.

Releasing of N-linked glycans. N-linked glycans were released by trypsin and PNGase F [33]. The isolated glycoproteins (200 µg) from each pooled sample were concentrated by a size-exclusion spin filter (Amicon Ultra 10K device, Millipore). The concentrated solutions were rinsed three times by 300 µl urea solution (8 M urea, 40 mM NH_4HCO_3), then incubated with 200 µl DTT solution (200 mM DTT, 40 mM NH_4HCO_3) for 45 min at 55 °C and 200 µl IAM solution (200 mM IAM, 40 mM NH_4HCO_3) for 30 min at 25 °C in the dark. The mixture was rinsed three times with 50 mM NH_4HCO_3, then proteomics grade trypsin (1:100 [w/w] of the enzyme to protein) was added to incubate overnight at 37 °C. The enzyme activity was deactivated by heating the mixture at 80 °C for 5 minutes. Thereafter, N-glycans were released from the glycopeptides by incubating with 2 µl PNGase F (New England BioLabs) at 37 °C overnight. Finally, the mixtures of N-linked glycans and peptides were collected by centrifugation at 12,000×g for 10 min and washed twice with 40 mM NH_4HCO_3.

Purification of N-glycans. HyperSep Hypercarb SPE cartridges (25mg, 1ml; Thermo Scientific) were washed three times with water, and equilibrated with 0.1% TFA in 50% ACN and 0.1% TFA in 5% ACN (Sigma-Aldrich), sequentially. Then, the samples were loaded onto the SPE cartridges and washed with HPLC grade water and 0.1% TFA in 5% ACN three times, respectively. Finally, The N-glycans were eluted three times with 0.5 ml of elution solution (0.1% TFA in 50% ACN), then lyophilized.

Characterization of N-glycans. N-glycans were characterized by Matrix-assisted laser desorption ionization time-of-flight/time-of-flight mass spectrum (MALDI-TOF/TOF-MS, UltrafleXtreme, Bruker Daltonics; Bremen, Germany). The lyophilized N-glycans were resuspended in 5 µl of the eluting solution (methanol: H_2O=1:1), and 2 µl of the N-glycans solution was spotted on an MTP AnchorChip sample target and air-dried. Then, 2 µl of 20 mg/ml 2,5-dihydroxybenzoic acid (DHB) was spotted to recrystallize the N-glycans. Ionization was performed by irradiation of a nitrogen laser (337 nm) operating at 1 kHz and mass calibration was performed using peptide calibration standards (250 calibration points; Bruker). Measurements were taken in positive and reflectron mode. Subsequently, the intense ions from MS spectra were selected and subjected to MS/MS with higher energy (80–90%). FlexAnalysis software (Bruker Daltonics) was used to take the original data of each peak, and representative MS spectra of N-glycans with signal-to-noise ratios >6 were selected and annotated by using the GlycoWorkbench program. Relative intensity was calculated by dividing the intensity of a given N-glycan peak intensity by the sum of total peaks intensity.

Results

N-glycans profiles from the isolated glycoproteins in pooled saliva. In order to detect the N-glycans recognized by PHA-E+L on salivary glycoproteins, the glycoproteins were isolated from the pooled saliva of HV (n=66), BB (n=25), BC-I (n=25), and BC-II (n=25) by PHA-E+L-magnetic particle conjugates and released by the enzymolysis of PNGase F, respectively. Then, isolated N-glycans were characterized by MALDI-TOF/TOF-MS. The basic characteristics of healthy volunteers and patients are summarized in Table 1. The proposed structures of identified N-linked glycans and MS profiles from the pooled saliva are shown in Figure 2. A total of 19, 25, 21, and 26 N-glycan peaks from HV, BB, BC-I, and BC-II groups were identified and annotated with proposed structures, respectively (Table 2 and Figure 3). Of these, 10 N-glycan peaks (e.g., m/z 1581.5823 $(Man)_7(GlcNAc)_2$, 1776.6291 $(NeuAc)_1(GalNAc)_1(GlcNAc)_1+(Man)_3(GlcNAc)_2(Fuc)_1$, and 1834.6709 $(Fuc)_1(GalNAc)_1(GlcNAc)_2+(Man)_3(GlcNAc)_2(Fuc)_1$) were presented in all groups but with

