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Original Article

Assessment of new biomarkers for ovarian carcinoma with serum N-glycan profiling

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Abstract: Background: To explore and validate the value of N-glycan markers in ovarian carcinoma and to unveil their underlying molecular mechanism. Methods: In total, 70 ovarian carcinoma, 111 ovarian benign tumor and 137 healthy controls were profiled. Serum N-glycan was profiled by DNA sequencer-assisted fluorophore-assisted carbohydrate electrophoresis (DSA-FACE). Two diagnostic models were constructed based on N-glycan markers with logistic stepwise regression. Lectin blot was used to analyze the β1,6-N-acetylglucosamine (β1,6-GlcNAc) residues, and the expression of N-acetylglucosaminy transferase V (GNT-V) was analyzed by quantitative reverse transcription-polymerase chain reaction. Results: We identified 12 N-glycan structures and we found the levels of β1,6-GlcNAc residues and GNT-V expression were obviously increased in ovarian carcinoma. Two multiparameter diagnostic models designated Model_Sugar and Model_Complex could discriminate ovarian carcinoma from ovarian benign tumor and healthy control. The AUC values for CA125, HE4, ROMA, Model_Sugar and Model_Complex were 0.860, 0.879, 0.913,0.903 and 0.963, respectively. Compared with HE4, ROMA, the sensitivity of Model_Sugar for discriminating ovarian cancer from benign tumors was increased 28.6% and 2.9%. The accuracy of Model_Sugar was increased 7.7% and 0.5% compared with CA125 and HE4. For Model_Complex, the sensitivity was increased 5.7%, 34.3% and 8.6% compared with CA125, HE4 and ROMA. The accuracy of Model_Complex was increased 17.7%, 10.5% and 10.0% compared with CA125, HE4 and ROMA. Conclusion: We conclude that the diagnostic models based on N-glycan markers are valuable and noninvasive means for identifying ovarian carcinoma. Combining N-glycan markers with CA125 and HE4 could improve the diagnostic efficacies for ovarian cancer significantly.

Keywords: Ovarian carcinoma, N-glycan, DSA-FACE, diagnostic model, β1,6-GlcNAc, biomarker

Introduction

Ovarian carcinoma is one of the commonest malignant tumors in female genital. It has been a serious threat to women's health and life. According to the latest estimates, there are 225500 new cases of ovarian carcinoma every year, the death cases of 140200. In 2013, 14030 patients with ovarian carcinoma were death within the new 22240 cases in the United States [1, 2]. Some 70% of women with ovarian carcinoma present at stage III or IV, the five-year survival rate is still only 20%-40% despite the increasing knowledge about surgery and chemotherapy. When caught at stage I, the five-year survival rates are as high as 90% [3, 4]. At present, many biomarkers such as CA125, HE4 and pelvic imaging examination can effectively help to identify the occurrence of ovarian carcinoma. In addition,risk ovarian malignancy algorithm(ROMA) [5, 6] has been proved to be more efficient in predicting the risk of ovarian carcinoma with pelvic tumor. And yet for all that, considering the prognosis of advanced ovarian carcinoma, we need a noninvasive and effective means to further improve the efficiency of the diagnosis of ovarian carcinoma.

The ideal biomarkers are detected by noninvasive means such as blood, urine, saliva, and cervical mucous are possibilities [7]. There is growing evidence that N-linked glycan could be regarded as a potential biomarker for diagnosing cancer. The glycans have an important structural and functional element of the majority of proteins, which participate in virtually all

physiological processes [8]. Glycosylation is one of the most common post-translational, and one of the most important regulating ways in many cells, membrane and secreted proteins. During progressions of the tumor, glycans participate in major pathophysiology events [9, 10]. With the development of DNA sequencer-assisted fluorophore-assisted carbohydrate electrophoresis (DSA-FACE), high performance liquid chromatography (HPLC), matrix-assisted laser desorption/ionization time offlight mass spectrometry (MALDI-TOF-MS) and lectin microarray, providing us with rapid, high sensitive, high throughputand reliable quantitative methods for N-glycan profiling. Many studies observed a significant change of N-glycan in few cancers and cancer cell lines such as breast cancer, ovarian cancer, prostate cancer, and pancreatic cancer [11-16]. There is evidence to indicate various histological subtypes of ovarian carcinoma exhibit different glycoproteins [17]. This is encouraging given in the challenge of ovarian carcinoma diagnosis.

Previous research has revealed the increases in levels of core fucosylated, agalactosyl biantennary glycans (FA2) and sialyl Lewis x (SLe^x) by HPLC and MALDI-TOF-MS in ovarian carcinoma [18]. In this study, by using DSA-FACE, we retrospectively profiled serum N-glycan in sample from patients with ovarian carcinoma or ovarian benign tumor and in samples from healthy individuals. Then, we assessed the identified ovarian carcinoma N-glycan markers with receiver operating characteristic (ROC) curves and validated the markers in prospective. Our purpose was to identify a promising biomarker for prediction and detection of ovarian carcinoma with improved sensitivity and specificity.

Materials and methods

Patient selection

In total, 181 patients with ovarian carcinoma (n=70) and ovarian benign tumor (n=111) were recruited between 2012 and 2014. All enrolledpatients who underwent surgery were histopathologically confirmed by two pathologists at Changhai Hospital of the Second Military Medical University (Shanghai, China). Patients with other gynecological malignant tumor were excluded from the study. All the serum detection was finished before any therapies. In the control group, 137 healthy volunteers (cancer-free) were enrolled who volunteered to join the research during the same period. All enrolled cases were confirmed to out of other important organs disorders, and no family history of cancer. Laboratory and clinical data for all participants were obtained from clinical medical records, pathology reports, and personal interviews. The ovarian carcinoma stage was classified according to the FIGO criteria. Accordingly, 8 patients (11.43%) presented at stage I, 4 patients (5.71%) presented at stage II, 41 patients (58.57%) presented at stage III and 17 patients (24.29%) presented at stage IV. All the serum samples were obtained before surgical resection by using a standard protocol from whole blood, treated by centrifugation at 3000 r/min for 10 minutes, and stored at -80°C. And all tissue samples were used in accordance with the Institutional Review Board Regulations of the Second Military Medical University. Paired tumor and adjacent tissue samples were obtained from 16 of 70 patients with ovarian carcinoma, and benign tissue samples were obtained from 11 of 111 patients with ovarian benign tumor. Tissue samples were immediately frozen at liquid nitrogen, or stored in RNA preservation solution (Ambion, USA) for 24 hours at 4°C and then frozen at -80°C, which were used for lectin blotting and reverse transcriptase-polymerase chain reaction (RT-PCR), respectively.

