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Research Paper

Salivary Glycopatterns as Potential Biomarkers for Screening of Early-Stage Breast Cancer

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ABSTRACT

Objective: We systematically investigated and assessed the alterations of salivary glycopatterns and possibility as biomarkers for diagnosis of early-stage breast cancer.
Design: Alterations of salivary glycopatterns were probed using lectin microarrays and blotting analysis from 337 patients with breast benign cyst or tumor (BB) or breast cancer (I/II stage) and 110 healthy humans. Their diagnostic models were constructed by a logistic stepwise regression in the retrospective cohort. Then, the performance of the diagnostic models were assessed by ROC analysis in the validation cohort. Finally, a double-blind cohort was tested to confirm the application potential of the diagnostic models.
Results: The diagnostic models were constructed based on 9 candidate lectins (e.g., PHA-E + L, BS-I, and NPA) that exhibited significant alterations of salivary glycopatterns, which achieved better diagnostic powers with an AUC value >0.750 ($p < 0.001$) for the diagnosis of BB (AUC: 0.752, sensitivity: 0.600, and specificity: 0.835) and I stage breast cancer (AUC: 0.755, sensitivity: 0.733, and specificity: 0.742) in the validation cohort. The diagnostic model of I stage breast cancer exhibited a high accuracy of 0.902 in double-blind cohort.
Conclusions: This study could contribute to the screening for patients with early-stage breast cancer based on precise alterations of salivary glycopatterns.

1. Introduction

Breast cancer (BC) is one of the commonly diagnosed malignancies worldwide, threatening to female health seriously. According to the last statistics, overall BC incidence among women in China presents a significant upward trend and age-adjusted mortality remains stubbornly high (Chen et al., 2016). Whereas a stable trend is observed in incidence and mortality has been continuously declining for the past decade in America (Siegel et al., 2015; Smith et al., 2016; Torre et al., 2016). The estimates from US National Cancer Institute-supported CISNET (Kalager et al., 2010) and UK (Marmot et al., 2013) suggest that at least 20% of the observed reduction are benefit from mammographic screening, showing the necessity of early detection for breast lumps. However, the high cost burden and adverse effects caused by over-diagnosis or false positives limit the implementation of regular screening, particularly in women younger than 50 years, in low and middle income countries (Corbex et al., 2012). The recent studies therefore, are targeted in exploring molecular changes that occur in an early phase of BC in order to discover biomarkers to aid routine clinical decisions.

Currently, the definitive diagnosis in BC requires biopsy and histopathology, such the best-validated markers are all tissue-based and include ER, PR, HER-2, uPA, and PAI-1. Assay of ER, PR, and HER-2 is now mandatory for all newly diagnosed BC patients. The measurement of uPA and PAI-1 is not presently in widespread clinical use, mainly due to the requirement of a minimum amount of fresh or freshly frozen tissue. Although, the use of serial levels of serum tumor markers is potentially useful in deciding whether to persist in using a particular type of therapy, terminate its use, or switch to an alternative therapy, the available blood-based biomarkers are of no value in the early diagnosis of BC (Sturgeon et al., 2008). Thus, the discoveries of markers in body fluid for early breast tumor diagnosing progressed slowly. Recently, there are numerous proteomics-based reports to attempt to find novel protein markers. Such, the BC patients and healthy controls were compared for differences in serum proteome via SELDI-TOF-MS, and identified that apolipoprotein CI and complement C3a desArg were up-regulated in sera of patient set (Chung et al., 2014; Opstal-van Winden et al., 2011). In order to estimate the discriminatory power of putative markers sufficiently, the investigators added benign disease controls comprising of breast fibroadenoma patients to study sets (Meng et al.,

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2011; Yang et al., 2015). Furthermore, the diagnostic accuracy of potential serum biomarkers were also evaluated with the multivariate logistic regression analysis (Lumachi et al., 2016).

Despite the late start of glycomics-related researches, significant change of plasma glycome in normal and sick women has been discovered by Mass Spectrometry (MS) and UPLC, such as an increase in sialyl Lewis x (sLe^x) structure, branching glycans and a decrease in biantennary core-fucosylated glycans (de Leoz et al., 2011; Saldova et al., 2014). The potential of aberrant glycome has been recognized serving as early sentinel for cancer detection (Adamczyk et al., 2012; Drake et al., 2010). Additional opportunities to profile glycosylation modifications of proteins was provided by lectin-based approaches, such as lectin microarray—a convenient high throughput screening approach (Kuno et al., 2005). Rely on the specific combination with glycans of glycoconjugates, this technology was used to analyze the variation of protein glycosylation or cell surface glycopatterns for different types of malignancies (Syed et al., 2016) including breast cancer (Guo et al., 2017; Zhou et al., 2015).

As a complex oral fluid originating from salivary glands, saliva is composed of multitudinous secretory proteins, electrolytes and other substances. Salivary composition and protein concentration depend on the body situation of individual, and are modified in pathological conditions. At present, saliva with the advantages in collection and storage has become a resource for assessment pathological situations in human and contributed to the development of cancer-related biomarkers for clinical application (Kaczor-Urbanowicz et al., 2017; Marti-Alamo et al., 2012; Nunes et al., 2015; Wang et al., 2017). The significant protein markers, such as VEGF, EGF, CEA and HER2, were discovered in saliva of patients with BC (Brooks et al., 2008; Streckfus et al., 2000). Similarly, the autoantibodies against HER2, MUC1 and ATP6AP1 were also indicated in saliva of patients with BC (Arif et al., 2015; Laidi et al., 2016). Besides salivary proteins, mRNA (Zhang et al., 2010), free amino acids (Cheng et al., 2015) and polyamines including their acetylated derivatives (Takayama et al., 2016) were investigated through transcriptomic and metabonomic technologies in order to present a new examination index. Our previous study revealed the sex/age-associated differences in the glycopatterns of healthy human salivary glycoproteins. Furthermore, healthy elderly individuals are found to have stronger resistance to influenza A virus (IAV) partly by presenting more terminal α2-3/6-linked sialic acid residues in their saliva to inhibit the activities of IAV and provided the evidence that elderly individuals with chronic diseases, such as diabetes and liver disease, might be more susceptible to avian influenza viruses due to the decreased expression of terminal α2-3-linked sialic acids in their saliva (Qin et al., 2013; Zhong et al., 2015).

The purpose of this study was to investigate the correlation of alterations in salivary protein glycosylation related to BC and compare different or similar alterations of protein glycopatterns between healthy volunteers, patients with benign breast cyst or tumor and BC patients (stage I/II), and assess the possibility of salivary-based protein glycopatterns as biomarkers for screening of early-stage BC as well as facilitates the discovery of biomarkers for BC during its early stages based on precise alterations of salivary glycopatterns.