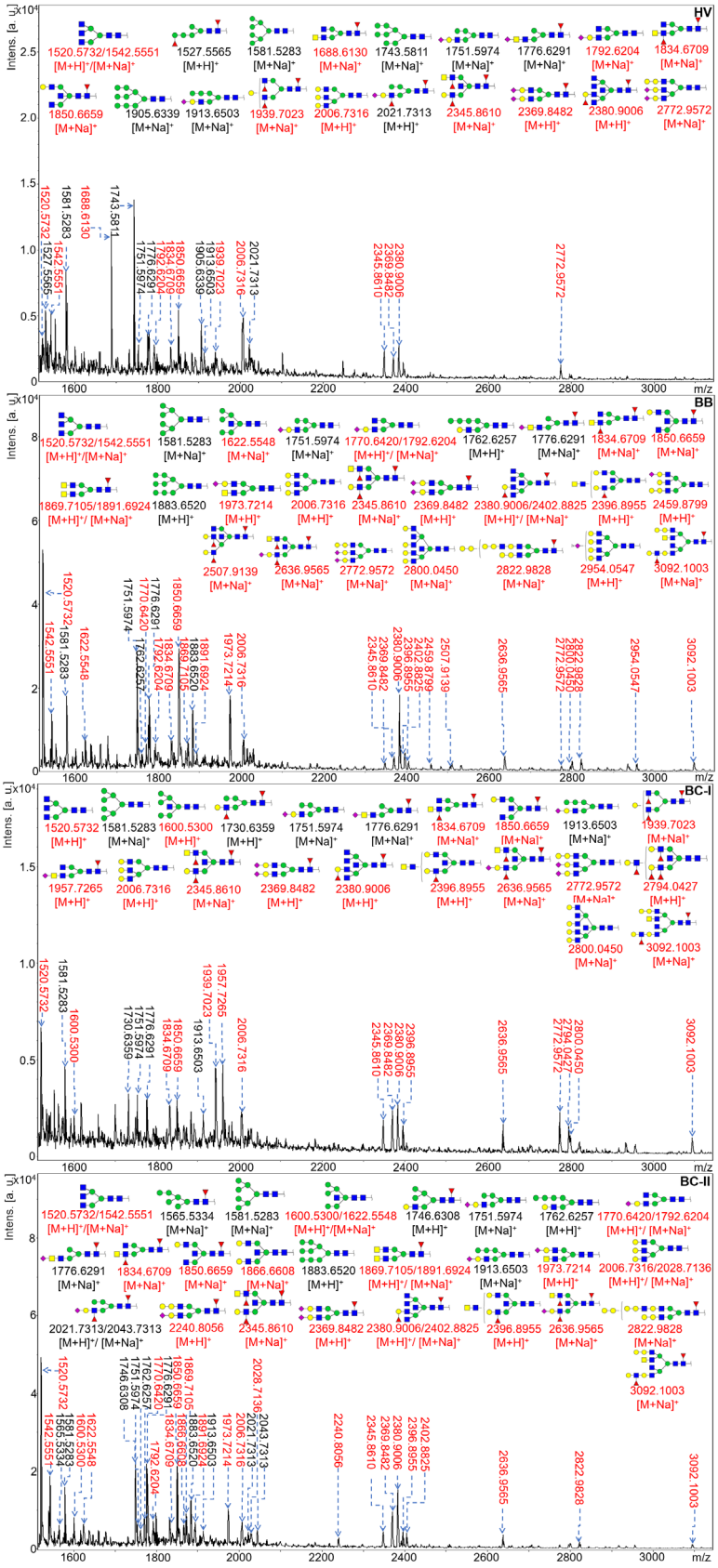


Figure 2. MALDI-TOF/TOF-MS spectra of salivary N-glycan peaks from HV, BB, BC-I, and BC-II groups. Detailed glycan structures were analyzed using the GlycoWorkbench software. Proposed structures and their m/z values are shown for each peak. The N-glycans recognized by PHA-E+L are labeled in red words. GlcNAc, blue square; GalNAc, yellow square; Gal, yellow circle; Fuc, red triangle; NeuAc, purple diamond.

Table 2. Proposed structures for 38 N-glycan signals detected in the present study.