The study protocol was approved by The Chinese Ethics Committee of Human Resources at the Second Military Medical University. Written informed consent was obtained from the patients and the healthy controls.

Laboratory tests

The main clinical and biochemical data from the patients were summarized in **Table 1**. Routine biochemical tests were measured using standard methods and matched reagents. CA125 levels were determined on a Roche Cobas E601 by ECLIA. HE4 levels were determined on a BioTek Epoch by ELISA. The cut-off values for CA125 and HE4 were 35 U/L and 150 pmol/L, respectively. The tests were finished by the Department of Laboratory Medicine, Changhai hospital, the Second Military Medical University, Shanghai.

Risk ovarian malignancy algorithm(ROMA)

We used the equations by Moore RG [5]: Premenopausal: Predictive Index (PI) =-12.0+ 2.38*LN(HE4)+0.0626*LN (CA125); Postme-

Table 1. Characteristics of the subjects in the disease and control groups

Characteristic	Control (n=137)	Ovarian benign tumor (n=111)	Ovarian carcinoma (n=70)
Age, years	43.31±11.75	41.02±13.31	52.34±10.13
>40 y (n, %)	80 (58.39)	54 (48.65)	61 (87.14))
Menopause (n, %)	43 (31.39)	21 (18.91)	38 (54.29)
CA125-positive (n, %)	0	35 (31.53)	58 (82.86)
HE4-positive (n, %)	0	1 (0.90)	38 (54.29)
ROMA-high risk (n, %)	0	18 (16.22)	56 (80.0)
TNM stage			
I			8 (11.43)
II			4 (5.71)
III			41 (58.57)
IV			17 (24.29)

The values supplied in **Table 1** were means with SD range.

nopausal: Predictive Index (PI) =-8.09+1.04*LN (HE4)+0.732*LN(CA125). Predicted Probability (PP) =exp(PI)/ [1+exp(PI)].

The equations were derived from the WIHRI and MGH combined pilot study. These cut-points were 13.1% PP for premenopausal patients and 27.7% PP for postmenopausal patients. The ROMA indexes were summarized in **Table 1**.

Serum protein N-glycan profiling

Serum protein N-glycan analyses were performed as described previously described [19]. Briefly, the N-glycans present on the proteins in 2 μl of serum were released with peptide N-glycosidase-F (PNGaseF) (New England Biolabs, Boston, Mass) and then labeled with APTS (8-aminonaphtalene-1, 3, 6-trisulphonic acid) (Invitrogen, Carlsbad, Calif). Sialic acid was removed with arthrobacter ureafaciens sialidase (RocheBioscience, Palo Alto, Calif), and the processed samples were analyzed with DSA-FACE technology using a capillary electrophoresis (CE)-based ABI3500 Genetic Analyzer (Applied Biosystems, Prism, Hitachi, America). The 12 most intense peaks that were detected in all samples (together, these peaks accounted for >90% of total serum N-glycans) were analyzed using GeneMapper V4.1 software (Applied Biosystems). Each structure of N-glycan was described numerically by normalizing its height to the sum of the heights of all peaks.

Tissue protein extraction and lectin blots

The tissues were homogenized using a mortar and pestle on the ice and suspended and pestled in lysis buffer containing a protease inhibitor cocktail (Roche Diagnostics, Meylan, France). Unlysed parts were removed twice by centrifugation (×12000 g for 15 minutes at 4°C). The concentration of solubilized proteins was determined using the Bradford assay (Bio-Light, Shanghai) and the samples were stored at -80°C until use.

In total, 25 μg of serum proteins or 50 μg of tissue proteins extracted from frozen samples were separated by electrophoresis in 10% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE). Gels were stained with CBB G250 or the proteins in the gel were transferred to a nitrocellulose membrane (Whatman/Schleicher&Schuell France, Versailles, France) for the detection of glycoproteins with β1,6-N-acetylglucosamine (β1,6-GlcNAc) branched residues. The membranes were blocked overnight at 4°C with 5%BSA (bovine serum albumin) in Tris-buffered saline (TBS, 140 mM NaCl, 10 mM Tris-HCl) and then incubated for 2 hour at room temperature with 5 μg/ml biotinylated Phytohemagglutinin-lymphocyte type (PHA-L) (Vector Laboratories, Burlingame, Calif) in TBS containing 0.05% Tween-20 (TBST buffer). After 4 washes for 10 minutes each with TBST, the membranes were incubated with a 1:10,000 dilution of IRDye 800 CW-streptavidin (LI-COR Biosciences, Lincoln, Neb) for 1 hour at room temperature, then washed 4 times with TBST and developed by Odyssey Infrared Imaging System (LI-COR Biosciences). Purified albumin (Sigma, St. Louis Mo) was used as negative control for lectin blot and total protein stained with Coomassie blue was used to calculate the percentage of PHA-L binding proteins.

Tissue total RNA extraction and quantitative reverse transcription PCR (qRT-PCR)

RNA was extracted from frozen tissues with RNeasy Plus Mini Kit according to the manufacturer's instruction (Qiagen, Hilden, Germany).