2. Materials and Methods

2.1. Retrospective Cohort

The collection and use of female whole saliva for the research presented here were approved by the Ethical Committee of Northwest University (Xi'an, China), the first Affiliated Hospital of Xi'an Jiaotong University. Written informed consent was received from participants for the collection of their whole saliva. This study was conducted in accordance with the ethical guidelines of the Declaration of Helsinki.

Exclusion criteria of study population covered smoking, pregnancy, lactation, diabetes and hepatopathy. In total, 259 eligible subjects were recruited between September 2014 and October 2015, healthy volunteers (HV, n = 66) and female patients with breast benign cyst or tumor (BB, n = 65), and female patients diagnosed with early (stage I, II) BC (BC-I/BC-II, n = 66/62) (Xu et al., 2015). The diagnoses for all enrolled patients were histopathologically confirmed. The above HV and patients were used in the retrospective cohort to construct the diagnostic model. A summary of the patient and HV clinical characteristics and the detailed information of the study cohorts with respect to social behavior, medical and gynecological history were provided in Table 1 and Supplementary Table S1, respectively. The patients who received preoperative radiotherapy, chemotherapy, or chemoradiotherapy were excluded from the study.

The collection of human whole saliva was performed in accordance with approved guidelines. The collection protocol has been described in previous reports (Bandhakavi et al., 2011; Qin et al., 2013; Ramachandran et al., 2006). Briefly, unstimulated saliva was collected between 3 p.m. and 5 p.m. at least 2 h after the last intake of food. The mouth was rinsed three times with physiological saline immediately before the beginning of the collection and then, with the head down and mouth slightly open, saliva was allowed to drip from the lower lip

Table 1
Baseline characteristics of healthy volunteers and patients with benign breast cyst, early-stage breast cancer.

	Retrospective cohort	Validation cohort	Double-blind cohort
Number of study population			
HV/HH	66	31	13
BB	65	30	21
BC-I	66	30	11
BC-II	62	36	12
BC-III	–	–	4
Age (mean ± SD)			
HV/HH	48.37 ± 7.13	48.30 ± 7.27	40.69 ± 6.30
BB	40.93 ± 10.93	42.26 ± 12.09	39.14 ± 11.11
BC-I	54.47 ± 8.46	55.59 ± 10.51	48.55 ± 15.00
BC-II	47.60 ± 10.23	49.79 ± 11.31	50.50 ± 11.42
BC-III	–	–	49.25 ± 12.28
Patient characteristics			
Breast cancer			
Histologic type			
Noninvasive carcinoma	2	4	0
Infiltrative specific carcinoma	6	3	0
Infiltrative non-specific carcinoma	117	58	27
Missing	3	1	0
Grade			
G1	10	2	1
G2	24	17	13
G3	80	33	10
Missing	6	7	3
Tumor size			
$T \leq 2$ cm	73	37	15
$T > 2$ cm	55	29	12
Lymph node involvement			
Positive	28	16	10
Negative	100	50	17
Estrogen/progesterone receptor			
Positive	85	45	14
Negative	41	17	5
Missing	2	4	8
HER2 overexpression			
Positive	70	45	15
Negative	56	17	4
Missing	2	4	8

HV/HH: healthy volunteer/healthy human; BD: breast disease; BB: benign breast cyst or tumor; BC-I: I stage of breast cancer; BC-II: II stage of breast cancer; HER2: human epidermal growth factor receptor 2.

into a weighed, dried and sterile plastic test tube within 5 min. Whole saliva (about 1 mL) was collected and placed on ice. Protease Cocktail Inhibitor (1 μL/mL of whole saliva) was added to the saliva immediately after collection to minimize protein degradation. Whole saliva was then centrifuged at 12000 rpm at 4 °C for 10 min to remove the insoluble materials. The supernatant of each sample was collected and filtered with a filter pore size of 0.45 μm. The filtered supernatant was collected and stored at −80 °C.

2.2. Validation Cohort

To evaluate the predictive value of the models established in the retrospective study described above, further, an additional cohort (n = 127) of HV (n = 31) and patients with BB (n = 30), BC-I (n = 30), and BC-II (n = 36) were investigated from October 2015 to November 2016 at the same hospital. The procedures and strategies were the same as those described above. And the validation results were compared with clinical information to evaluate the diagnosis power for BC. A summary of the patient and HV clinical characteristics is also provided in Table 1.

2.3. Double-Blind Cohort

To properly confirm the availability of the diagnostic models, a double-blind cohort (n = 61) was enrolled from March 2017 to May 2017 at the same hospital. Whole saliva of subjects were collected prior to the diagnosis/staging-related examinations in a clinical setting (Xu et al., 2015). An independent test was performed in double-blind cohort. Finally, the results of the double-blind test were compared with clinical final diagnosis.

2.4. Lectin Microarrays

The salivary proteins were labeled with Cy3 fluorescent dye (GE Healthcare, Buckinghamshire, UK) and purified using Sephadex G-25 columns according to the manufacturer's instructions. Subsequently, the Cy3-labeled salivary proteins were quantified and stored at −20 °C in the dark until use. The lectin microarrays were produced as previously described (Qin et al., 2013; Zhong et al., 2015). Briefly, 37 lectins with different binding preferences covering N- and O-linked glycans were spotted on homemade epoxysilane-coated slides. Each lectin was spotted in triplicate per block, with quadruplicate blocks on one slide. After immobilization, the slides were blocked with blocking buffer containing 2% BSA in 1 × PBS (0.01 mol/L phosphate buffer containing 0.15 mol/L NaCl, pH 7.4) for 1 h and rinsed twice with 1 × PBS. Then the blocked slide was incubated with Cy3-labeled salivary proteins diluted in 0.6 mL of incubation buffer for 3 h at room temperature. After incubation, the microarray was rinsed twice with 1× PBST (0.2% Tween 20 in 1× PBS) for 5 min each, and finally rinsed in 1× PBS before drying. The microarrays were scanned using a Genepix 4000B confocal scanner (Axon Instruments, Foster City, Calif., USA) set at 70% photomultiplier tube and 100% laser power. The acquired images were analyzed at 532 nm for Cy3 detection by Genepix 3.0 software.

2.5. Lectin Blotting Analysis

The pooled salivary proteins from HV, BB, BC-I, and BC-II subjects of the retrospective cohort were analyzed by SDS-PAGE and subsequently lectin blotting as previously described (Qin et al., 2013; Zhong et al., 2015). Briefly, For SDS-PAGE, samples were boiled for 4 min at 100 °C mixed with 5 × loading buffer, and run on a 10% polyacrylamide resolving gel and a 3% stacking gel. Molecular mass standards (Thermo Scientific, Waltham, USA) were run with all gels. Some gels were then stained directly with alkaline silver. For lectin blotting, the proteins in gels were then transferred to a PVDF membrane (Immobilon-P; Millipore Corp., Bedford, MA, U.S.A.) with a wet transfer unit (Hoefer Scientific) for 1.5 h at 32 mA. After transfer, the membranes were washed twice with TTBS (150 mM NaCl, 10 mM Tris-HCl, 0.05% v/v Tween20, pH 7.5) and then blocked for 1 h with Carbo-Free Blocking Solution (Vector, Burlingame, CA) at room temperature. The membranes were then washed again and incubated with Cy5 (GE Healthcare, Buckinghamshire, UK) labeled lectins (2 μg/mL in Carbo-Free Blocking Solution) with gentle shaking overnight at 4 °C in the dark. The membranes were then washed twice each for 10 min with TTBS and scanned by red fluorescence channel (635 nm excitation/650LP emission) with the voltage of 800 PMT using a phosphor imager (Storm 840, Molecular Dynamics).