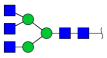
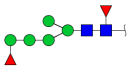
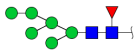
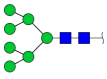
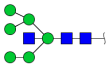
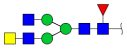
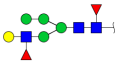
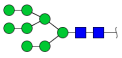
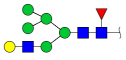
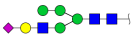
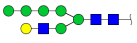
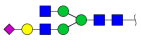
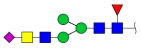
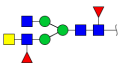
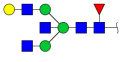
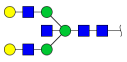
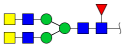
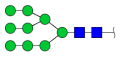
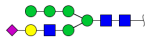
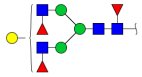
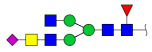
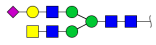
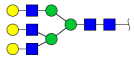
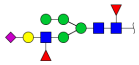
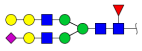
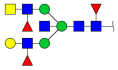
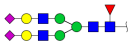
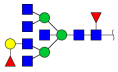
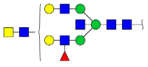
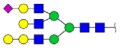
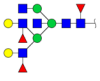
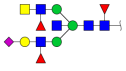
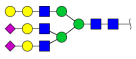
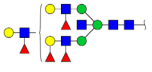
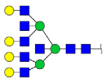
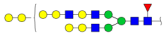
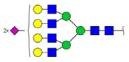
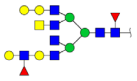
No.	Calculated m/z	Glycan structure[a]	Charge	Relative Intensities of N-Glycans (%)			
				HV	BB	BC-I	BC-II
1.	1520.5732 1542.5551		[M+H]+ [M+Na]+	2.9 3.2	18.5 4.3	11.9 0	15.7 5.5
2.	1527.5565		[M+H]+	5.2	0	0	0
3.	1565.5334		[M+Na]+	0	0	0	1.3
4.	1581.5283		[M+Na]+	8.8	5.4	8.5	4.3
5.	1600.5300 1622.5548		[M+H]+ [M+Na]+	0 0	0 2.3	4.1 0	1.7 1.8
6.	1688.6130		[M+Na]+	10.2	0	0	0
7.	1730.6359		[M+H]+	0	0	5.0	0
8.	1743.5811		[M+Na]+	12.5	0	0	0
9.	1746.6308		[M+H]+	0	0	0	6.7
10.	1751.5974		[M+Na]+	2.6	9.1	5.1	1.6
11.	1762.6257		[M+H]+	0	1.3	0	1.2
12.	1770.6420 1792.6204		[M+H]+ [M+Na]+	0 2.6	1.8 1.5	0 0	2.4 1.4
13.	1776.6291		[M+Na]+	3.5	4.6	4.9	6.5
14.	1834.6709		[M+Na]+	2.4	2.1	4.2	2.3
15.	1850.6659		[M+Na]+	4.2	8.9	4.4	6.3
16.	1866.6608		[M+Na]+	0	0	0	1.7
17.	1869.7105 1891.6924		[M+H]+ [M+Na]+	0 0	2.0 1.2	0 0	2.4 1.7
18.	1883.6520 1905.6339		[M+H]+ [M+Na]+	0 3.8	4.3 0	0 0	3.6 0
19.	1913.6503		[M+Na]+	1.9	0	3.2	1.1
20.	1939.7023		[M+Na]+	1.9	0	8.3	0
21.	1957.7265		[M+H]+	0	0	7.4	0
22.	1973.7214		[M+H]+	0	5.5	0	2.7
23.	2006.7316 2028.7136		[M+H]+ [M+Na]+	4.5 0	2.4 0	4.1 0	1.5 1.0
24.	2021.7313 2043.7313		[M+H]+ [M+Na]+	2.5 0	0 0	0 0	1.0 1.2
25.	2240.8056		[M+H]+	0	0	0	0.6
26.	2345.8610		[M+Na]+	1.8	0.5	2.4	1.0
27.	2369.8482		[M+H]+	1.5	0.9	3.2	2.1
28.	2380.9006 2402.8825		[M+H]+ [M+Na]+	2.1 0	4.0 0.5	3.9 0	3.5 0.9
29.	2396.8955		[M+H]+	0	1.0	5.7	0.9
30.	2459.8799		[M+H]+	0	0.4	0	0
31.	2507.9139		[M+Na]+	0	0.4	0	0
32.	2636.9565		[M+Na]+	0	0.8	4.6	0.7
33.	2772.9572		[M+Na]+	0.8	0.3	2.8	0
34.	2794.0427		[M+H]+	0	0	1.8	0