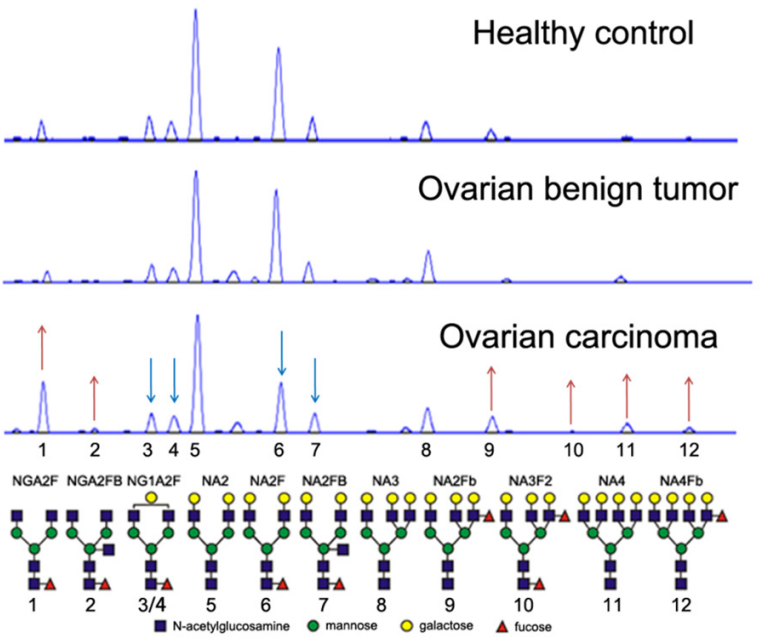

Figure 1. The typical desialylated N-glycan profiles from total serum proteins. At least 12 peaks can be identified. Peak1, 2, 9, 10, 11 and 12 are elevated, and Peak 3, 4, 6 and 7 are decreased in ovarian carcinoma compared with those levels in ovarian benign tumor and normal controls. The structures of the N-glycan Peaks are shown below the chart.

The purity and concentration of RNA were determined by spectrophoto-meter (Epoch, Biotek, Ameri-ca). The cDNA was synthesized starting from 1 μg of total RNA by using Rever-Tra Ace-α-RT-PCR kit (Toyobo, Osaka, Japan). The quantitative real time PCR of mannosyl (alpha-1,6-)-glycoproteinbeta-1, 6-N-acetylglucosaminyl transferase (*MGAT5*) was performed using the SYBR Green Real-time PCR Master Mix kit (TOYOBO, Japan) and was analyzed on ABI system 7300 (Life Technologies, USA). The gene *GAPDH* was used as an internal control. Each reaction was performed in triplicate. The PCR cycling was performed by denaturation at 95°C for 5 min followed by 40 cycles of 15 s at 95°C, 15 s at 55°C or 59°C, and detection for 45 s at 72°C. The primer for MGAT5 and GAPDH are listed as followed: MGAT5-forward, 5'TCTGCACTTTACCATCCAGCA3', MGAT5-reverse, 5'CCAATGCGCTGCAAAATGTTAT3', GAPDH-forward, 5'AGGGCTGCTTTTAACTCTGGT3' and GAPDH-reverse, 5'CCCCACTTGATTTTGGAGGGA3'. The relative abundance of MGAT5 transcripts was normalized to GAPDH using the Delta-Delta Ct method.

Statistical analysis

All quantitative variables are expressed as means ± standard deviations unless stated otherwise. The quantitative variables were compared with Student t tests, analyses of variance (ANOVA) and multiple comparisons, or nonparametric tests. Pearson coefficients of correlation and the associated probabilities (P) were used to evaluate the correlations between parameters. And Spearman coefficients of correlation were calculated for ordinal categorical variables. Diagnostic models were identified and constructed based on forward stepwise logistic regression analysis. The diagnostic performances of single marker and the diagnostic models were evaluated by ROC curve analysis. Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and accuracy were calculated using cut-off values optimally selected with the ROC curves. All reported *P* values were 2-tailed, and *P* values <0.05 were considered statistically significant. Statistical analyses were performed with SPSS16.0 for Windows statistical software (SPSS Inc.).

Results

Different profiling patterns of N-glycan in ovarian carcinoma, ovarian benign tumor and healthy controls

By using DSA-FACE, at least 12 N-glycan structures (Peaks) were identified in serum samples from patients with ovarian carcinoma (n=70), patients with ovarian benign tumor (n=111), and healthy controls (n=137). Representative N-glycan profiling patterns of the three groups were shown in **Figure 1**. Callewaert *et al* and Liu *et al* published the structural analysis of these N-glycans previously [19, 20]. The average relative abundances of these N-glycan structures were shown in **Table 2** and all structure abundance had been quantified.

The abundance of structures in Peak1, 2, 3, 4, 6, 7, 9, 10, 11 and 12 were statistically significant different between the ovarian carcinoma, ovarian benign tumor and healthy controls,

Table 2. Abundence of N-Glycan profiling by DNA sequencer-assisted fluorophore- assisted capillary electrophoresis in all groups

Variable	Control (n=137)	Ovarian benign (n=111)	Ovarian carcinoma (n=70)	*F*	*P*
Peak1	6.25±2.14	6.94±2.49	10.48±4.64	49.00	<0.001
Peak2	1.00±0.41	0.83±0.44	1.21±0.64	14.04	<0.001
Peak3	6.35±1.10	5.91±1.89	5.60±2.02	5.30	0.005
Peak4	5.24±1.00	5.72±1.37	5.18±1.41	6.14	0.002
Peak5	38.93±3.10	37.97±4.06	39.24±6.09	2.43	0.090
Peak6	22.50±3.20	23.04±3.32	16.89±3.94	80.94	<0.001
Peak7	6.26±1.38	5.96±1.19	5.09±1.36	18.47	<0.001
Peak8	7.95±1.57	8.08±1.64	7.93±1.91	0.24	0.79
Peak9	2.09±0.92	2.04±1.10	4.08±2.26	57.77	<0.001
Peak10	0.28±1.17	0.30±0.15	0.36±0.22	5.29	0.006
Peak11	1.85±0.50	1.84±0.44	2.17±0.61	11.41	<0.001
Peak12	0.42±0.20	0.38±0.20	0.90±0.55	69.32	<0.001

which indicated the difference of N-glycan patterns in different pathophysiologic conditions. Compared with ovarian benign tumor and healthy controls, the abundance of Peak1, 2, 9, 10, 11, and 12 was increased ($P<0.01$), and Peak3, 4, 6 and 7 was decreased ($P<0.01$) (**Table 2**). Among these abundance of the structures Peak1, 6, 9 and 12 were significantly different in ovarian carcinoma (**Figure 2**), while not significant different between the ovarian benign tumor and healthy controls.Compared with ovarian benign tumor and healthy controls, CA125, HE4, ROMA were obviously increased in ovarian carcinoma, while they were not significant different between the ovarian benign tumor and healthy controls (**Figure 2**).