2.6. Statistical Analysis

The original data of microarrays need to be normalized by median normalization method for minimizing the possible systematic variation. The average background was subtracted, and values less than the average background ± 2standard deviations (SD) were removed from each data point. The median of the effective data points for each lectin was globally normalized to the sum of medians of all effective data points for each lectin in a block. Each sample was observed consistently on three repeated slides. The normalized medians of each lectin from 9 repeated blocks were averaged, and its SD was counted. The normalized data of the parallel groups were compared with each other based on fold change, according to the following criteria: fold change ≥2.0 or ≤0.50 in pairs indicated up-regulation or down-regulation, respectively. Differences between multiple data sets were tested using one-way ANOVA in SPSS version 19. The original data were further analyzed using Expander 6.0 (http://acgt.cs.tau.ac.il/expander/) to perform a hierarchical clustering analysis (HCA).

Statistical differences between groups were first assessed using a Kruskal-Wallis test, followed by a Dunn's Multiple Comparison Test to correct for multiple comparisons using GraphPad Prism5.0 software. Differences were considered statistically significant for values of $^{*}P < 0.05$, $^{**}P < 0.01$ or $^{***}P < 0.001$. Five diagnostic models of breast disease (Model BD), BB (Model BB), BC (Model BC), BC-I (Model BC-I), and BC-II (Model BC-II) were constructed according to the glycopattern abundances based on a forward stepwise logistic regression analysis using SPSS statistics 21.0 software, respectively. The diagnostic performance of candidate lectins and diagnostic models was evaluated by ROC curve analysis using Origin 8.0 software (Qin et al., 2013; Shu et al., 2017).

3. Results

3.1. Alteration of Salivary Glycopatterns in Pooled Saliva from Subjects

Because there are significant age differences in the glycopatterns of human salivary glycoproteins (Qin et al., 2013), the subjects were chosen at the same or similar age stage and 30 salivary samples of HV, BB, BC-I, and BC-II subjects in the retrospective cohort were pooled and detected using a lectin microarray, respectively. The layout of the lectin microarrays and glycopatterns of Cy3-labeled salivary proteins from HV, BB, BC-I, and BC-II subjects bound to the lectin microarrays are shown in Fig. 1A and B. Their normalized fluorescent intensities (NFIs) for each lectin were summarized as the mean values ± SD in Supplementary Table S2. Lectin signal patterns were classified into three categories to evaluate whether the glycopatterns