Table 2. *Continued ...*

No.	Calculated m/z	Glycan structure[a]	Charge	Relative Intensities of N-Glycans (%)			
				HV	BB	BC-I	BC-II
35.	2800.0450		$[M+Na]^+$	0	0.5	1.0	0
36.	2822.9828		$[M+Na]^+$	0	0.5	0	0.3
37.	2954.0547		$[M+H]^+$	0	0.3	0	0
38.	3092.1003		$[M+Na]^+$	0	0.4	0.9	0.2

Notes: [a]Monosaccharides are represented according to MS-tools from the GlycoWorkbench software (GlcNAc, blue square; Man, green circle; Gal, yellow circle; GalNAc, yellow square; Fuc, red triangle; NeuAc, purple diamond; 0, not detected in the sample). Red words indicate the bisecting GlcNAc, bi-antennary N-glycans, tri-, or tetra-antennary complex-type N-glycans which recognized by PHA-E+L identified in the present study

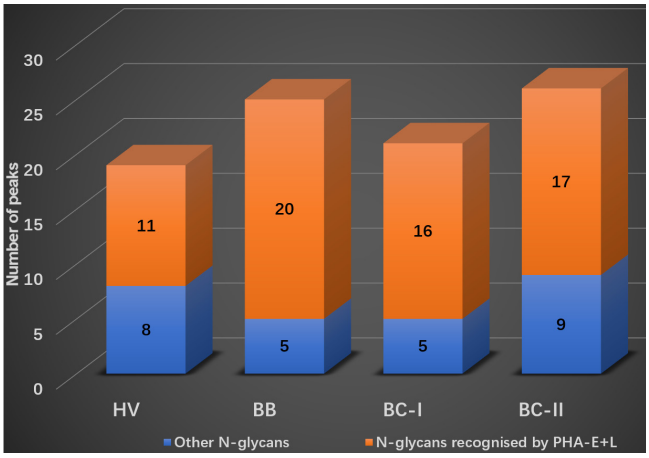


Figure 3. The number of N-glycan peaks released from PHA-E+L-isolated glycoproteins in HV, BB, BC-I and BC-II groups.

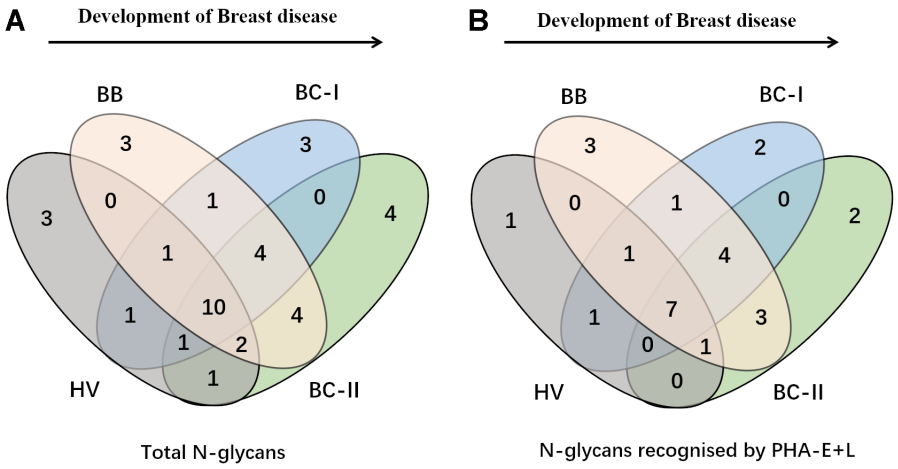


Figure 4. Cross-correlation of the salivary A) Total N-glycan peaks; B) N-glycan peaks recognized by PHA-E+L from groups of HV, BB, BC-I, and BC-II presented in the Venn diagram.