In addition, the abundance of Peak1 was elevated with the increasing of age in ovarian carcinoma ($P<0.05$), whereas the other structures were not statistically significant different in different age.

Designation and assessment of diagnosis model based on N-glycan markers to differentiate ovarian carcinoma from normal controls

We screened ovarian carcinoma-related N-glycan alterations based on logistic regression analysis.Logistic regression coefficients were used to estimate odds ratios for each of the independent variables. Two diagnosis models were designated with or without HE4 and CA125. The mathematic formula named Model_Sugar and Model_Complex were constructed to differentiate ovarian carcinoma from ovarian benign tumor (Model_Sugar=0.204×Peak1+3.560×Peak12-0.226×Peak6+0.528; Model_Complex=3.611×Peak12+0.003×CA125+0.023×HE4-0.177×Peak6-1.311). Compared with ovarian benign tumor and healthy controls, Model_Sugar and Model_Complex were obviously elevated in ovarian carcinoma. They were significantly different in ovarian carcinoma, while not significant different between the ovarian benign tumor and healthy controls (**Figure 3**).

To assess the contributions of Model_Sugar, Model_Complex, N-glycan markers, CA125, HE4 and ROMA, we used ROC analysis. The area under the ROC curve (AUC) of Peak1, Peak6, Peak9 and Peak12 were 0.754, 0.878, 0.795 and 0.830, respectively, which were closer to the AUC of HE4 (0.879) and CA125 (0.860) (**Figure 4A**). The AUC of Model_Sugar (AUC=0.903) was higher than those of individual markers. Whereas, the ROC curve of Model_Sugar was a bit lower than that of ROMA (AUC=0.913) (**Figure 4B**). Further improvement was achieved with the involvement of HE4 and CA125 (Model_Complex) to improve the diagnosis efficiency. Compared with ROMA and Model_Sugar, the area under the ROC curve of Model_Complex (AUC=0.963) was the highest, indicating that Model_Complex was the most effective in distinguishing ovarian carcinoma from ovarian benign tumor (**Figure 4B**).

The sensitivity, specificity, PPV, NPV and accuracy of CA125, HE4, ROMA, Model_Sugar, and Model_Complex in predicting ovarian carcinoma were listed in **Table 3**. CA125 at the recommended cut-off value of 35 U/L had a sensitivity of 82.9% and a specificity of 69.4%. HE4 at the recommended cut-off value of 150pmol/L had a sensitivity of 54.3% and a specificity of 99.1%. ROMA had a sensitivity of 80.0% and a specificity of 83.8% at the premenopausal recommended cut-off value of 13.1% and postmenopausal recommended cut-off value of 27.7%.

An optimal cut-off value of -0.8203 was selected for Model_Sugar using ROC curve analysis. At this cut-off value, Model_Sugar had a sensi-