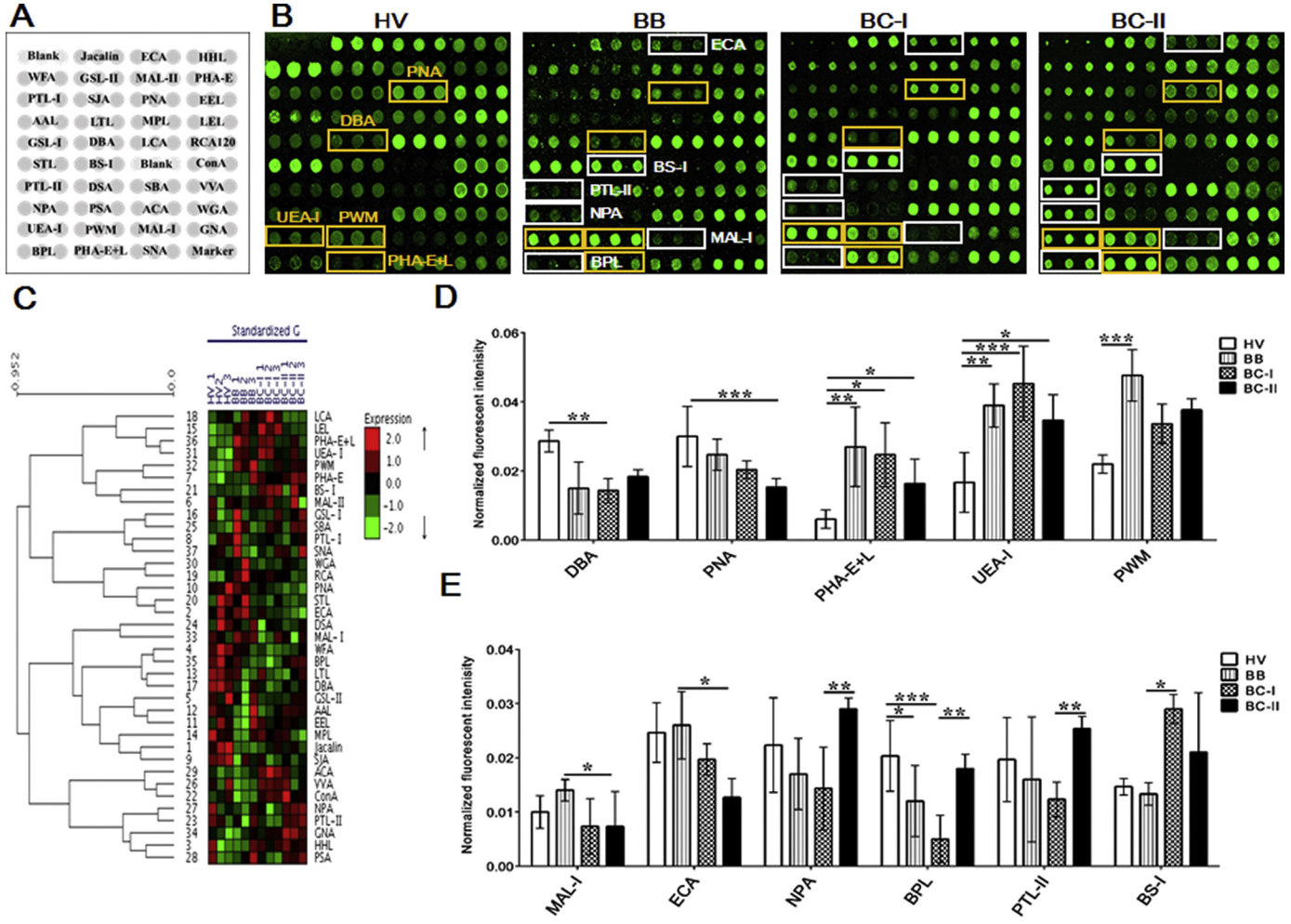

Fig. 1. The different salivary glycopatterns in HV, BB, BC-I and BC-II using a lectin microarray. (A) The layout of the lectin microarrays. Each lectin was spotted in triplicate per block, with quadruplicate blocks on one slide. Cy3-labeled BSA was spotted as a location marker and BSA as a negative control. (B) The glycopatterns of a Cy3-labeled pooled salivary sample bound to the lectin microarrays. The lectin microarrays revealed significant signal differences between HV, BB, BC-I and BC-II marked with yellow frames. While the significant differences among three mixture saliva from disease groups marked with white frames. (C) Unsupervised average linkage HCA of the lectin microarray responses to saliva. The samples were listed in columns, and the lectins were listed in rows. The color and intensity of each square indicated expression levels relative to the other data in the row. Red, high; green, low; black, medium. (D) Five lectins revealed significant differences between HV and patients with breast disease, but found no differences between patients with breast disease. (E) Six lectins revealed significant differences among breast diseases groups. Lectins showing increase of NFIs (fold change ≥ 2, $p < 0.05$) or decrease of NFIs (fold change ≤ 0.5, $p < 0.05$) between HV, BB, BC-I and BC-II according to one-way ANOVA ($^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$).

of the salivary glycoproteins were altered between HV, BB, BC-I, and BC-II subjects: (1) results showing significant increases in NFIs (fold change ≥ 2.0, $p < 0.05$), (2) results showing significant decreases in NFIs (fold change ≤ 0.50, $p < 0.05$), and (3) results showing almost even level in NFIs (fold change range from 0.50 to 2.0, no significant difference). All based on fold change in pairs (with p-values lower than 0.05) with the NFIs of each lectin

Table 2
Fold change of glycopatterns in the pooled saliva of the retrospective cohort based upon ratio of the NFIs of 11 lectins.

Lectin	Compared with HV (p value)[a]			Compared with each other (p value)[b]		
	BB/HV	BC-I/HV	BC-II/HV	BC-I/BB	BC-II/BB	BC-II/BC-I
DBA	—	0.43 (0.002)	—	—	—	—
PNA			0.50 (<0.001)			
PHA-E + L	3.48 (0.020)	3.58 (0.025)	2.74 (0.028)			
UEA-I	2.19 (0.004)	2.48 (<0.001)	2.15 (0.026)	—	0.61 (0.011)	—
PWM	2.15 (<0.001)	—	—	—	—	—
MAL-I	—	—	—	—	0.44 (0.016)	—
ECA	—	—	—	—	0.50 (0.014)	—
NPA	—	—	—	—	—	2.78 (0.009)
BPL	0.42 (0.033)	0.18 (<0.001)	—	—	—	5.30 (0.001)
PTL-II	—	—	—	—	—	2.59 (0.004)
BS-I	—	—	—	2.15 (0.023)	—	—

—, no significant difference.
[a] The NFIs of each lectin from BB, BC-I, and BC-II patients were compared with that from HV based on their fold change (i.e., BB/HV, BC-I/HV, and BC-II/HV), respectively.
[b] The NFIs of each lectin from patients with breast disease were compared with each other based upon fold change in pairs, i.e., BC-I/BB, BC-II/BB, and BC-II/BC-I.

from HV, BB, BC-I, and BC-II subjects were showed in Table 2. The results showed that there were 11 lectins to give significant signal differences in pooled saliva from the retrospective cohort. The generated data from three biological replicates were imported into EXPANDER 6.0 to perform a hierarchical clustering analysis (Fig. 1C).

The results showed that the αGalNAc and GalNAcα1-3(Fucα1-2)Gal binder DBA and the Galβ1-3GalNAcα binder PNA exhibited significantly decreased NFIs in BC-I and BC-II compared with HV subjects (fold change = 0.43 and 0.50, $p \le 0.002$), respectively. The bisecting GlcNAc, multiple-antennary complex-type *N*-glycan binder PHA-E + L and the Fucα1-2Galβ1-4Glc (NAc) binder UEA-I showed increased NFIs in BB, BC-I, and BC-II, as well as the branched (LacNAc)n binder PWM showed increased NFIs in BB compared with HV subjects (fold change ≥ 2.15, $p \le 0.028$). However, the NFIs of these lectins were not significantly different between BB, BC-I, and BC-II subjects (Fig. 1D).

The NFIs of the Galβ1-4/3GlcNAc binder MAL-I and ECA decreased significantly in BC-II compared with BB subjects (all fold change ≤ 0.50, $p \le 0.016$), and the high-Man and Manα1-6Man binder NPA increased significantly in BC-II compared with BC-I subjects (fold change = 2.78, $p = 0.009$). The Galβ1-3GalNAc binder BPL decreased significantly in BC-I compared with HV, BB, and BC-II subjects (all fold change ≤ 0.42, $p \le 0.033$). However, the NFIs of the Galβ1-3GalNAcα-Ser/Thr (T antigen) binder PTL-II increased significantly in BC-II compared with BC-I subjects (fold change = 2.59, $p = 0.004$), and the Galα1-3/6Gal/Glc binder BS-I increased significantly in BC-I compared with BB subjects (all fold change = 2.15, $p = 0.023$) (Fig. 1E).

3.2. Lectin Blotting Analysis

Lectin blotting analyses were performed using four lectins (PHA-E + L, NPA, MAL-I, and BS-I) selected randomly to confirm the different abundances of glycopatterns in pooled saliva from HV, BB, BC-I, and BC-II subjects of the retrospective cohort. The results of SDS-PAGE demonstrated that the salivary protein bands from patients were similar, except for four apparent different bands with molecular weights (Mr) of approximately 100 kDa, 70 kDa, 48 kDa, and 22 kDa, as compared with the HV (Supplementary Fig. S1A). PHA-E + L showed stronger binding to four apparent bands ranging from b1 to b4 in BB, BC-I, and BC-II than in HV subjects, however, NPA showed weaker binding to four apparent bands ranging from b1 to b4 in BC-I than in HV, BB, and BC-II subjects. MAL-I showed a stronger binding to an apparent band (b1) in HV and BB than in BC-I and BC-II subjects. BS-I displayed stronger binding to five bands ranging from b1 to b5 in BC-I than in HV, BB, and BC-II subjects (Supplementary Fig. S1B). The relative binding strength of these lectins to saliva samples was almost coincident with the results from the lectin microarrays.