different intensities (Figure 4A). Besides, four N-glycan peaks (m/z 1600.5300/1622.5548 $(GlcNAc)_1(Man)_3$+$(Man)_3(GlcNAc)_2$, 2396.8955 $(Fuc)_1$ $(Gal)_2(GalNAc)_1(GlcNAc)_4$+$(Man)_3(GlcNAc)_2$,2636.9565$(NeuAc)_1(Fuc)_2(Gal)_1(GalNAc)_1(GlcNAc)_3$+$(Man)_3(GlcNAc)_2(Fuc)_1$, and 3092.1003 $(Fuc)_1(Gal)_4(GalNAc)_1(GlcNAc)_5$+$(Man)_3(GlcNAc)_2(Fuc)_1$) existed in BB, BC-I, and BC-II group. Notably, there were 3 N-glycan peaks (m/z 1527.5565 $(Fuc)_1(Man)_2$+$(Man)_3(GlcNAc)_2(Fuc)_1$, 1688.6130 $(GalNAc)_1(GlcNAc)_2$+$(Man)_3(GlcNAc)_2(Fuc)_1$, and 1743.5811 $(Man)_8(GlcNAc)_2$) in HV group merely, 3 N-glycan peaks (m/z 2459.8799 $(NeuAc)_1(Gal)_4(GlcNAc)_3$+$(Man)_3(GlcNAc)_2$, 2507.9139 $(Fuc)_2(Gal)_2(GlcNAc)_4$+$(Man)_3(GlcNAc)_2(Fuc)_1$, and 2954.0547 $(NeuAc)_2(Gal)_4(GlcNAc)_4$+$(Man)_3(GlcNAc)_2$) existed in BB group only, 3 N-glycan peaks (m/z 1730.6359 $(Fuc)_1(Gal)_1(GlcNAc)_1(Man)_1$+$(Man)_3(GlcNAc)_2(Fuc)_1$, 1957.7265 $(NeuAc)_1(GalNAc)_1(GlcNAc)_2$+$(Man)_3(GlcNAc)_2(Fuc)_1$, and 2794.0427 $(Fuc)_4(Gal)_3(GlcNAc)_4$+$(Man)_3(GlcNAc)_2$) existed in BC-I group only, and 4 N-glycan peaks (m/z 1565.5334 $(Man)_6(GlcNAc)_2(Fuc)_1$, 1746.6308 $(Gal)_1(GlcNAc)_1(Man)_2$+$(Man)_3(GlcNAc)_2(Fuc)_1$, 1866.6608 $(Gal)_2(GlcNAc)_3$+$(Man)_3(GlcNAc)_2$, and 2240.8056 $(NeuAc)_1(Gal)_3(GlcNAc)_2$+$(Man)_3(GlcNAc)_2(Fuc)_1$) existed in BC-II group merely.

Alterations of salivary N-glycans recognized by PHA-E+L among HV, BB, BC-I, and BC-II group. N-glycans with the specific structures (bisecting GlcNAc, bi-antennary