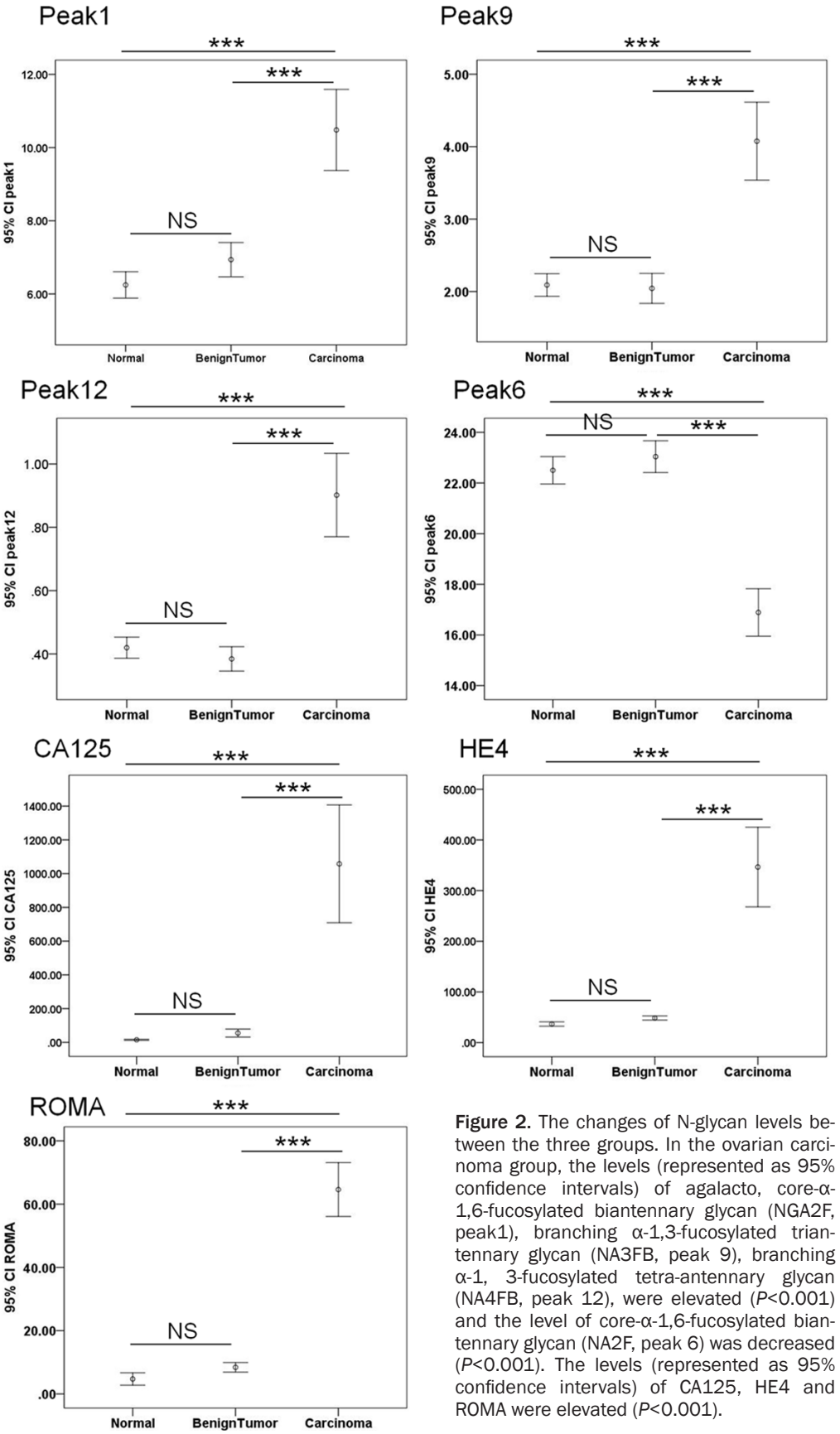

Figure 2. The changes of N-glycan levels between the three groups. In the ovarian carcinoma group, the levels (represented as 95% confidence intervals) of agalacto, core-α-1,6-fucosylated biantennary glycan (NGA2F, peak1), branching α-1,3-fucosylated triantennary glycan (NA3FB, peak 9), branching α-1, 3-fucosylated tetra-antennary glycan (NA4FB, peak 12), were elevated (P<0.001) and the level of core-α-1,6-fucosylated biantennary glycan (NA2F, peak 6) was decreased (P<0.001). The levels (represented as 95% confidence intervals) of CA125, HE4 and ROMA were elevated (P<0.001).

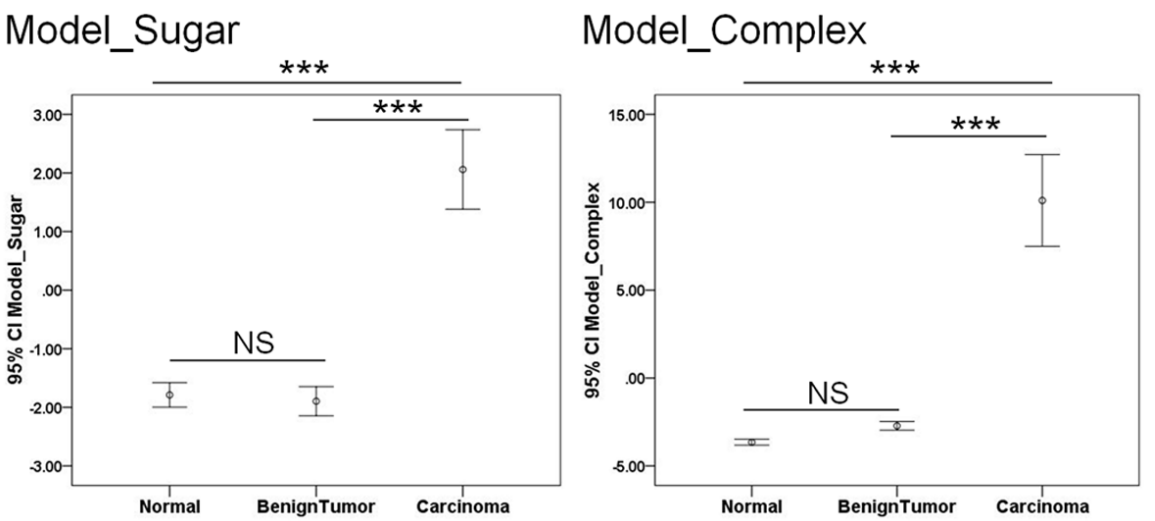

Figure 3. The changes of model levels between the three groups.In ovarian carcinoma group, the levels (represented as 95% confidence intervals) of Model_Sugar and Model_Complex were obviously elevated (P<0.001).