3.3. Individual Validation of the Different Glycopatterns in Saliva

All remainder salivary samples (36 HV, 35 BB, 36 BC-I, and 32 BC-II) in the retrospective cohort were tested using the lectin microarrays independently. The NFIs of each candidate lectin showed variable expression levels of salivary glycopatterns were further represented in scatter diagram by Kruskal-Wallis test. As shown in Fig. 2, totally, there were 8 lectins to exhibit a high degree of consistency with the pooled

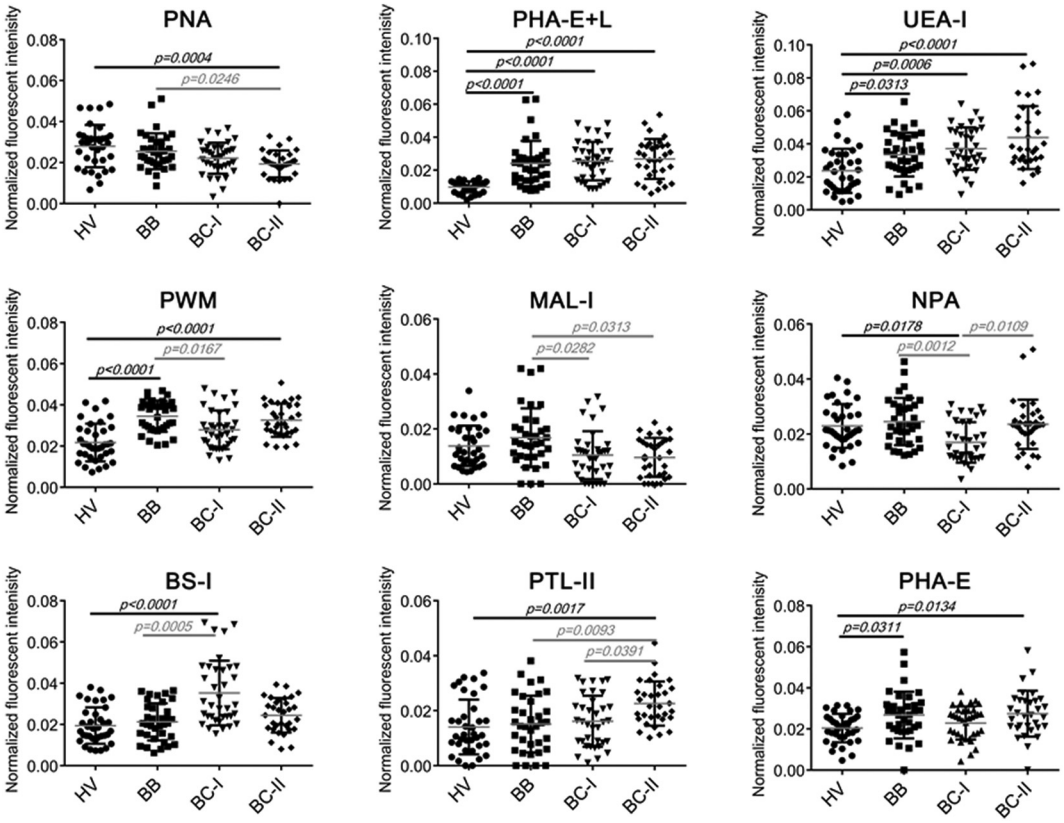

Fig. 2. The variable expression levels of salivary glycopatterns in the saliva with breast diseases represented in scatter diagram by Kruskal-Wallis test. The p value indicating the difference between HV and patients with breast diseases ($p < 0.05$) marked with black, and the difference between BB, BC-I and BC-II ($p < 0.05$) marked with gray.

samples of HV, BB, BC-I, and BC-II subjects. Such PNA exhibited significantly decreased NFIs in BC-I and BC-II compared with HV subjects ($p \leq 0.025$), PHA-E + L and UEA-I showed increased NFIs in BB, BC-I, and BC-II ($p \leq 0.031$), PWM showed increased NFIs in BB and BC-II compared with HV subjects ($p \leq 0.025$), MAL-I decreased significantly in BC-I and BC-II compared with HV and BB subjects ($p \leq 0.031$), NPA decreased significantly in BC-I compared with HV, BB, and BC-II subjects ($p \leq 0.018$), BS-I increased significantly in BC-I compared with HV and BB subjects ($p \leq 0.001$), as well as PTL-II increased significantly in BC-II compared with HV, BB, and BC-I subjects ($p \leq 0.039$). Besides, a bisecting GlcNAc, biantennary complex-type *N*-glycan with outer Gal binder PHA-E exhibited significantly increased NFIs in BB and BC-II compared with HV subjects ($p \leq 0.031$).