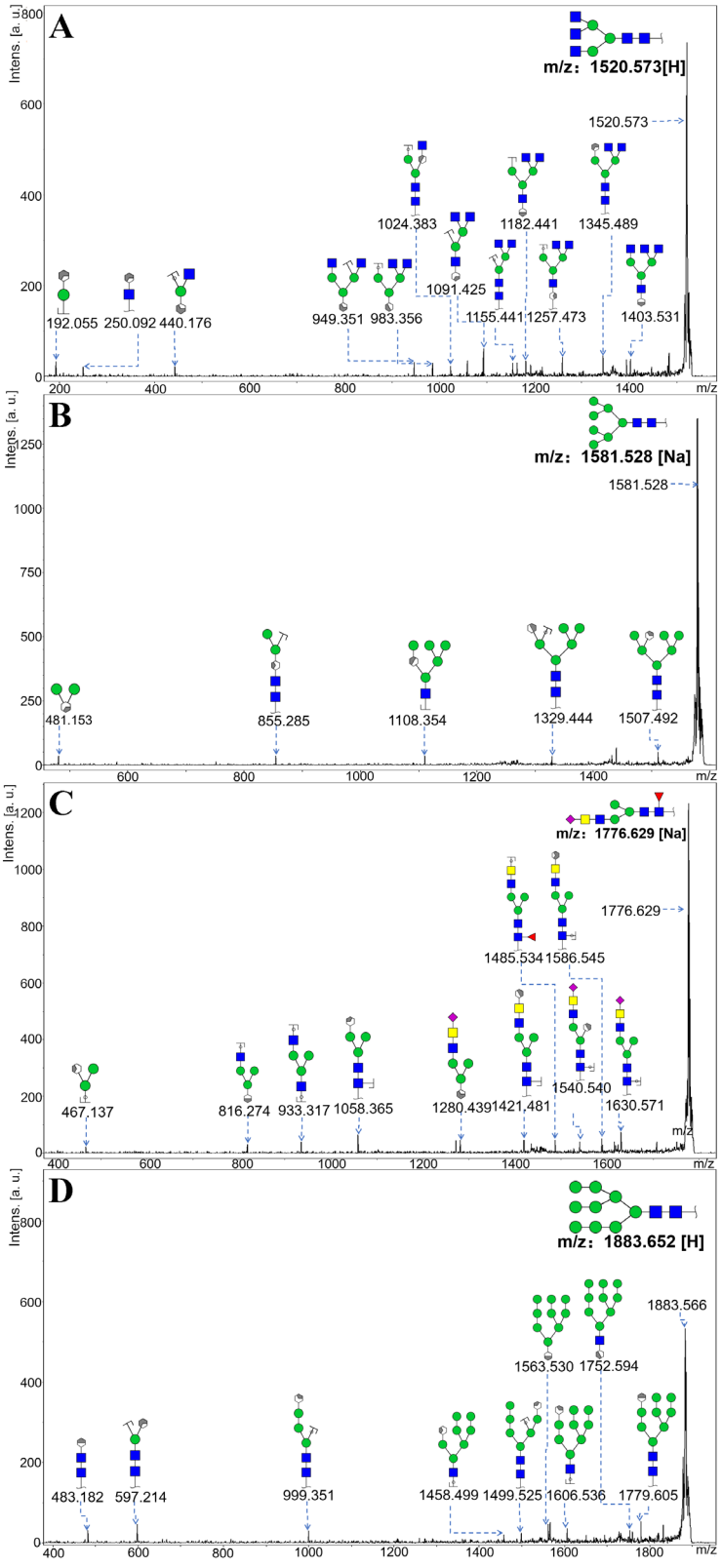


Figure 5. MALDI-TOF/TOF-MS/MS analyzing the N-linked glycan precursor ion from MS spectra. The Four N-glycan peaks A) m/z 1520.573; B) m/z 1581.528; C) m/z 1776.629 and D) m/z 1883.652 subjected to MS/MS analysis.

N-glycans, tri-, and tetra-antennary complex-type N-glycans) could be recognized by PHA-E+L. And there were 11, 20, 16, and 17 N-glycan peaks recognized by PHA-E+L that were identified and annotated in groups of HV, BB, BC-I, and BC-II, respectively (Table 2 and Figure 3). Among these, 7 N-glycan peaks recognized by PHA-E+L (e.g., m/z 1501.529 $(Gal)_1(GlcNAc)_2+(Man)_3(GlcNAc)_2$, 2129.811 $(GalNAc)_2$ $(GlcNAc)_4+(Man)_3(GlcNAc)_2$, and 3188.115 $(Gal)_6(GlcNAc)_4+(Man)_3(GlcNAc)_2(Fuc)_1$) were presented in both groups but with different intensities (Figure 4B). Besides, four N-glycan peaks recognized by PHA-E+L (m/z 1600.5300/1622.5548 $(GlcNAc)_1(Man)_3+(Man)_3(GlcNAc)_2$, 2396.8955 $(Fuc)_1(Gal)_2$ $(GalNAc)_1(GlcNAc)_4+(Man)_3(GlcNAc)_2$, 2636.9565 $(NeuAc)_1(Fuc)_2(Gal)_1(GalNAc)_1$ $(GlcNAc)_3+(Man)_3(GlcNAc)_2(Fuc)_1$, and 3092.1003 $(Fuc)_1(Gal)_4(GalNAc)_1(GlcNAc)_5+(Man)_3(GlcNAc)_2(Fuc)_1$) existed in BB, BC-I, and BC-II group. More importantly, there was 1 N-glycan peak recognized by PHA-E+L (m/z 1688.6130 $(GalNAc)_1(GlcNAc)_2+(Man)_3(GlcNAc)_2(Fuc)_1$) in HV group merely, 3 N-glycan peaks recognized by PHA-E+L (m/z 2459.8799 $(NeuAc)_1(Gal)_4$ $(GlcNAc)_3+(Man)_3(GlcNAc)_2$, 2507.9139 $(Fuc)_2(Gal)_2(GlcNAc)_4+(Man)_3(GlcNAc)_2(Fuc)_1$, and 2954.0547 $(NeuAc)_2(Gal)_4(GlcNAc)_4+(Man)_3(GlcNAc)_2$) existed in BB group only, 2 N-glycan peaks recognized by PHA-E+L (m/z 1957.7265 $(NeuAc)_1(GalNAc)_1(GlcNAc)_2+(Man)_3(GlcNAc)_2(Fuc)_1$ and 2794.0427 $(Fuc)_4(Gal)_3(GlcNAc)_4+(Man)_3(GlcNAc)_2$) existed in BC-I group only, and 2 N-glycan peaks recognized by PHA-E+L (m/z 1866.6608 $(Gal)_2(GlcNAc)_3+(Man)_3(GlcNAc)_2$ and 2240.8056 $(NeuAc)_1$ $(Gal)_3(GlcNAc)_2+(Man)_3(GlcNAc)_2(Fuc)_1$) existed in BC-II group merely.