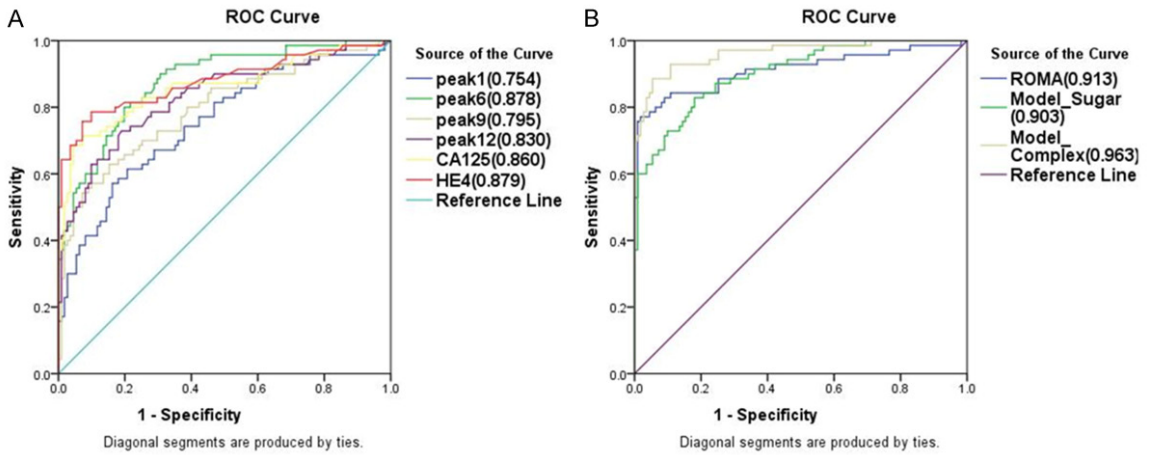

Figure 4. Receiver operating characteristic (ROC) curve analyses for the prediction of ovarian carcinoma. A.The ROC analysis for distinguishing between ovarian carcinoma and ovarian benign tumor using individual biomarker. The areas under the ROC curve (AUCs) indicate the diagnostic power: Peak1 (0.754), Peak6 (0.878), Peak9 (0.795), Peak12 (0.830), CA125 (0.860) and HE4 (0.879). B. The ROC analysis for distinguishing between ovarian carcinoma and ovarian benign tumor using multiparameter models. The areas under the ROC curve (AUCs) indicate the diagnostic power: ROMA (0.913), Model_Sugar (0.903) and Model_Complex (0.963). The diagnostic model was constructed by using forward stepwise logistic regression analysis: Model_Sugar = 0.204×Peak1+3.560×Peak12-0.226×Peak6+0.528. Model_Complex = 3.611×Peak12+0.003×CA125+0.023×HE4-0.177×Peak6-1.311. ROMA: Predicted Probability (PP) = exp (PI)/[1+exp (PI)]. Premenopausal: Predictive Index (PI) = −12.0 + 2.38 * LN (HE4) + 0.0626 * LN (CA125); Postmenopausal: Predictive Index (PI) = -8.09 + 1.04 * LN (HE4) + 0.732 * LN (CA125).

tivity of 82.9%, which was increased 28.6% and 2.9% compared with HE4 and ROMA. The specificity of Model_Sugar (90.1%) was increased 20.7% and 6.3% compared with CA125 and ROMA. The accuracy of Model_Sugar (82.3%) was increased 7.7% and 0.5% compared with CA125 and HE4, while was the same as that of ROMA. An optimal cut-off value of -0.7445 was selected for Model_Complex using ROC curve analysis. At this cut-off value, Model_Complex had a sensitivity of 88.6%, which was increased 5.7%, 34.3% and 8.6% compared with CA125, HE4 and ROMA, respectively. The specificity of Model_Complex (94.6%) was increased 25.2% and 10.8% compared with CA125 and ROMA. The accuracy of Model_Complex (92.3%) was

Table 3. The diagnostic power for ovarian carcinoma

Cut-off value	Test result	Pathological diagnosis		Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)
		+	-					
CA125 (35 U/mL)	+	58	34	82.9	69.4	63.0	86.5	74.6
	-	12	77					
HE4 (150 pmol/L)	+	38	1	54.3	99.1	97.4	77.5	81.8
	-	32	110					
ROMA*	+	56	18	80.0	83.8	75.7	86.9	82.3
13.1%, 27.7%	-	14	93					
Model_sugar	+	58	10	82.9	90.1	85.3	88.3	82.3
(-0.8203)	-	12	91					
Model_Complex	+	62	6	88.6	94.6	91.2	92.9	92.3
(-0.7445)	-	8	105					

Abbreviations: PPV, positive predictive value; NPV, negative predictive value. *: the cut-off of premenopausal is 13.1%; the cut-off of postmenopausal is 27.7%;+: ovarian carcinoma positive; -: ovarian carcinoma negative.

increased 17.7%, 10.5% and 10.0% compared with CA125, HE4 and ROMA, respectively.

Increased levels of β1,6-GlcNAc branchedglycosylated proteins in ovarian carcinoma

The branching α-1, 3-fucosylated tetra-antennary glycan (NA4Fb, Peak12) was significantly higher in ovarian carcinoma than that in ovarian benign tumor and healthy controls, which containing a β1,6-N-acetylglucosamine (β1,6-GlcNAc) branched residues. To confirm this finding, we use Phytohemagglutinin-lymphocyte type(PHA-L) lectin to probed the serum and tissue β1,6-GlcNAc branched proteins from patients with ovarian carcinoma, because PHA-L can specifically recognize the glycoproteins with β1,6-GlcNAc. In serum, the level of PHA-L-binding β1,6-GlcNAc branched residues was higher in the ovarian carcinoma group than that in the ovarian benign tumor and healthy group. There was no significant difference between benign tumor and healthy group (**Figure 5A**). In tissue, the abundance of PHA-L-binding β1,6-GlcNAc branched residues was higher in ovarian carcinoma tissues than in paired adjacent tissues and ovarian benign tumor tissues. There was no significant difference between adjacent tissues and benign tumor tissues (**Figure 5B**).

To determine whether the change of β1,6-GlcNAc in ovarian carcinoma tumor tissue is relevant to alteration of the glycosylation biosynthesis pathway, we analyzed the abundance of mammalian N-acetylglucosaminy transferase V (GNT-V) in ovarian carcinoma tumors, adjacent tissues and ovarian benign tumor tissues by using qRT-PCR. GNT-V is a key enzyme catalyzing the reaction of adding β1,6-GlcNAc on asparagine-linked oligosaccharides of proteins, and relevant to increase metastasis. The enzymes are encoded by *MGAT5* genes [21]. The result revealed that *MGAT5* mRNA expression was higher in tumors than that in adjacent tissues and benign tumor tissues. There was no significant difference between adjacent tissues and benign tumor tissues (**Figure 5C**).

Correlations between the N-glycan markers, multiparameter models and clinical parameters

At present, CA125 and HE4 have been widely used for screening and monitoring of ovarian carcinoma. Therefore, we analyzed the correlations between individual N-glycan markers, diagnostic models and CA125, HE4, ROMA and menopause in ovarian carcinoma (**Table 4**). The Pearson correlation analysis indicated that HE4 was positive correlated with Peak1, Peak11, Peak12, and Model_Sugar, and negative correlated with Peak6 and Peak7 ($P<0.05$). ROMA showed positive correlation with Peak1, Peak11, Model_Sugar and Model_Complex, and negative correlation with Peak6 ($P<0.05$). Menopause was positive correlated with Peak1 and Peak2 ($P<0.05$).