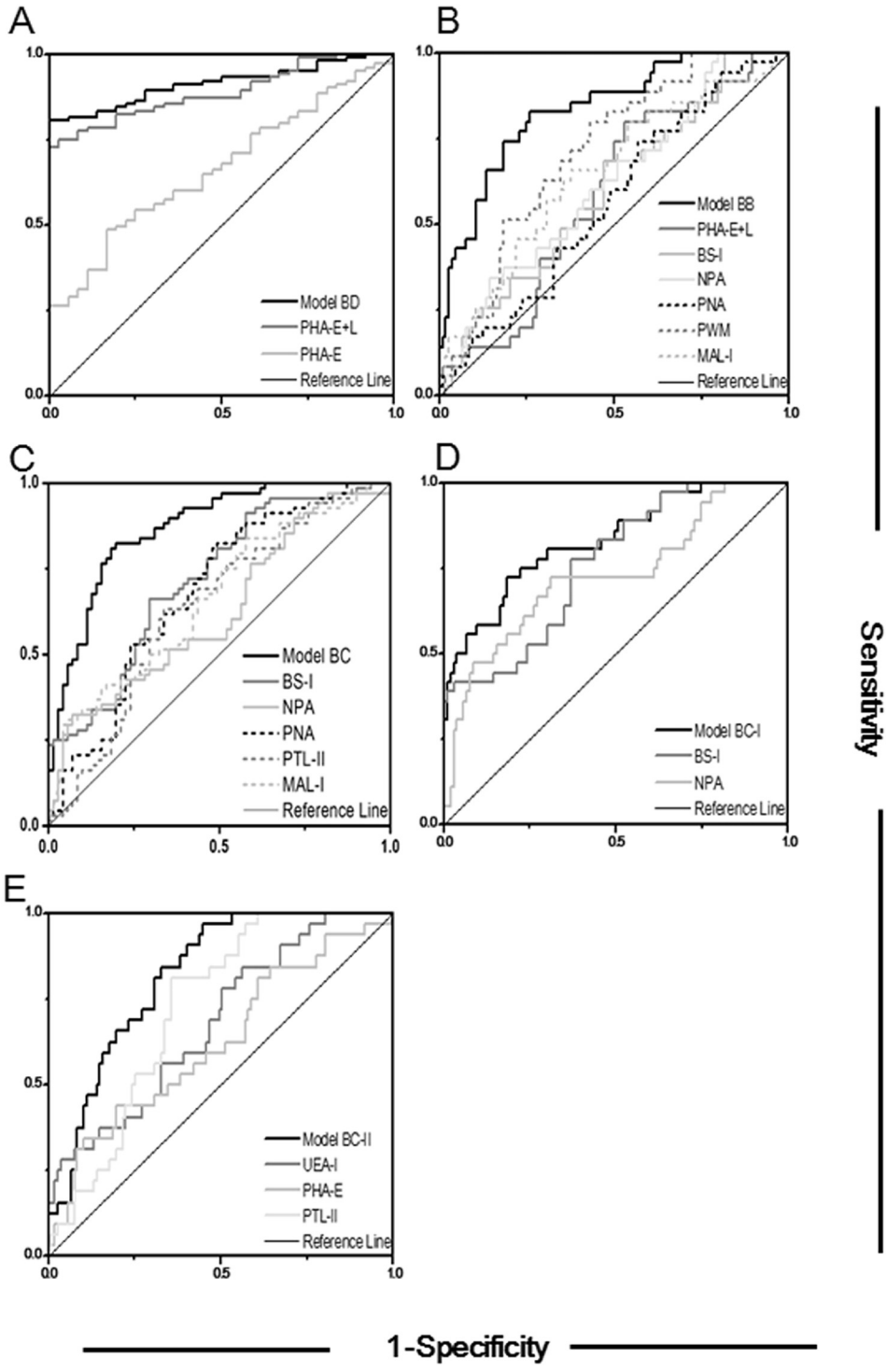

Fig. 3. The diagnosis accuracy of the diagnostic models and selected lectins analyzed by ROC analysis. (A), (B), (C), (D) and (E), The ROC analysis for Model BD, Model BC, Model BB, Model BC-I and Model BC-II as well as the selected lectins in the retrospective cohort, respectively. ROC-AUC values were expressed by (1-value) if lectins showed the decreased signal.

3.4. Construction of Diagnostic Models Based on Glycopattern Abundances

The salivary glycopatterns of BB, BC-I, and BC-II subjects were evaluated based on the above 9 candidate lectins that exhibited significantly alterations of protein glycopatterns in saliva with breast diseases (BD) in the retrospective cohort. Firstly, the Model BD mathematic formula with a precision average of 0.836 was constructed to differentiate BD from HV using a logistic regression analysis.

$$\text{Model BD} = \frac{1}{1 + e^{-(-5.142+315.606*(\text{PHA}-\text{E}+\text{L})+74.311*(\text{PHA}-\text{E}))}}$$

The diagnostic accuracy of Model BD referred to two lectins (PHA-E + L and PHA-E) in the retrospective cohort was analyzed by a receiver operating characteristic (ROC) curve (Fig. 3A), which indicated that Model BD had a higher diagnostic accuracy for distinguishing BD from HV (AUC: 0.913, sensitivity: 0.806, and specificity: 0.972) than two single lectins, such as PHA-E + L (AUC: 0.891, sensitivity: 0.738, and specificity: 1.000), PHA-E (AUC: 0.664, sensitivity: 0.485, and specificity: 0.833) (Supplementary Table S3).

The Model BB mathematic formula with a precision average of 0.785 was constructed to diagnose the BB from HV and patients with BC using a logistic regression analysis.

$$\text{Model BB} = \frac{1}{1 + e^{-(-8.198+55.912*(\text{PHA}-\text{E}+\text{L})-73.966*(\text{BS}-\text{I})+105.732*(\text{PWM})+65.789*(\text{NPA})+83.279*(\text{PNA})+60.380*(\text{MAL}-\text{I}))}}$$

The diagnostic accuracy of Model BB referred to six lectins (PHA-E + L, BS-I, PWM, NPA, PNA, and MAL-I) in the retrospective cohort were analyzed by a ROC curve, which indicated that Model BB had higher diagnostic accuracy for distinguishing BB (AUC: 0.835, sensitivity: 0.829, and specificity: 0.740) than these single lectins (Fig. 3B), such as PHA-E + L (AUC: 0.590, sensitivity: 0.800, and specificity: 0.471), BS-I (AUC: 0.600, sensitivity: 0.686, and specificity: 0.529), PWM (AUC: 0.712, sensitivity: 0.800, and specificity: 0.567), NPA (AUC: 0.618, sensitivity: 0.514, and specificity: 0.606), PNA (AUC: 0.567, sensitivity: 0.743, and specificity: 0.433), and MAL-I (AUC: 0.651, sensitivity: 0.657, and specificity: 0.625).

The Model BC mathematic formula with a precision average of 0.806 was constructed to diagnose the BC from other non-malignant breast lesions using a logistic regression analysis.

$$\text{Model BC} = \frac{1}{1 + e^{-(1.907+74.375*(\text{BS}-\text{I})-142.687*(\text{NPA})-62.848*(\text{PNA})+112.867*(\text{PTL}-\text{II})-91.867*(\text{MAL}-\text{I}))}}$$

The diagnostic accuracy of Model BC referred to five lectins (BS-I, NPA, PNA, PTL-II, and MAL-I) in the retrospective cohort was analyzed by a receiver operating characteristic (ROC) curve (Fig. 3C), which indicated that Model BC had a higher diagnostic accuracy for distinguishing BC from other non-malignant breast lesions (AUC: 0.869, sensitivity: 0.824, and specificity: 0.803) than five single lectins, such as BS-I (AUC: 0.719, sensitivity: 0.662, and specificity: 0.704), NPA (AUC: 0.626, sensitivity: 0.324, and specificity: 0.930), PNA (AUC: 0.682, sensitivity: 0.824, and specificity: 0.507), PTL-II (AUC: 0.639, sensitivity: 0.632, and specificity: 0.662), MAL-I (AUC: 0.662, sensitivity: 0.309, and specificity: 0.944).

The Model BC-I mathematic formula with a precision average of 0.819 was constructed to differentiate the BC-I from the HV, BB and other stages of BC using a logistic regression analysis.

$$\text{Model BC}-\text{I} = \frac{1}{1 + e^{-(-1.267+97.991*(\text{BS}-\text{I})-123.015*(\text{NPA}))}}$$

The diagnostic accuracy of Model BC-I referred to two lectins (BS-I and NPA) in the retrospective cohort was analyzed by a ROC curve, which indicated that Model BC-I had higher diagnostic accuracy for distinguishing BC-I (AUC: 0.831, sensitivity: 0.750, and specificity: 0.777) than two single lectins (Fig. 3D), such as BS-I (AUC: 0.763, sensitivity: 0.778, and specificity: 0.631), and NPA (AUC: 0.726, sensitivity: 0.722, and specificity: 0.689).