The MS/MS analysis of specific N-glycan structures. The MS/MS analysis was further performed in order to determine the exact N-glycan structures. The glycosidic cleavages that generated B-, Y-, C-, and Z-type ions, and cross ring cleavages that generated A- and X-type ions were observed. The MS/MS spectra of 4 randomly selected N-glycan precursor ions with m/z 1520.573, 1581.528, 1776.629, and 1883.652 are illustrated in Figure 5.

Discussion

Protein N-glycosylation is one of the most ubiquitous post-translational modifications and contains high mannose, hybrid, and complex type sugar chains. Alternations in N-glycans have attracted particular interest because of their important roles in cellular mobility, signaling, metastatic capability, and immune properties

[34]. N-glycans are associated with angiogenesis, which is necessary for the progression and metastasis of breast and other solid tumors [35]. The expression of complex β-1,6 branched N-linked glycans are increased to promote tumor migration, invasion, and metastasis [36]. N-linked glycosylation alternation of 2-deoxyglucose is contributed to the antiproliferative action in breast cancer cells [37]. N-glycan structures are associated with breast malignancies and reflected the potential events underlying tumor progression, suggesting that N-glycans may be used as predictive or prognostic biomarkers to improve the diagnostic and prognostic stratification of patients with breast disease [38]. Serum tumor marker CA 15.3, as a carbohydrate antigen, is a powerful predictor of survival in primary breast cancer [39]. The prevalence of tumor-associated N-glycan polylactosamine in primary and metastatic breast cancer tissues could be used as a potential prognostic biomarker [40]. In our previous study, the expression levels of glycan structures recognized by PHA-E+L were significantly increased in BB, BC-I, and BC-II compared with HV [30].

There were 24 salivary N-glycan peaks identified in HV, BB, BC-I, and BC-II in our previous study, of these, there were 19 galactosylated N-glycans [31]. This study further investigated N-glycan profiles of HV, BB, BC-I, and BC-II salivary glycoproteins isolated by the PHA-E+L-magnetic particle conjugates. In this study, there were 38 salivary N-glycan peaks identified in HV, BB, BC-I, and BC-II, of these, there were 26 N-glycans recognized by PHA-E+L. There was no identical salivary N-glycan peak in the present study compared with the previous study, and this study could provide more specific glycan structure information based on selective glycoprotein fractions from saliva by lectin-magnetic particle conjugates [41]. Saliva from breast cancer patients expresses various types of N-glycans. The specific N-glycans that appear during breast cancer may reflect the progress of the disease and are expected to be used as a biomarker.