Discussion

Glycosylation is one of the most common and important post-translational modifications, and more than half of all known proteins are thought to be glycoproteins. Changes in N-linked glycosylation are known to wide spread during auto-

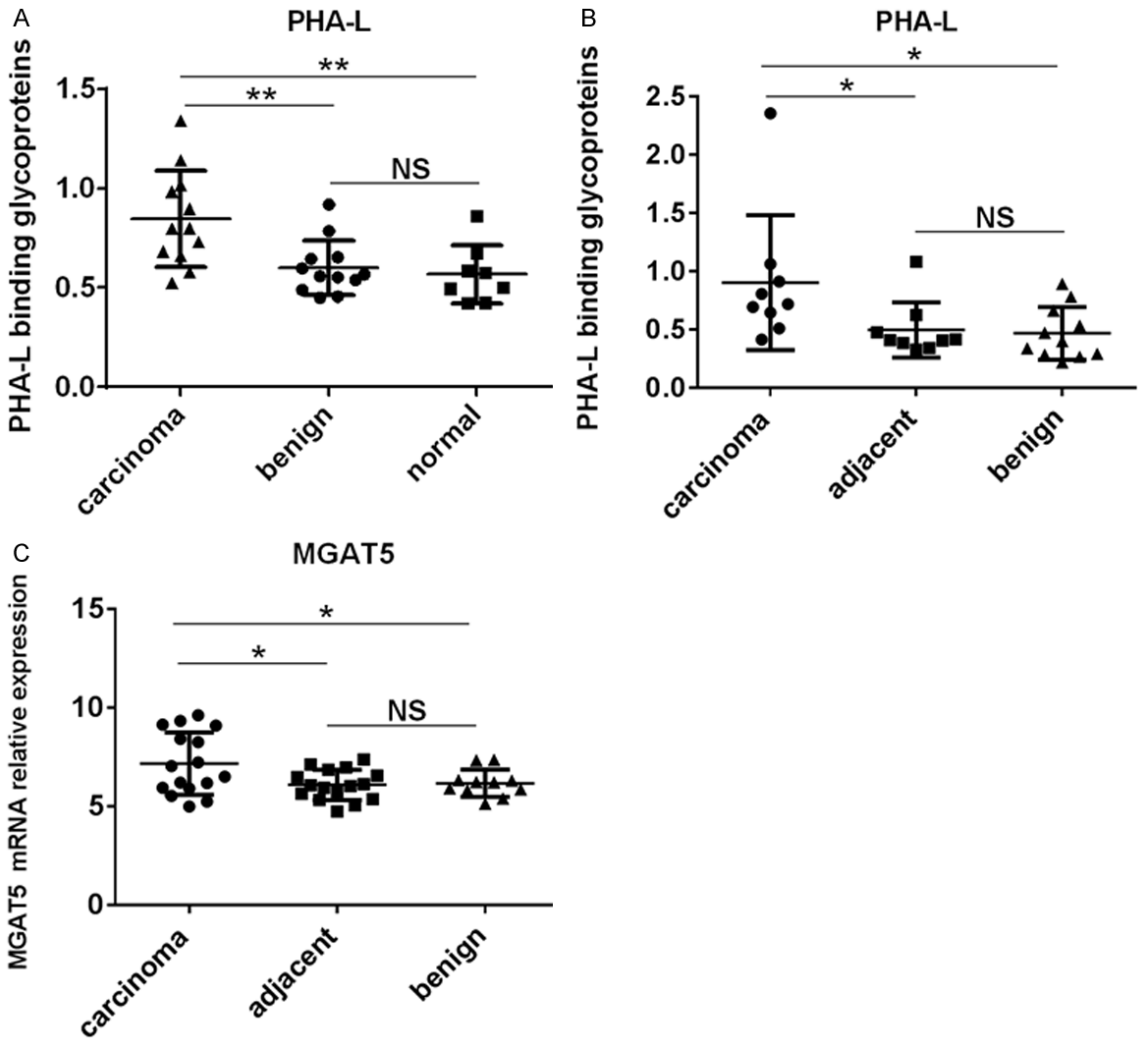

Figure 5. The abundance of glycoproteins with β1,6-GlcNAc residues detected by lectin bolts and the mRNA level of *MGAT5* is illustrated using reverse transcriptase-polymerase chain reaction (RT-PCR) analyses. A. Lectin blots of serum proteins were probed with phytohemagglutinin-lymphocyte type (PHA-L). The horizontal axis represents the experimental groups: normal (n=8), benign (n=12), and ovarian carcinoma (n=12). The vertical axis indicates the ratio of β1,6-GlcNAc proteins to total proteins. The difference between ovarian carcinoma and other two groups was statistically significant ($P<0.01$). The difference between normal and benign was not statistically significant ($P>0.05$). B. Lectin blots from tissue proteins were probed with PHA-L. The horizontal axis represents the experimental groups: carcinoma tissues (n=9), adjacent tissues (n=9) and the benign tissues (n=11) vertical axis indicates the ratio of β1,6-GlcNAc proteins to total proteins. The difference between carcinoma groups and other groups was statistically significant ($P<0.05$). The difference between normal groups and adjacent groups was not statistically significant ($P>0.05$). C. The relative messenger RNA (mRNA) expression levels of in tissues were measured by qRT-PCR. The horizontal axis represents the experimental groups: carcinoma tissues (n=16), adjacent tissues (n=16) and benign tissues (n=11). The vertical axis separately indicates the relative mRNA levels of *MGAT5*. The difference between carcinoma tissues and other two groups was statistically significant ($P<0.05$). The difference between adjacent tissues and benign groups was not statistically significant ($P>0.05$).

immune diseases, hematological cancers, inflammatory diseases and malignant tumor [22, 23]. The technology of glycobiology can obviously increase sensitivity and specificity of the noninvasive diagnostic. DSA-FACE can obviously detect the change of N-glycan in serum total protein and some particular proteins [20, 24]. And now, this technique has been applied to the diagnoses such as hepatocellular carcinoma, colorectal cancer, gastric cancer and breast cancer [11, 25-27].