The Model BC-II mathematic formula with a precision average of 0.739 was constructed to differentiate the BC-II from HV, BB and other stages of BC using a logistic regression analysis.

$$\text{Model BC}-\text{II} = \frac{1}{1 + e^{-(-6.717+46.576*(\text{UEA}-\text{I})+80.113*(\text{PHA}-\text{E})+87.625*(\text{PTL}-\text{II}))}}$$

The diagnostic accuracy of Model BC-II referred to three lectins (UEA-I, PHA-E and PTL-II) in the retrospective cohort was analyzed by a ROC curve, which indicated that Model BC-II had higher diagnostic accuracy for distinguishing BC-II from HV, BB and other stages of BC (AUC: 0.818, sensitivity: 0.844, and specificity: 0.673) than three single lectins (Fig. 3E), such as UEA-I (AUC: 0.679, sensitivity: 0.563, and specificity: 0.673), PHA-E (AUC: 0.621, sensitivity: 0.438, and specificity: 0.804) and PTL-II (AUC: 0.724, sensitivity: 0.813, and specificity: 0.645).

3.5. Validation of the Diagnostic Models

The constructive models in the retrospective cohort were then applied to the validation cohort of HV (n = 31) and patients with BB (n = 30), BC-I (n = 30) and BC-II (n = 36), in order to evaluate the diagnostic power. The NFIs of 9 candidate lectins exhibited variable expression levels of salivary glycopatterns were further represented in box diagram by Kruskal-Wallis test. As shown in Supplementary Fig. S2, all candidate lectins that showed variable expression levels of salivary glycopatterns were generally coincident with the results in the retrospective cohort. Meanwhile, ROC analyses were performed to show the diagnostic accuracy of the constructive models in Supplementary Table S3. The ROC curves indicated that the Model BD (cutoff value: 0.401, AUC: 0.902, sensitivity: 0.823, and specificity: 0.839) had high diagnostic accuracy for distinguishing BD from HV (Supplementary Fig. S3A). 79 cases of 96 BD and 26 cases of 31 HV were correctly classified by Model BD. The ROC curves indicated that the Model BB (cutoff value: 0.133, AUC: 0.752, sensitivity: 0.600, and specificity: 0.835) had high diagnostic accuracy for distinguishing BB from HV and patients with BC (Supplementary Fig. S3B), 18 cases of 30 BB and 80 cases of 97 other subjects were correctly classified by Model BB. While the ROC curves indicated that the Model BC (cutoff value: 0.405, AUC: 0.802, sensitivity: 0.742, and specificity: 0.754) had high diagnostic accuracy for distinguishing BC

Table 3
The results of the double-blind test compared with clinical final diagnosis.

Total cases	Clinical final diagnosis[a]	Prediction Method	False-positive	False-negative	Sensitivity	Specificity	Accuracy
61	HH: 13	–	–	–	–	–	–
	BD: 48	Model BD: 45	4	7	0.854 (41/48)	0.692 (9/13)	0.820 (50/61)
	BB: 21	Model BB: 25	10	6	0.714 (15/21)	0.750 (30/40)	0.738 (45/61)
	BC: 27	Model BC:	4	8	0.704 (19/27)	0.882 (30/34)	0.803 (49/61)
	BC-I: 11	Model BC-I: 11	3	3	0.727 (8/11)	0.940 (47/50)	0.902 (55/61)
	BC-II: 12	Model BC-II: 30	21	3	0.750 (9/12)	0.571 (28/49)	0.607 (37/61)
	BC-III: 4	–	–	–	–	–	–

—, no statistics.
[a] Baseline characteristics of 61 subjects in the double-blind cohort were shown in Table 1. HH: healthy human; BD: breast disease; BB: benign breast cyst or tumor; BC: breast cancer; BC-I: I stage of BC; BC-II: II stage of BC; BC-III: III stage of BC.

from other non-malignant breast lesions (Supplementary Fig. S3C). 49 cases of 66 BC and 46 cases of 61 HV and BB were correctly classified by Model BC. The ROC curves indicated that the Model BC-I (cutoff value: 0.268, AUC: 0.755, sensitivity: 0.733, and specificity: 0.742) had high diagnostic accuracy for distinguishing BC-I from HV, BB, and BC-II (Supplementary Fig. S3D), 22 cases of 30 BC-I and 72 cases of 97 other subjects were correctly classified by Model BC-I. However, the model BC-II showed a lower diagnostic accuracy (cutoff value: 0.023, AUC: 0.680, sensitivity: 0.944, and specificity: 0.460) for distinguishing BC-II from HV, BB, and BC-I (Supplementary Fig. S3E), 34 cases of 36 BC-II and 40 case of 91 other subjects were correctly classified by Model BC-II.

3.6. Double-Blind Test Based on the Diagnostic Models

An independent test was performed in double-blind cohort with 61 subjects in order to further confirm the diagnostic accuracy of these diagnostic models for differential diagnosis of BD, BB, BC, BC-I, and BC-II. The results of the double-blind test were compared with clinical final diagnosis in Table 3. Forty-five cases of 61 salivary specimens were identified as BD-positive by Model BD. Actually, 41 cases of 48 BD and 9 cases of 13 healthy humans were correctly classified by Model BD with an accuracy of 0.820 (50/61). Twenty-five cases of 61 salivary specimens were identified as BB-positive by Model BB. Actually, 15 case of 21 BB and 30 case of 40 other subjects were correctly classified by Model BB with an accuracy of 0.738 (45/61). Moreover, 23 cases of 61 salivary specimens were identified as BC-positive by Model BC. Actually, 19 case of 27 BC and 30 case of 34 other subjects were correctly classified by Model BC with an accuracy of 0.803 (49/61). Eleven cases of 61 subjects were identified as BC-I-positive by Model BC-I. Actually, 8 case of 11 BC-I and 47 case of 50 other subjects were correctly classified by Model BC-I with an accuracy of 0.902 (55/61). But, 30 cases of 61 subjects were identified as BC-II-positive by Model BC-II. Actually, 9 cases of 12 BC-II and 28 cases of 49 other subjects were correctly classified by model BC-II with an accuracy of 0.607 (37/61).

4. Discussion

Glycosylation and related processes play important roles in cancer development and progression. The previous studies explained the abilities of aberrant glycosylation and the expression patterns of glycosyltransferase genes for classifying different types of cancer or proposing subtypes (Ashkani and Naidoo, 2016; Hua et al., 2014). Several studies have shown that *N*-glycans have potential diagnostic value as cancer serum biomarkers, such as the decreased high mannosylated glycans, the increased out-fucosylated monoantennary glycans and the increased multi-antennary glycans in serum of BC patients compared with HV (Kyselova et al., 2008; Saldova et al., 2014). The abundance of particular serum *N*-glycan structures as important features of breast tumor biology have been explored by studying the serum glycome and tumor transcriptome (mRNA and miRNA) of BC patients, which describe the tri-antennary tri-galactosylated tri-sialylated glycans in serum as being associated with lower levels of tumor transcripts to involve in focal adhesion and integrin-mediated cell adhesion. These glycan structures are also linked to poor prognosis in patients with ER negative tumors. High abundance of simple monoantennary glycan structures are associated with increased survival, particularly in the basal-like subgroup (Haakensen et al., 2016).