N-glycans branches are usually related to more aggressive tumor behavior, such as enhanced migration, invasion, and metastatic potential [42]. Expression levels of biantennary N-glycan chains as well as α-2,3- linked sialic acid-modified N-glycans are often decreased in sera of patients with breast cancer compared to healthy controls [43]. Changes in several N-glycan patterns are caused by either a primary tumor or from other organs in response to a neoplastic process [44]. PHA-E+L recognizes bisecting GlcNAc, bi-antennary N-glycans, tri-, and tetra-antennary complex-type N-glycans. In this study, a total of 11, 20, 16, and 17 N-linked glycans recognized by PHA-E+L were identified and annotated from the pooled salivary samples of HV, BB, BC-I, and BC-II, respectively. The proportion of N-linked glycans recognized by PHA-E+L was increased in BB (61%), BC-I (70.7%), and BC-II (58.3%) compared with HV (38.1%). Interestingly, the tri-antennary N-glycans peak (m/z 1520.5732) was the highest in the N-linked glycan profiles of BB (18.5%), BC-I (11.9%), and BC-II (15.7%), but it decreased apparently in the N-linked glycan profiles of HV (2.9%). The glycan peaks of m/z 1520.5732 ($[M+H]^+$) and 1542.5551 ($[M+Na]^+$) with the same structure, and their total relative intensity was also the highest in the N-linked glycan profiles of BB (22.8%), BC-I (11.9%), and BC-II (21.2%), but it decreased apparently in the N-linked glycan profiles of HV (6.1%). Notably, the bi-antennary N-glycans peak (m/z 1688.6130) was the highest in the N-linked glycan profiles recognized by PHA-E+L of HV (10.2%), however, it disappeared in BB, BC-I, and BC-II. The expression of N-glycans in different stages of breast cancer and healthy volunteers is not identical, which may be due to the influence of disease state on glycosylation. Bisecting GlcNAc has unique characteristics different from other GlcNAc branches, it is usually not elongated, whereas other GlcNAc branches in N-glycan are further modified with galactose, sialic acid, fucose, and others [45]. Bisecting GlcNAc is involved in diseases, such as cancer and Alzheimer's disease [46]. The stability of adherens junctions is improved by bisecting N-linked glycans on E-cadherin, which leads to tumor suppression [47]. The signaling activity of integrins is modulated by the expression of branched versus bisected N-glycans on integrins [48].

Protein N-glycosylation is a diversified process that involves not only a series of genes or gene products but also extracellular signaling and the intracellular environment [35]. N-glycans are assembled in the endoplasmic reticulum (ER). The quality control mechanism is involved in ensuring that only properly folded proteins can leave the ER, while misfolded proteins can be retained and eventually degraded [49]. However, tumor cells always undergo abnormal glycosylation pathways due to the incomplete synthesis and neo-synthesis processes [50]. In this study, there was 1 N-glycan peak recognized by PHA-E+L (m/z 1688.6130) in HV group merely, 3 N-glycan peaks recognized by PHA-E+L (m/z 2459.8799, 2507.9139, and 2954.0547) existed in BB group only, 2 N-glycan peaks recognized by PHA-E+L (m/z 1957.7265 and 2794.0427) existed in BC-I group only, and 2 N-glycan peaks recognized by PHA-E+L (m/z 1866.6608 and 2240.8056) existed in BC-II group merely. Complex types of N-glycans are prone to abnormal glycosylation in the process of diseases because of their many branches. In the breast cancer cells, the expression of ST6GAL1 participated in the reduction of cell-cell adhesion and enhanced invasion capacity [51]. N-glycosylation can be used as a metabolic master regulator of cell proliferation and arrest through modulation of receptor tyrosine kinases (RTK) glycosylation [52].

Our analyses have two limitations. First, we have only investigated the specific N-glycans recognized by PHA-E+L in the saliva of breast cancer patients. Larger and more replicable trials need to be researched further to evaluate their clinical value. Another limitation of our study is that our investigation has not referred to the mechanism leading to the specific N-glycans in the saliva of breast cancer.

In conclusion, the present study systematically compared the alternations of N-glycans from the salivary proteins isolated by PHA-E+L-magnetic particle conjugates among HV, BB, BC-I, and BC-II. Totally, there were 11, 20, 16, and 17 N-linked glycans recognized by PHA-E+L were identified and annotated from the pooled salivary samples of HV, BB, BC-I, and BC-II, respectively. There were 3 N-glycan peaks recognized by PHA-E+L (m/z 2459.8799, 2507.9139, and 2954.0547) existed in BB group only, 2 N-glycan peaks recognized by PHA-E+L (m/z 1957.7265 and 2794.0427) existed in BC-I group only, and 2 N-glycan peaks recognized by PHA-E+L (m/z 1866.6608 and 2240.8056) existed in BC-II group merely. In the present study, the N-glycans MALDI-TOF/TOF-MS analysis represented a comprehensive assessment of the tumor N-glycans recognized by PHA-E+L from the salivary proteins among HV, BB, BC-I, and BC-II.

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