In current, the glycoprotein biomarker CA125 and HE4 are the most commonly used to screen

Table 4. The correlation between N-glycan markers and clinical indicators in ovarian carcinoma

Correlation	CA125		HE4		ROMA		menopause	
	r[a]	P	r[a]	P	r[a]	P	r[b]	P
Peak1	0.108	0.373	0.326	0.006	0.304	0.010	0.307	0.010
Peak2	0.085	0.485	0.080	0.512	0.162	0.181	0.346	0.003
Peak3	0.180	0.135	-0.082	0.499	-0.043	0.723	-0.141	0.246
Peak4	-0.120	0.324	-0.156	0.198	-0.075	0.537	0.150	0.214
Peak5	-0.137	0.258	-0.065	0.593	-0.181	0.133	-0.045	0.709
Peak6	0.026	0.829	-0.296	0.013	-0.282	0.018	-0.202	0.094
Peak7	-0.015	0.899	-0.261	0.029	-0.140	0.246	-0.006	0.963
Peak8	0.037	0.763	0.222	0.065	0.174	0.151	-0.132	0.276
Peak9	-0.040	0.742	0.221	0.065	0.183	0.128	0.112	0.355
Peak10	-0.034	0.786	0.186	0.124	0.206	0.088	0.055	0.649
Peak11	0.041	0.738	0.288	0.016	0.245	0.041	0.018	0.879
Peak12	-0.027	0.823	0.318	0.007	0.202	0.094	0.142	0.241
Model_Sugar	0.009	0.941	0.377	0.001	0.330	0.005	0.236	0.050
Model_Complex	0.649	<0.001	0.918	<0.001	0.709	<0.001	0.054	0.657

r: Correlation coefficient; a, Pearson's correlation analysis; b: Spearman's correlation analysis.

for early detection of ovarian carcinoma. However, CA125 has high false positive rate and serum levels may be in the high range in 50%-60% of symptomatic stage I patients of symptomatic stage I patients [28]. HE4 has been widely used in distinguishing ovarian carcinoma from other benign gynecological diseases. The diagnostic accuracy of HE4 was superior to that of CA125, and the combination of HE4 and CA125 may enhance the diagnostic sensitivity [29]. In the screening of ovarian carcinoma, it is unlikely that an individual biomarker can reach a specificity of 99.6% and sensitivity greater than 75% [30]. ROMA is superior to individual biomarker and could help to distinguish in cases with any doubt with a high diagnostic accuracy [31]. However, to further improve the early diagnosis of ovarian carcinoma, and to reduce the death rate, we still hope to find new biomarker with higher sensitivity and specificity. Therefore, we believed that N-glycan profiling might be more effective than the detection of a single glycosylated molecule.

In this study, we analyzed serum N-linked glycan and identified specific structures of N-glycan in ovarian carcinoma by DSA-FACE. After serum N-glycan profiling, two mathematic diagnostic models were constructed with statistical analysis and step logistic regression. Model_Sugar based on N-glycan markers could differentiate ovarian carcinoma from ovarian benign tumor. Model_Complex based on both N-glycan markers, CA125 and HE4 could not only differentiate ovarian carcinoma from ovarian benign tumor but also improved the diagnostic efficiency. The AUC of Model_Sugar was improved as compared to that of CA125 and HE4 (0.903 vs 0.860, 0.879). The accuracy of Model_Sugar was increased 7.7% and 0.5% as compared to that of CA125 and HE4, and the same as that of ROMA. The AUC of Model_Complex was improved as compared to that of ROMA (0.963 vs 0.913). The accuracy of Model_Complex was improved to further increased 17.7%, 10.5% and 10.0% as compared to that of CA125, HE4 and ROMA. Both models demonstrated better diagnostic performance with higher the accuracy (>80.0%). It was worth noting that Model_Complex could further improve the diagnostic performance than ROMA and Model_Sugar.

In the analysis of individual N-glycan structure abundance, we observed that the abundance of tetra-antennary glycan (Peak12, NA4Fb) was significant increased in ovarian carcinoma. This is similar to what occur in pancreatic cancer [32]. NA4Fb is a branching α-1, 3-fucosylated tetra-antennary glycan which contained β1,6-GlcNAc branched residues. To further confirm this finding, we performed lectin blot analysis in both serum and tissue samples. By using ovarian benign tumor and healthy groupserum as positive control, β1,6-GlcNAc was obviously increased in ovarian carcinoma serum lectin blot. By using ovarian carcinoma, paired adjacent tissues and ovarian benign tumor tissues,

β1,6-GlcNAc was obviously increased in ovarian carcinoma tumor tissue.

In view of Peak12 increased in ovarian carcinoma, we further detected related glycosyltransferases to verify the glycosylation change by RT-PCR. GNT-V is an enzyme encoded by *MGAT5* gene that catalyzes the addition of β1,6-GlcNAc branching of N-glycans, and has been associated to increase metastasis [21]. We tested expression levels of GNT-V to validate the relevant steps for changes in β1,6-GlcNAc. We observed that the expression of *MGAT5* gene was significantly higher in ovarian carcinoma than that in adjacent tissue and benign tumor tissue. The result suggested that the high expression of GNT-V in carcinoma tissues could lead to increased levels of β1,6-GlcNAc.Takahashi N *et al* [33] reported that higher GNT-V expression was founded in ovarian carcinoma, and was positively correlated with early FIGO staging. This is consistent with our findings. In addition,increased activity or expression of GNT-V and β-1, 6 GlcNAc-branched N-glycans has been found in several tumors, such as prostate cancer, melanoma, breast cancers [34-36]. This suggests that GNT-V may participate in the pathogenesis of malignant transformation. The molecular mechanism for alterations of N-glycosylation in ovarian carcinoma is still a critical challenge for our further study.

In conclusion, this study analyzed that alteration of glycosylation in serum N-glycan profiling exited in the development and progression in ovarian carcinoma. Model_Complex and Model_sugar are promising to improve efficiency ovarian carcinoma diagnosis as noninvasive serologic markers. They are also valuable supplements to the serologic markers already in use. The current study preliminary exposition the progression of ovarian carcinoma in molecular and cellular biology process, glycosylation is involved in the pathogenesis of malignancies. In future studies, glycobiologicalmethod will be focused on glycosylation changes in each step of development and progression with ovarian carcinoma, which is more conducive to improve the diagnosis, prediction and monitoring of ovarian carcinoma.

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Disclosure of conflict of interest

None.

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