In the present study, 259 saliva samples were collected for screening candidate lectins and constructing diagnostic models in retrospective cohort. While another 127 saliva samples were collected for examining diagnostic accuracy of the diagnostic models in the validation cohort. There were 9 lectins (e.g., PHA-E + L, BS-I, PWM, and NPA) to reveal significant alterations of the salivary glycopatterns between HV, BB, BC-I and BC-II using lectin microarrays, lectin blotting analysis, and statistical analysis. We found that the bi- and multi-antennary complex-type *N*-glycans recognized by PHA-E + L and PHA-E have increased in patients with BD. The model BD referred to PHA-E + L and PHA-E was constructed in the retrospective cohort, which had a higher diagnostic accuracy (AUC: 0.902, sensitivity: 0.823, and specificity: 0.839) to differentiate the BD from the HV in the validation cohort. The Galβ1-4/3GlcNAc recognized by MAL-I and ECA decreased significantly in BC-II compared with BB subjects, and the high-Man and Manα1-6Man recognized by NPA increased significantly in BC-II compared with BC-I subjects, the Galβ1-3GalNAc recognized by BPL decreased significantly in BC-I compared with HV, BB, and BC-II subjects. However, the Galβ1-3GalNAcα-Ser/Thr (T antigen) recognized by PTL-II increased significantly in BC-II compared with BC-I subjects, and the Galα1-3/6Gal/Glc recognized by BS-I increased significantly in BC-I compared with BB subjects. The diagnostic models of BB, BC, BC-I, and BC-II were further constructed according to these results from the retrospective cohort, which indicated that Model BB referred to six lectins (PHA-E + L, BS-I, PWM, NPA, PNA and MAL-I) had higher diagnostic accuracy (AUC: 0.752, sensitivity: 0.600, and specificity: 0.835) and a blind-test accuracy of 0.738 for distinguishing BB from HV and patients with BC. The Model BC referred to five lectins (BS-I, NPA, PNA, PTL-II and MAL-I) had higher diagnostic accuracy (AUC: 0.802, sensitivity: 0.742, and specificity: 0.754) and a blind-test accuracy of 0.803 for distinguishing BC from other non-malignant breast lesions. The Model BC-I referred to two lectins (BS-I and NPA) had higher diagnostic accuracy (AUC: 0.755, sensitivity: 0.733, and specificity: 0.742) and a blind-test accuracy of 0.902 for distinguishing BC-I from HV, BB, and other stages of BC. However, the model BC-II referred to three lectins (UEA-I, PHA-E and PTL-II) showed a lower diagnostic accuracy (AUC: 0.680, sensitivity: 0.944, and specificity: 0.460) and a blind-test accuracy of 0.607 for distinguishing BC-II from HV, BB, and other stages of BC.

We observed that the candidate lectins (e.g., PHA-E + L, BS-I, PWM, and NPA) and the diagnostic models (Model BC-I) associated with clinical pathologic variables like histological grade, tumor size, lymph node status and specific receptors, respectively. Here, the multiple linear regression was used to test their correlation by analyzing data of 128 samples from patients with BC in the retrospective cohort. The results were summarized in Supplementary Table S4, which showed BS-I and UEA-I associated with tumor size and lymph node status ($p = 0.001$ and $p = 0.011$), respectively. And PHA-E + L, PWM, NPA, and MAL-I had a significant correlation with hormone receptor (ER or PR) or HER2 status ($p \leq 0.031$). Furthermore, the Model BC-I and Model BC-II associated with tumor size ($p < 0.001$) and lymph node involvement ($p = 0.041$), respectively, which implied their potential to reflect the tumorigenesis and tumor progression. And, an analysis was performed by the breast cancer subtypes (Luminal A/B, $HER2^+$, and triple negative) in comparisons with non-malignant breast lesions. The results were summarized in Supplementary Table S5, which showed the Model BC and Model BC-II had a higher potential for the diagnosis of the breast cancer subtypes, such as Luminal A/B and $HER2^+$, respectively.

Saliva is a mirror of body health and good indicator of the plasma levels of various substances such as hormones and drugs. Saliva contains many disease markers which reflect the state of health of not only the salivary glands and oral cavity, but also the whole body. Saliva contains numerous cells (exfoliated epithelial cells, leucocytes, bacteria), and the majority of blood disease markers (antibodies, interleukins, neoplasma markers) that may be applied in the detection of early pathological changes in humans (e.g. viral infections, autoimmunological diseases, cancers) (Chojnowska et al., 2017). Recent studies have elucidated that many diseases could be detected by saliva, such as cardiovascular disease, gastric disease and BC (Foley et al., 2012; Porto-Mascarenhas et al., 2017; Streckfus et al., 2000; Zhang et al., 2010). Our previous studies also provided the evidence that the correlation of alterations in salivary glycosylation related to gastrosis, which provided with two diagnostic models and achieved high diagnostic power for diagnosis of atrophic gastritis and gastric cancer (Shu et al., 2017).

One limitation for this study is that our investigation has not referred to the mechanisms that cause the alterations of salivary protein glycosylation in patients with BC. Second limitation is lack of enough clinical samples from patients with the subtypes (such as Luminal A/B, $HER2^+$, and triple negative) of BC. Another concern is lack of enough data from patients with BC-II, we should elaborate the heterogeneity of BC by comparing the clinicopathological data of the cases in order to detect the patients with BC-II accurately. Studies in larger cohorts enrolling more patients with the different subtypes and stage II of BC need to be carried out in future.

In conclusion, the present study investigated the correlation of alterations in salivary protein glycosylation related to BC and compare different or similar alterations of protein glycopatterns between HV, patients with BB, BC-I and BC-II, as well as the diagnostic models (Model BD, Model BB, Model BC, Model BC-I, and Model BC-II) were constructed based on the selected 9 lectins that exhibited significantly alterations of protein glycopatterns in saliva with development of breast diseases. Model BD, Model BB, Model BC, and Model BC-I achieved better diagnostic powers with an AUC value >0.750 ($p < 0.001$) for the diagnosis of BD, BB, BC, and BC-I. Furthermore, the Model BC-I exhibited a high accuracy of 0.902 in double-blind cohort. This study provides pivotal information to distinguish women with BD, BB, BC, especially in BC-I based on precise alterations in salivary glycopatterns, which may contribute to screen patients with early-stage BC.

Declaration of Interests

All authors declare no commercial nor associative interest that represents a conflict of interest in connection with the work submitted.

Contributors

Study concept and design: ZL, XL, JH; Acquisition of data: XL, HY, YQ, JY, JS, JZ, ZZ; Analysis and interpretation of data: XL, ZL, HY, YQ, JY, JH; Writing the manuscript: XL, ZL, JY; Critical revision of the manuscript for important intellectual content: ZL, XL, JH, HY, YQ, JY, JS, JZ, ZZ; Statistical analysis: XL, ZL, YQ, JY.

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Appendix A. Supplementary data

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